Amino acid substitution Ser83Ile in GyrA of DNA gyrases confers high-level quinolone resistance to nontyphoidal *Salmonella* without loss of supercoiling activity

Running title: Ser83Ile confers high quinolone resistance

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Abstract

Aims: Quinolone-resistant nontyphoidal *Salmonella* having serine replaced by isoleucine at the 83rd. amino acid in GyrA (GyrA-Ser83Ile), has recently been found in Asian countries. Here, we aimed to examine the direct effect of substitution Ser83Ile on DNA gyrase activity and/or resistance to quinolones.

Materials and Methods: Using 50% of the maximal inhibitory concentrations (IC_{50}s) of quinolones, recombinant wild type (WT) and seven mutant DNA gyrases having amino acid substitutions, including Ser83Ile, were screened for enzymatic activity that causes supercoils in relaxed plasmid DNA and resistance to quinolones.

Results: Little differences in supercoiling activity were observed between WT and mutant DNA gyrases. By contrast, the IC_{50}s of ciprofloxacin and norfloxacin against GyrA-Ser83Ile/WT-GyrB were 11.6 and 73.3 μg/mL, respectively, which were the highest used against the DNA gyrases examined in the present study.

Conclusion: Ser83Ile in GyrA was shown to confer high-level quinolone resistance to DNA gyrases of nontyphoidal *Salmonella*, with no loss of supercoiling activity. *Salmonella* strain carrying GyrA with Ser83Ile may emerge under a high-concentration pressure of quinolones and easily spread even with no selection bias by quinolones. Hence, avoiding the overuse of quinolones is needed to prevent the spread of *Salmonella* with Ser83Ile in GyrA.

Keywords: Antimicrobial resistance, Nontyphoidal *Salmonella*, Quinolone, DNA gyrase

Introduction

Food illness caused by nontyphoidal *Salmonella* is a great public health threat worldwide[1]. Currently, more than 2,500 *Salmonella* serovars have been identified, with about half of them belonging to *Salmonella enterica subsp. enterica*, which cause the majority of *Salmonella* infections[2–4]. Among *Salmonella enterica*, *S.* Typhimurium and *S.* Enteritidis are the two epidemiologically most important serovars that infect humans[5]. In 2017 alone, global incidence of invasive nontyphoidal *Salmonella* was estimated at 535,000 cases [6]. Although most nontyphoidal *Salmonella* infections cause only uncomplicated gastroenteritis that hardly requires antimicrobial treatment, for cases presenting serious symptoms such as bacteremia and sepsis in immunocompromised individuals, ampicillin and trimethoprim-sulfamethoxazole are used as treatments [5,7]. When nontyphoidal *Salmonella* resistant to these drugs are identified, quinolones are used as the last resort medication[8]. As they are important antibacterial agents to treat both people and animals, many quinolones have been developed thus far. For example, ciprofloxacin has been used to treat *Salmonella* infections in humans and is listed as an essential medicine by the World Health Organization [9]. For animals, norfloxacin has been used not only as antibiotic but also as growth promoter, especially in Asian poultry farms[10–12]. In epidemiological studies, nalidixic acid is used as the standard antibiotic for screening quinolone-resistant bacteria such as *Salmonella* spp[13]. Unfortunately, in recent years, resistance to quinolones has been observed in *Salmonella* strains. In the last 20 years, a number of quinolone-resistant *Salmonella* isolates as well as the mechanisms by which quinolone resistance is acquired have been reported[14,15].

Quinolone resistance in *Salmonella* is believed to be initially caused by a single-point mutation in the gene-encoding subunits A of DNA gyrases[16,17]. A DNA gyrase consists of two subunits A and two subunits B encoded by *gyrA* and *gyrB*, respectively, with subunits GyrA and GyrB forming a functional tetrameric enyzme[18,19]. DNA gyrases introduce negative supercoils into the bacterial circular DNA in an ATP-dependent reaction. This mechanism is essential for DNA replication and transcription as it removes knots and tangles

from the bacterial chromosomes and maintains the structure of supercoiled DNA[18]. Quinolones inhibit DNA gyrase activity and exert bactericidal effects. In contrast, quinolone resistance is caused by occurrence of amino acid substitutions in DNA gyrases, which lowers the affinity with quinolones[19]. These mutations have been reported mostly at codons 83 and 87 in the amino acid sequence of GyrA in quinolone-resistant *Salmonella*. Amino acids at these positions are considered to be strongly involved in the binding to quinolones[20–22]. Although quinolone-resistant *Salmonella* isolates with amino acid substitutions at both positions 83 and 87 have been identified, these amino acid substitutions were not observed in the majority of the isolates[23]. This existing evidence seems to imply that not only occurrence of a single amino acid substitution may not compromise the survival of *Salmonella* in the environment, but also it may be enough to cause quinolone resistance.

