Amino Acid Substitutions in GyrA Affect Quinolone Susceptibility in *Salmonella* Typhimurium

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Running title: Amino Acid Substitutions Affect *Salmonella* Quinolone Susceptibility

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ABSTRACT

The prevalence of quinolone-resistant *Salmonella* has become a public health concern. Amino acid substitutions have generally been found within the quinolone resistance-determining region in subunit A of DNA gyrase (GyrA) of *Salmonella* Typhimurium. However, direct evidence of the contribution of these substitutions to quinolone resistance remains to be shown. To investigate the significance of amino acid substitutions in *S.* Typhimurium GyrA to quinolone resistance, we expressed recombinant wild-type (WT) and five mutant DNA gyrases in *Escherichia coli* and characterised them *in vitro*. WT and mutant DNA gyrases were reconstituted *in vitro* by mixing recombinant subunits A and B of DNA gyrase. The correlation between the amino acid substitutions and resistance to quinolones ciprofloxacin, levofloxacin, nalidixic acid and sitafloxacin was assessed by quinolone-inhibited supercoiling assays. All mutant DNA gyrases showed reduced susceptibility to all quinolones when compared with WT DNA gyrases. DNA gyrase with a double amino acid substitution in GyrA, serine to phenylalanine at codon 83 and aspartic acid to asparagine at 87 (GyrA-S83F-D87N), exhibited the lowest quinolone susceptibility amongst all mutant DNA gyrases. The effectiveness of sitafloxacin was shown by the low inhibitory concentration required for mutant DNA gyrases, including the DNA gyrase with GyrA-S83F-D87N. We suggest sitafloxacin as a candidate drug for the treatment of salmonellosis caused by ciprofloxacin-resistant *S.* Typhimurium.

Key words: *Salmonella* Typhimurium, Amino acid substitutions, Quinolone resistance

INTRODUCTION

Salmonella gastroenteritis is generally a self-limiting illness. However, antimicrobials are required for invasive or severe cases of *Salmonella* infection, particularly vulnerable groups such as young children, the elderly and immune-compromised individuals. Although chloramphenicol, ampicillin and trimethoprim-sulfamethoxazole have been used for the treatment of salmonellosis, resistance to these drugs has increased significantly in recent years. Currently, drug-resistant *Salmonella* is frequently encountered in most parts of the world, and the rate of resistance has increased significantly. As a result, quinolones, especially fluoroquinolones (FQs), have become the drugs of choice for the treatment of severe *Salmonella* infections. Quinolones have been categorised by the World Health Organization as critically important antimicrobials[1]. However, although minimising the occurrence of resistance to quinolones has become a public health priority, several reports have described limited progress [2-6].

The mechanisms responsible for quinolone resistance derive mainly from chromosomal mutations in genes encoding target enzymes, DNA gyrase (*gyrA* and *gyrB*) and DNA topoisomerase IV (*parC* and *parE*). Mutations result in conformational changes at the target site of the enzyme tetrameric interface, which lead to a decrease in binding affinity of quinolones and an increase in resistance against these agents [7]. Mutations are commonly located in the quinolone resistance-determining regions (QRDRs) [8-11] of these genes. Hopkins and his colleague summarized the association of amino acid substitutions in GyrA to quinolone resistance in *Salmonella* as well as in *E. coli* [12]. In *Salmonella*, mutations caused by amino acid substitutions in GyrA of serine to phenylalanine at codon 83 (S83F) and aspartic acid to asparagine (D87N), glycine (D87G) or tyrosine (D87Y) at codon 87 are frequently observed [13-19]. Moreover, *Salmonella* isolates with double mutation at both codons 83 and 87 (GyrA-S83F-D87N) have previously been documented to associate with

high levels of resistance to FQs, including ciprofloxacin (CIP) [20].

Barnard *et al.* investigated the effect of the substitutions serine and aspartic acid to alanine at position 83 (S83A) and 87 (D87A), respectively, on GyrA of *E. coli* with identical QRDR amino acid sequence as *Salmonella*, to study quinolone resistance [21]. However, no direct evidence of the contribution of amino acid substitutions to quinolone resistance has been shown by *in vitro* assay in *Salmonella*. Moreover, the elucidation of the effects of S83F, D87N, D87G or D87Y on GyrA is necessary, as these substitutions, not S83A or D87A, are commonly identified in *Salmonella*.

In the present study, we aimed to elucidate the role of amino acid substitutions in GyrA of *Salmonella* in reducing quinolone sensitivity against four quinolones using recombinant wild-type (WT) and mutant DNA gyrases and quinolone-inhibited supercoiling assays, which has been the classical approach to identify and quantitate the inhibition of DNA gyrase by quinolones.

MATERIALS AND METHODS

Reagents and kits. Non-fluorinated quinolone Nalidixic acid (NAL) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Fluoroquinolones CIP and levofloxacin (LVX) were purchased from LKT Laboratories, Inc. (St. Paul, MN, USA), and sitafloxacin (SIT) was a gift from Daiichi-Sankyo Pharmaceutical, Co., Ltd. (Tokyo, Japan). TaKaRa Mighty (Blunt End) Cloning Reagent sets and Ni-nitrilotriacetic acid (Ni-NTA) protein purification kits were obtained from Takara Bio Inc. (Shiga, Japan) and Thermo Fisher Scientific Inc. (Waltham, MA, USA), respectively. Restriction enzymes were obtained from New England Biolabs, Inc. (Ipswich, MA, USA). Relaxed pBR322 DNA and supercoiled pBR322 DNA were purchased from John Innes Enterprises Ltd. (Norwich, United Kingdom), and cOmplete™, Mini, EDTA-free protease inhibitor cocktail tablets from Roche Applied

Science (Mannheim, Germany).

Bacterial strains and plasmids. Quinolone-susceptible strain *S.* Typhimurium NBRC 13245 (*S.* Typhimurium LT2) was purchased from NITE Biological Resource Center (Tokyo, Japan). Bacterial DNA was extracted from the strain using the InstaGene^{TM} Matrix (Bio-Rad Laboratories Inc., Hercules, CA, USA) according to the manufacturer's protocol. *E. coli* strain TOP-10 (Thermo Fisher Scientific Inc.) was used as the host for cloning purposes. *E. coli* strain BL21(DE3)/pLysS and vector plasmid pET-20b (+) were purchased from Merck KGaA (Darmstadt, Germany) and used for recombinant GyrA and GyrB expression.

Construction of WT and mutant DNA gyrase expression vectors. WT recombinant GyrA and GyrB expression vectors were constructed as previously described [22-25]. Mutations were introduced into the WT *gyrA* gene of *S.* Typhimurium NBRC 13245 by PCR with pairs of complementary primers containing the nucleotide substitution of interest (Table 1). *gyrA* with base substitutions was digested with *Nde*I and *Xho*I, ligated into WT *gyrA* expression plasmid, digested with the same restriction endonucleases, and transformed in *E.coli* TOP-10 to obtain mutant *gyrA* expression plasmid. The nucleotide sequence of the DNA gyrase gene in the plasmid was confirmed using a BigDye Terminator (version 3.1) cycle sequencing kit and an ABI Prism 3130*xl* genetic analyser (Thermo Fisher Scientific Inc.) according to the manufacturer’s protocol.

Expression and purification of recombinant DNA gyrase. DNA gyrase subunits were purified as previously described [22-25]. Expression plasmids carrying WT and mutant *gyrA* and WT *gyrB* genes of *S.* Typhimurium were transformed in *E. coli* BL21(DE3)/pLysS. Expression of GyrA and GyrB was induced with the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (Wako Pure Chemical Industries Ltd.) and incubation at 18 °C for 13 h. Recombinant DNA gyrase subunits in the supernatant of the sonicated (Sonifier 250; Branson, Danbury, CT, USA) lysate were purified by column chromatography

using Ni-NTA agarose resin. After purification, protein fractions were diluted with glycerol to 50% solutions (wt/vol) and stored at -80 °C until use. Protein fractions were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

DNA supercoiling activities and inhibition by quinolones. ATP-dependent and quinolone-inhibited supercoiling assays were carried out as previously described [22-25] with the following modifications. The reaction mixture (30 μL) consisted of DNA gyrase assay buffer, relaxed pBR322 DNA (0.3 μg) and 30 ng of Gyr A and B proteins each. Reactions were run at 35 °C for 20 min and stopped by the addition of 30 μL of chloroform-isoamyl alcohol (24:1) and 3 μL of 10× DNA loading dye. Total reaction mixtures were subjected to electrophoresis using 1% agarose gel in 0.5× Tris-borate-EDTA (TBE) buffer. The gels were run for 60 min at 80 mA and stained with ethidium bromide (0.7 μg/mL). Supercoiling activity was evaluated by tracing the brightness of the bands with software ImageJ (http://rsbweb.nih.gov/ij). The inhibitory effect of quinolones on DNA gyrases was assessed by determining the drug concentrations required to inhibit the supercoiling activity of the enzyme by 50% (IC_{50}) in the presence or absence of 2-fold serial increases in quinolone concentration. To allow direct comparisons, all incubations with DNA gyrase were carried out and processed in parallel on the same day under identical conditions. All enzyme assays were carried out at least three times to confirm reproducibility.

RESULTS

Expression and purification of recombinant GyrA and GyrB proteins. Expressed recombinant WT and mutant DNA gyrase subunits were purified from 200-mL cultures as 0.5 to 16 mg soluble His-tagged 97-kDa and 89-kDa proteins of GyrA and GyrB, respectively. The purity of recombinant proteins was confirmed by SDS-PAGE (Figure 1). All recombinant proteins were obtained with high purity (>95%).

DNA supercoiling activity of recombinant DNA gyrases. Combinations of WT, GyrA-S83F, GyrA-D87N, GyrA-D87G, GyrA-D87Y, GyrA-S83F-D87N and WT GyrB subunits were examined for DNA supercoiling activities using relaxed pBR322 DNA as a substrate in the presence or absence of ATP (Figure 2). DNA supercoiling activities were observed in the presence of ATP and recombinant DNA gyrase subunits (Figure 2, lane 2), whilst neither subunit alone exhibited DNA supercoiling activity (Figure 2, lanes 3 and 4). In addition, no supercoiling activity was observed when ATP was omitted from the reaction mixture (Figure 2, lane 5).