Nontyphoidal *Salmonella* isolates with serine at the 83rd. amino acid of GyrA replaced by isoleucine (GyrA-Ser83Ile) has been found in Asian countries. For example, in China, Thailand and Malaysia, high-level, quinolone-resistant *Salmonella* Enteritidis strains with GyrA-Ser83Ile have been isolated from humans and animals[23–25]. Moreover, in China, Ser83Ile was found in *Salmonella* Derby, one of the most prevalent serovars in pork and poultry[26]. In addition, a possible clonal expansion of a *Salmonella* Enteritidis strain with Ser83Ile was also reported in Thailand[26]. However, no direct evidence of the contribution of amino acid substitutions to high-level quinolone resistance has been reported. Therefore, in the present study, we examined the effect of Ser83Ile in nontyphoidal *Salmonella* DNA gyrases on enzyme activity and quinolone resistance and compared it with those of other amino acid substitutions in GyrA.

Material and Methods

Reagents and quinolones used in the present study

Oligonucleotide primers were synthesized by Life Technologies (Carlsbad, CA). TaKaRa Mighty

Cloning Reagent Sets and a Ni-NTA agarose column were purchased from Life Technologies (Carlsbad, CA). Restriction enzymes and Lambda HindIII DNA size markers were purchased from New England Biolabs, Inc. (Ipswich, MA). Relaxed pBR322 DNA was purchased from John Innes Enterprises Ltd (Norwich, UK). Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Wako Pure Chemical Industries Ltd (Tokyo, Japan). *E. coli* BL21 (DE3) was purchased from Merck KGaA (Darmstadt, Germany).

For the inhibitory assay, three quinolones were used. Ciprofloxacin was purchased from LKT Laboratories Inc. (St Paul, MN). Norfloxacin and nalidixic acid were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Expression and purification of recombinant subunits of Salmonella Typhimurium

To evaluate the direct effect of amino acid substitutions on DNA gyrase activity, recombinant DNA gyrases were created using plasmid vectors. Plasmid vector pET-20b (Merck KGaA, Darmstadt, Germany) carrying wild type (WT) *gyrA* and *gyrB* of *S.* Typhimurium have been used in a previous study[27]. In addition to WT GyrA and GyrB, seven mutant GyrA were also produced by using the same expression vectors. As per a previously described method[28], the desired mutations were introduced by PCR using complementary primer sets containing the nucleotide substitutions and primers with restriction enzyme recognition sites. The nucleotide sequences of the primers are shown in Table 1. Single-base substitutions corresponding to amino acid substitutions Ser83Phe, Ser83Thr, Ser83Tyr, Asp87Asn, Asp87Gly and Asp87Tyr, were introduced into the WT *gyrA* in the plasmid vectors. To construct the plasmid vectors coding *gyrA* with two-base substitution causing mutation Ser83Ile, a second base substitution was introduced into the *gyrA* of Ser83Phe in the plasmid vectors. The nucleotide sequences of the gene were confirmed using a BigDye Terminator cycle sequencing kit version 3.1. An ABI Prism 3130xl Gene Analyzer (Thermo Fisher Scientific Inc., Waltham, Mass.) was used to confirm that no misconstruction occurred.

Recombinant GyrA and GyrB were separately produced using the constructed plasmids as previously described[29]. Briefly, plasmids were introduced into *E. coli* BL21 (DE3) (Merck KGaA) and the transformed *E. coli* was incubated at 37 °C in Luria-Bertani (LB) broth containing 100 μg/mL of ampicillin up to the log phase. The expression of the recombinant subunits was then induced by adding 1 mM IPTG, followed by further incubation. Different incubation periods and temperatures for the expression of GyrA and GyrB were used to improve the purity and yield. The recombinant *E. coli* carrying the GyrA expression plasmid was incubated for 40 h, and the temperature after the addition of IPTG was 16 °C. By contrast, recombinant *E. coli* containing GyrB was incubated at 18 °C for 13 h. Afterwards, the *E. coli* was centrifuged and sonicated to separate the expressed protein from cells. The recombinant DNA gyrase subunit was purified by Ni-NTA agarose column chromatography and dialyzed against a DNA gyrase dilution buffer (50 mM Tris-HCl, pH 7.5; 100 mM KCl, 2 mM DTT, 1 mM EDTA). Glycerol was added to the obtained protein and the mixture was stored at -80 °C until further use. The purity of the protein was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