Inhibition of DNA gyrase activities by quinolones. The IC_{50} of quinolones were determined using the quinolone-inhibited supercoiling assay. Representative data demonstrating the inhibitory effects of SIT on DNA gyrase are shown in Figure 3, and those of other quinolones in Supplementary Figures S1-S3. The IC_{50} of each quinolone required for WT and mutant DNA gyrases are summarised in Table 2. Each quinolone showed dose-dependent inhibition, with IC_{50} ranging from 0.2 to 1610 μg/mL. DNA gyrases bearing GyrA-S83F-D87N showed significantly higher IC_{50} than WT gyrase (Table 2) and higher resistance than DNA gyrases bearing GyrA-S83F, D87N, D87G or D87Y. The inhibitory effects of quinolones against GyrA-S83F-D87N were ranked SIT > LVX > CIP > NAL (Figure 4).

DISCUSSION

Whilst FQs (SIT, LVX and CIP) inhibited the DNA supercoiling activities of WT DNA gyrase at considerably lower concentrations than NAL, all quinolones were required at high concentrations to inhibit these activities (Table 2). In addition, inhibition of single mutation activity needed 2- to 11-fold and 26- to 30-fold IC_{50} for FQs and NAL, respectively. In contrast, double mutation GyrA-S83F-D87N exhibited markedly higher resistance, as 19- to as much as 1466-fold IC_{50} was required for all quinolones to cause a similar level of

176 inhibition as that for WT DNA gyrase. The striking difference in concentrations of
177 quinolones required for the double mutation as compared with the single mutations suggest
178 that this unique combination of amino acid substitutions may drastically alter the tertiary
179 structure of QRDR without disturbing DNA gyrase activity.

180 Past work on *E. coli* DNA gyrase, which has an identical QRDR amino acid sequence
181 as that of *Salmonella*, demonstrated a 34.8-fold increase in the IC_{50} of CIP due to the
182 S83A-D87A substitution [20]. To our knowledge, DNA gyrase with GyrA-S83A-D87A has
183 never been identified in clinical *Salmonella* isolates. In the present work, we examined amino
184 acid substitutions commonly identified in quinolone-resistant clinical isolates of *S.*
185 Typhimurium. Substituents in the structures of fluoroquinolone at R1, R7 and R8 have been
186 proposed to associate with GyrB [24, 26] and contribute to the affinity with DNA gyrase.
187 Amongst FQs, SIT was found to have relatively strong binding with WT, single and double
188 mutants, and also had the lowest IC_{50}. Moreover, the fluorinated cyclopropyl residue at R1
189 and the chlorine atom at R8 in SIT are believed to contribute to a high affinity [26]. This strong
190 binding seems to keep the quinolone-DNA gyrase interaction and maintain the inhibitory
191 activity of SIT against GyrA mutants. By contrast, CIP and LVX showed significantly lower
192 inhibitory activity against S83F-D87N, although they and SIT required a similar IC_{50} for WT.
193 This difference in inhibitory activity may be due to the halogen atoms found at R1 and R8 in
194 SIT, but not in CIP or LVX, possibly contributing to a strong interaction with DNA gyrase [24],
195 which in turn may enhance the inhibitory activity against S83F-D87N.

196 In the present study, the IC_{50} of SIT for *S.* Typhimurium WT DNA gyrase and DNA
197 gyrase carrying GyrA-S83F-D87N were 0.22 and 3.8 μg/mL, respectively (Table 2).
198 Moreover, the minimum inhibition concentration (MIC) of SIT for *S.* Typhimurium with WT
199 DNA gyrase genes was previously found in our premises to be 0.008 μg/mL [22], and
200 elsewhere the maximum concentration (C_{max}) in a 200 mg dose was determined to be 1.9

μg/mL [28]. Furthermore, a previous study showed that the MIC values of the SIT tested on *Salmonella* isolates with reduced CIP susceptibility and CIP resistance ranged from 0.016 to 2 μg/mL [27]. SIT has been approved in Japan for the treatment of bacterial infections, with the majority of adverse reactions being mild or moderate in severity during clinical trials [29]. Thus, considering the ratio between IC_{50} and MIC, it is very likely that SIT could safely be used to inhibit CIP-resistant *S.* Typhimurium with GyrA-S83F-D87N at clinical level. However, fluoroquinolone resistance arises in a stepwise process, involving a result of accumulating mutations in the genes encoding the target enzymes DNA gyrase and the topoisomerase IV. It is probable that *Salmonella* resistant to SIT will be developed during treatment, despite its initially low MIC value, thus, careful usage of SIT is recommended.

In conclusion, we elucidated the contribution of amino acid substitutions in GyrA to quinolone resistance in *S.* Typhimurium using recombinant DNA gyrase. The amino acid substitutions at position 83 and 87 of GyrA and the structural variations at R1 and R8 of quinolones seemed to affect the interaction between DNA gyrases and drugs. Moreover, the halogen atoms at R1 and R8 in SIT caused a stronger effect on DNA gyrase with a double amino acid substitution in GyrA than did other FQs. Due to the low concentration needed to cause inhibition and the mild adverse reactions observed at clinical level, we suggest that SIT may be a good choice for the treatment of salmonellosis caused by CIP-resistant *S.* Typhimurium.

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FIGURE LEGENDS

Figure 1. SDS-PAGE analysis of purified *Salmonella* Typhimurium DNA gyrases. His-tagged recombinant DNA gyrases were overexpressed in *E. coli* and purified by Ni-NTA affinity resin chromatography. Lanes: M, protein marker (NEB); 1, wild-type GyrA; 2, GyrA-S83F; 3, GyrA-D87N; 4, GyrA-D87G; 5, GyrA-D87Y; 6, GyrA-S83F-D87N; 7, wild-type GyrB. Three hundred ng of each protein were loaded onto a 5-20% gradient polyacrylamide gel.

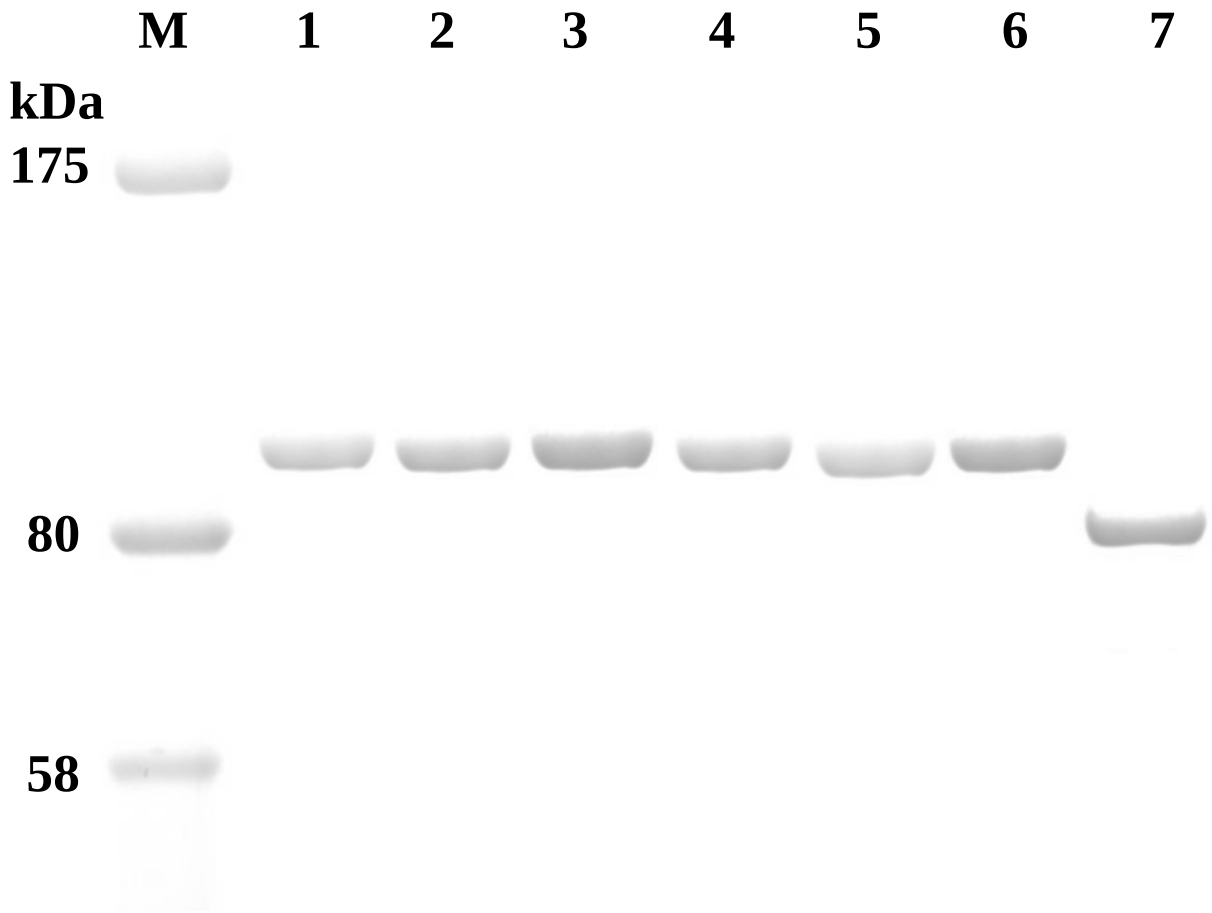
Figure 2. GyrA and GyrB proteins of *Salmonella* Typhimurium generate ATP-dependent DNA supercoiling activity.
R, relaxed pBR322 DNA. SC, supercoiled pBR322 DNA. Lanes: 1, relaxed pBR322 DNA; 2, relaxed pBR322 DNA and both recombinant GyrA and GyrB proteins; 3, relaxed pBR322 DNA and only GyrA protein; 4, relaxed pBR322 DNA and mutants GyrB protein; 5, absence of ATP. Relaxed pBR322 DNA (0.3 μg) was incubated with mutant DNA gyrase reconstituted from GyrA (30 ng) and GyrB (30 ng) in the presence or absence of 1 mM ATP. The reactions were stopped, and the DNA products were separated by electrophoresis in 1% agarose gels. DNA was stained with ethidium bromide and photographed under UV illumination.

Figure 3. Inhibitory activities of SIT on the supercoiling activities of *Salmonella* Typhimurium DNA gyrases.
Relaxed pBR322 DNA (0.3 μg) was incubated with 30 ng of each DNA gyrase in the presence of the indicated amounts of SIT (μg/mL). The reaction was stopped, and the DNA products were analysed by electrophoresis in 1% agarose gels.