DNA gyrase supercoiling activity assay

A time-course assay was carried out to differentiate the supercoiling activity caused by the amino acid substitutions. Purified GyrA and GyrB were mixed with relaxed pBR322 (Inspiralis Ltd., Norwich, UK) in gyrase assay solution [35 mM Tris-HCl, 6 mM $MgCl_2$, 1.8 mM spermidine, 24 mM KCl, 5 mM DTT, 0.36 mg/mL of BSA and 6.5% glycerol (w/v)]. The molar concentration of DNA gyrase and relaxed pBR322 was set at 18 nM and 1.5 nM, respectively (ratio of DNA gyrase to DNA: 12:1). The reaction mixture was incubated at 35 °C and the reaction was stopped by adding 8 μL of a stop solution (5% SDS, 25% glycerol and 0.25mg/mL of bromophenol blue) after 0, 15, 30, 45, 60, 75, 90, 105 and 120 min. Next, 10 μL from each time-point reaction mixture was loaded onto 1% agarose gels. Ten μL of Lambda HindIII was also

loaded onto the gels, and gel electrophoresis was run at 40 mA for 2 h. Gels were stained using 0.5xTBE buffer with 5 μg/mL of GelRed for 30 min. The supercoiled DNA bands were confirmed under an LED light. The supercoiled DNA was observed to be distinctly separated from the relaxed DNA because the supercoiled DNA had a more compact conformation and moved faster in the gel than the relaxed DNA. The brightness of the bands of supercoiled DNA and lambda *Hind* III were measured using ImageJ (http://rsbweb. nih.gov/ij). Since the lambda *Hind*III contains 6 DNA fragments and the mass of DNA in each of the 6 bands is known, a calibration curve was created by plotting the mass of DNA and the brightness of the band as a set. The amount of supercoiled DNA was then quantified based on the calibration curve. Each time-course assay was run in triplicate to eliminate experimental bias and to confirm reproducibility.

Supercoiling inhibitory assay using quinolones

The quinolone resistance of WT and mutant DNA gyrases was evaluated using the minimum concentration of quinolone needed to inhibit supercoiling activity. Quinolone inhibition of DNA gyrases was confirmed with gel electrophoresis and assessed by 50% of the maximal inhibitory concentrations (IC_{50}) of ciprofloxacin, norfloxacin and nalidixic acid. The procedure for this inhibition assay was same as that described above for the supercoiling activity assay, except that the quinolones were in the gyrase assay solutions. The concentrations of the quinolones in the mixtures ranged from 0.032 ($\approx 10^{-1.5}$) to 3,200 ($\approx 10^{3.5}$) μg/mL. Reaction mixtures containing different concentrations of quinolones were incubated for 1 hour and afterwards, analyzed with agarose gel electrophoresis. The brightness of supercoiled DNA was measured with imageJ and quantified by comparing it with that of pure quinolones, which were defined as 100% control. The supercoiling inhibitory assay was carried out in triplicate and the obtained data were plotted with the quinolone concentrations. Dose-response patterns from the supercoiling inhibitory assay were described by fitting a four-parameter logistic model. Thus, IC_{50} was determined after fitting the patterns into the model.

All statistical analysis was carried out using software R with the add-on package “drc”.

Results

Supercoiling activity of recombinant DNA gyrases

Expressed and purified recombinant WT and mutant DNA gyrase subunits GyrA and GyrB with molecular weight of 97-kDa and 89-kDa, respectively (Figure 1), were subjected to the time-course supercoiling activity assay. The results confirmed time-dependent supercoiling activity in all recombinant DNA gyrases (Figure 2A). The band intensity was measured, and the sequential change of each DNA gyrase activity was estimate. A uniform increase of supercoiled DNA was confirmed in all DNA gyrases but no clear differences between WT and mutant DNA gyrases were observed (Figure 2B).

Quinolone resistance of WT and mutant DNA gyrases

Unlike supercoiling activity, quinolone resistance of DNA gyrase was considerably affected by amino acid substitutions. The electrophoresis images obtained from the supercoiling inhibitory assay are shown in Figure 3A. When compared with mutant DNA gyrases, generation of supercoiled DNA bands by the WT DNA gyrase, which is quinolone susceptible, was inhibited at lower quinolone concentrations. In addition, supercoiled DNA bands of mutant DNA gyrases tended to differ from each other. The dose-response curves corresponding to the electrophoretic patterns are shown in Figure 3B, and the IC_{50}s calculated from the results are summarized in Table 2. For the dose-response curves of ciprofloxacin, the results showed that DNA gyrases consisting of GyrA with Ser83Ile and WT GyrB (GyrA-Ser83Ile/GyrB-WT) were on the right end (higher concentration) whereas WT and DNA gyrases with serine at position 83 substituted with threonine (GyrA-Ser83Thr/GyrB-WT) were on the left end (lower concentration). The IC_{50} of ciprofloxacin