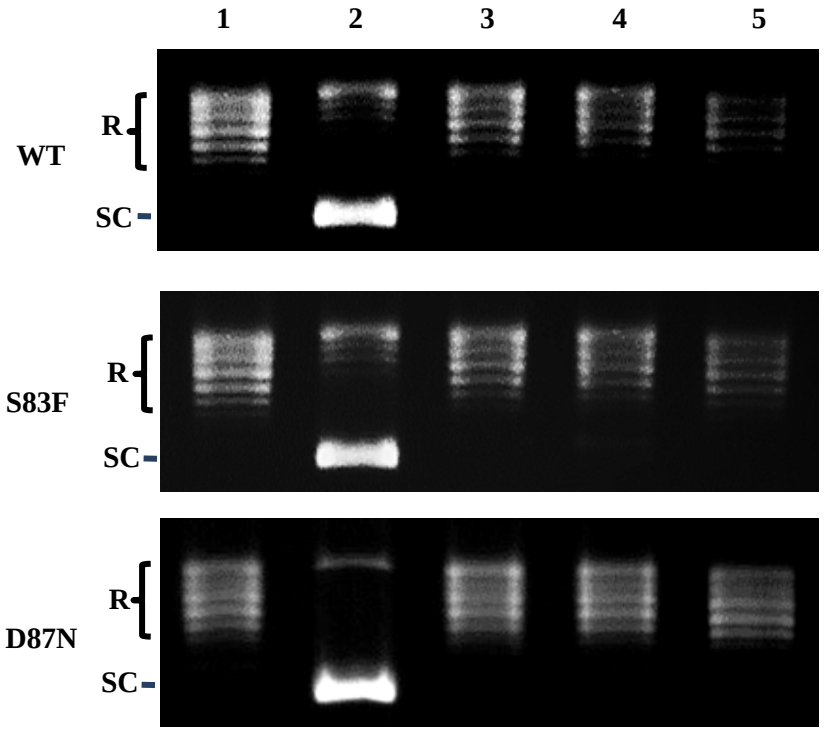
Figure 4. Inhibitory activities of SIT, LVX, CIP and NAL on the supercoiling activities of *S*. Typhimurium DNA gyrases with GyrA-S83F-D87N

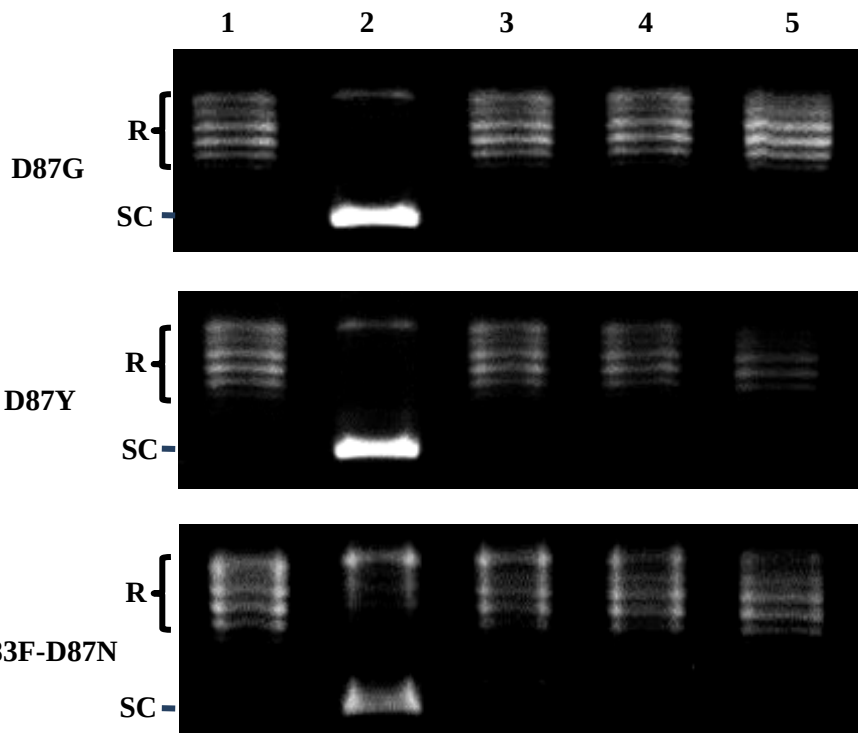
Relaxed pBR322 DNA (0.3 μg) was incubated with 30 ng of each DNA gyrases in the presence of the indicated amounts (in μg/ml) of each quinolone. The reactions were stopped, and the DNA products were analyzed by electrophoresis in 1% agarose gels. R and SC denote relaxed and supercoiled pBR322 DNA, respectively.

Kongsoi *et al*. Fig. 1



Kongsoi *et al*. Fig. 2

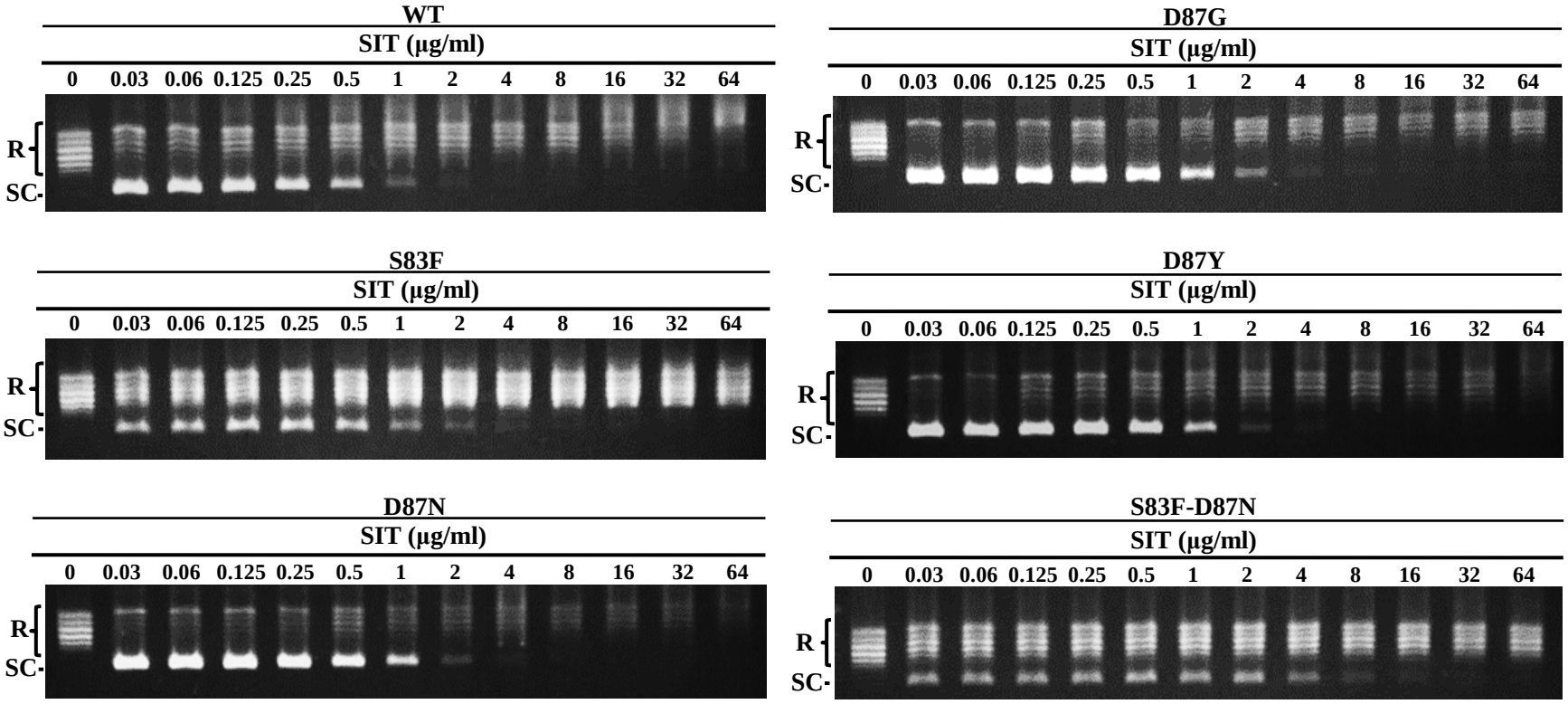




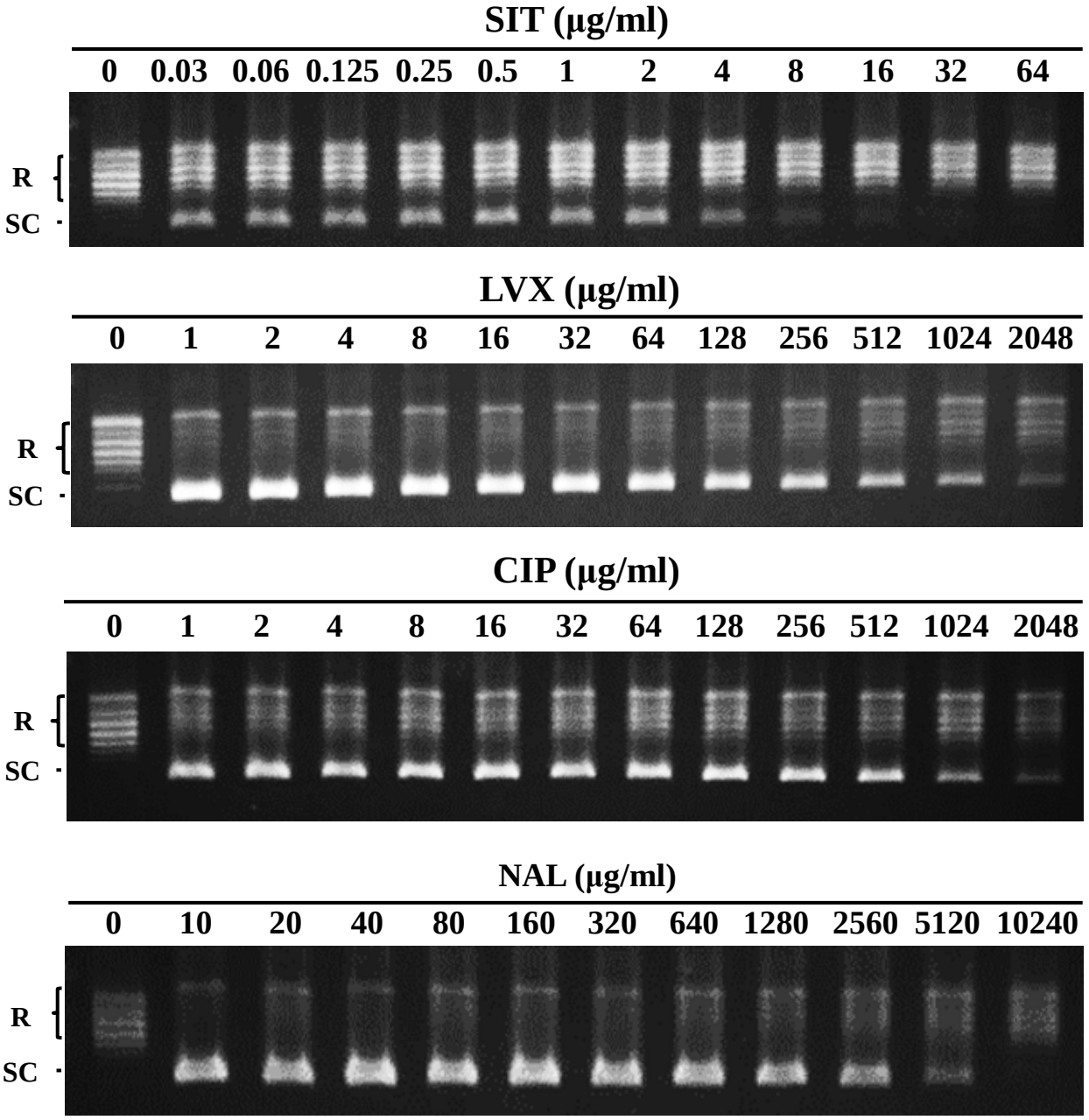
GyrA	-	+	+	-	+
GyrB	-	+	-	+	+
ATP	-	+	+	+	-
pBR322	+	+	+	+	+

GyrA	-	+	+	-	+
GyrB	-	+	-	+	+
ATP	-	+	+	+	-
pBR322	+	+	+	+	+

Kongsoi *et al*. Fig. 3



Kongsoi *et al*. Fig. 4



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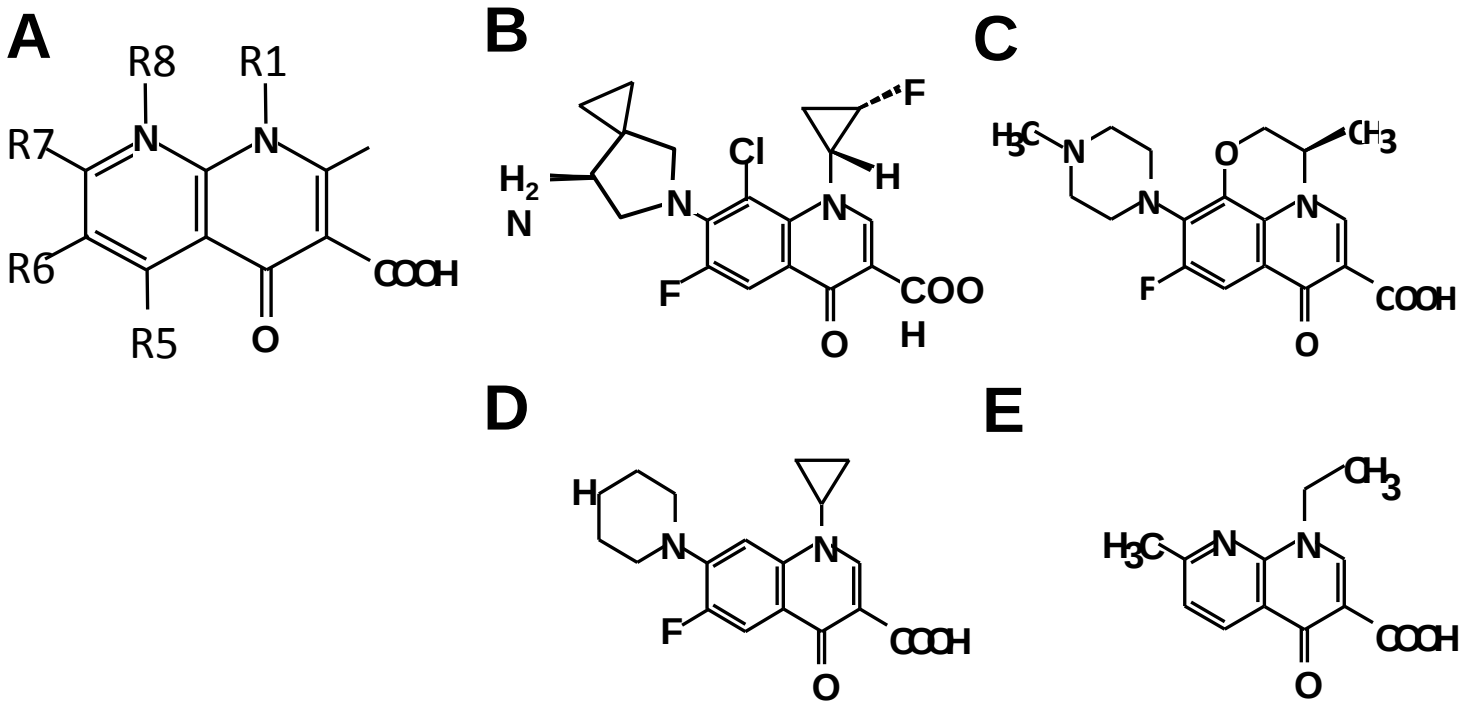

Quinolone	R-1	R-6	R-7	R-8	IC$_{50}$ (μg/ml)*					
					WT**	S83F	D87N	D87G	D87Y	S83F-D87N
SIT	fluorinated cyclopropyl	F	amino azaspiroheptane	Cl	0.2 ± 0.06	0.4 ± 0.15 (2.0)	0.5 ± 0.10 (2.5)	0.3 ± 0.06 (1.5)	0.3 ± 0.06 (1.5)	3.8 ± 0.89 (19.0)
LVX	bridge C1-C8	F	methylpiperazine	bridge C1-C8	0.4 ± 0.06	4.7 ± 0.90 (11.7)	2.7 ± 0.55 (6.7)	1.5 ± 0.10 (3.7)	1.4 ± 0.25 (3.5)	240 ± 7.4 (600.0)
CIP	cyclopropyl	F	piperazine	H	0.3 ± 0.00	1.7 ± 0.00 (5.6)	1.9 ± 0.35 (6.3)	1.7 ± 0.35 (5.6)	1.4 ± 0.46 (4.6)	440 ± 27 (1,460)
NAL	ethyl	H	methyl	N	53.5 ± 11	1580 ± 65 (29.5)	1590 ± 74 (29.7)	1380 ± 76 (25.7)	1380 ± 99 (25.7)	1610 ± 95 (30.0)

All mutant DNA gyrases of *S.* Typhimurium showed reduced susceptibility to all quinolones comparing to WT DNA gyrases. DNA gyrase with a double amino acid substitution in GyrA, GyrA-S83F-D87N, exhibited the lowest quinolone susceptibility. The effectiveness of sitafloxacin was shown by the low inhibitory concentration required for mutant DNA gyrases, including the DNA gyrase with GyrA-S83F-D87N.

TABLE 1 Oligonucleotide sequences of primers used in this study

Primer name	Sequence (Nucleotide position)[a]
GyrA_Nde_Fw	5'-ggcatatgagcgaccttgcgagaga-3' (1-20), *Nde* I site
GyrA_Xho_Rv	5'-ggctcgagctcgtcagcgtcatccgc-3' (2617-2634), *Xho* I site
GyrA_Ser83Phe_Fw	5'-acggcgatttcgcagtgt-3' (239-256)
GyrA_Ser83Phe_Rv	5'-acactgcgaaatcgccgt-3' (239-256)
GyrA_Asp87Asn_Fw	5'-cgcagtgtataacaccatcg-3' (249-268)
GyrA_Asp87Asn_Rv	5'-cgatggtgttatacactgcg-3' (249-268)
GyrA_Asp87Gly_Fw	5'-cgcagtgtatggcaccatcg-3' (249-268)
GyrA_Asp87Gly_Rv	5'-cgatggtgccatacactgcg-3' (249-268)
GyrA_Asp87Tyr_Fw	5'-cgcagtgtattacaccatcg-3' (249-268)
GyrA_Asp87Tyr_Rv	5'-cgatggtgtaatacactgcg-3' (249-268)
GyrA_Aat_Rv	5'-ggtcggcatgacgtccgg-3' (466-483), *Aat* II site
GyrB_Nde_Fw	5'-ggcatatgtcgaattcttatgactc-3' (1-20), *Nde* I site
GyrB_Xho_Rv	5'-ggctcgagaatatcgatattcgctgctttc-3' (2391-2412), *Xho* I site

[a]Restriction enzyme sites and mutated codons are underlined and double underlined, respectively.

TABLE 2. IC50 of quinolones against WT and mutant DNA gyrases

Quinolone[#]	R-1[$]	R-6[$]	R-7[$]	R-8[$]	IC_{50} (μg/ml)*					
					WT**	S83F	D87N	D87G	D87Y	S83F-D87N
SIT	fluorinated cyclopropyl	F	amino azaspiroheptane	Cl	0.2 ± 0.06	0.4 ± 0.15 (2.0)***	0.5 ± 0.10 (2.5)	0.3 ± 0.06 (1.5)	0.3 ± 0.06 (1.5)	3.8 ± 0.89 (19.0)
LVX	bridge C1-C8	F	methylpiperazine	bridge C1-C8	0.4 ± 0.06	4.7 ± 0.90 (11.7)	2.7 ± 0.55 (6.7)	1.5 ± 0.10 (3.7)	1.4 ± 0.25 (3.5)	240 ± 7.4 (600.0)
CIP	cyclopropyl	F	piperazine	H	0.3 ± 0.00	1.7 ± 0.00 (5.6)	1.9 ± 0.35 (6.3)	1.7 ± 0.35 (5.6)	1.4 ± 0.46 (4.6)	440 ± 27 (1,460)
NAL	ethyl	H	methyl	N	53.5 ± 11	1580 ± 65 (29.5)	1590 ± 74 (29.7)	1380 ± 76 (25.7)	1380 ± 99 (25.7)	1610 ± 95 (30.0)

*IC_{50}, drug concentration required to inhibit the supercoiling activity of DNA gyrase by 50%.

**WT, wild-type.

***(n), the value in bracket is n-fold increase to WT.

[#]Quinolones: SIT, sitafloxacin. LVX, levofloxacin. CIP, ciprofloxacin. NAL, nalidixic acid.

[$]Position of substituents on the structure of quinolo

X: N or C