against GyrA-Ser83Ile/GyrB-WT was 11.6 μg/mL (95% confidence interval (95%CI: 9.29 - 13.8), which was the highest ciprofloxacin concentration used against the DNA gyrases examined. In contrast, the lowest IC_{50} of ciprofloxacin was 0.185 μg/mL (95%CI: 0.167 – 0.212 μg/ml) against WT followed by 0.219 μg/mL (95%CI: 0.187 – 0.250 μg/ml) against GyrA-Ser83Thr/GyrB-WT. The third lowest IC_{50} of ciprofloxacin was that against DNA gyrase with serine at position 83 substituted with phenylalanine (GyrA-Ser83Phe/GyrB-WT; 0.917 μg/mL; 95%CI: 0.748 – 1.09 μg/mL). In the inhibitory assay using norfloxacin, the results were similar to those obtained for ciprofloxacin. For example, the highest and lowest IC_{50} of norfloxacin were 73.3 μg/mL (95%CI: 57.6 – 89.1 μg/mL) and 0.639 μg/mL (95%CI: 0.538 – 0.741 μg/mL) against GyrA-Ser83Ile/GyrB-WT and WT DNA gyrases, respectively. The IC_{50} of norfloxacin against GyrA-Ser83Thr (1.17 μg/mL, 95%CI: 0.989 – 1.35 μg/mL) was the second lowest. Most dose-response curves overlapped at higher concentrations at which two curves of WT and GyrA-Ser83Thr/GyrB-WT were farther left from other curves. The lowest IC_{50} of nalidixic acid was against GyrA-Ser83Thr/GyrB-WT (28.0 μg/mL, 95%CI: 22.3 – 33.7 μg/ml) followed by that against WT (66.3 μg/mL; 95%CI: 54.3 – 78.4 μg/mL). The IC_{50}s of nalidixic acid against the other mutants were higher than 1,000 μg/mL (Table 2).

Discussion

The emergence of foodborne diseases caused by quinolone-resistant nontyphoidal *Salmonella* has become one of the most significant global health concerns[28–30]. *Salmonella* becomes resistant to quinolones by acquiring an amino acid substitution in the DNA gyrase. Quinolone-resistant bacteria can be selected in the environment through quinolone concentration pressure, which can happen in both humans and animals. In the present study, quinolone resistance conferred by the amino acid substitution Ser83Ile in the DNA gyrase of *Salmonella* was examined using wild-type and mutant recombinant DNA gyrases.

The enzyme activity of DNA gyrases is considered to be highly related to bacterial growth because

DNA gyrases play a key role in bacterial replication and transcription[18,19]. In the present work, none of the wild-type and mutant recombinant DNA gyrases showed significant differences in supercoiling activity, regardless of the amino acid substitution. The enzyme activity of DNA gyrases can be estimated not only by the quantity but also by the quality of the supercoiling activity. The DNA gyrase binds to bacterial chromosomes to exert the supercoiling activity. The closer the position of the amino acid substitution that confers quinolone resistance is to the active site where DNA is bound[33], the higher is the binding affinity of the DNA gyrase with the bacterial chromosome[34–36]. Thus, the gene expression is differentially affected by the binding affinity with the chromosome[37]. The typical size of the nontyphoidal *Salmonella* chromosome is about 4.8 Mbp, which was 1,000-fold larger than the relaxed plasmid pBR322 (4,361 bp) used in the present study as the enzyme substrate. Hence, the topology of the nontyphoidal *Salmonella* chromosome is much more complex than pBR322, and therefore, it was difficult to estimate differences in the effect of the amino acid substitutions on the chromosome DNA topology using only the results obtained from the present work. Thus, further investigation is deemed necessary to elucidate the effect of the amino acid substitutions on the binding of DNA gyrases to nontyphoidal *Salmonella* chromosomes. In future studies, we plan to analyze the gene expression in the bacterial cells.

Although the supercoiling activity of DNA gyrases was not affected by the amino acid substitutions, quinolone resistance was greatly changed (Fig. 3). Increased IC_{50}s of quinolones against mutant DNA gyrases compared with those used against WT gyrases indicated that all tested amino acid substitutions on GyrA conferred quinolone resistance to DNA gyrase[38]. Quinolones bind to DNA gyrase and inhibit its enzymatic activity. Previous studies have shown that serine and acidic amino acids at positions 83 and 87 of GyrA, respectively, work as anchor points for DNA gyrase at this binding mechanism, forming a metal-water ion-mediated bridge between the quinolone and DNA gyrase[22,39,41]. Thus, amino acid substitutions at these positions weaken the binding of quinolones to DNA gyrase. The 87th amino acid of GyrA was aspartic acid,

which is an acidic amino acid, and the mutated amino acid was neutral or non-polar; the increase in IC_{50} for the mutant DNA gyrase with the amino acid substitution at position 87 was most likely due to a change in the property of amino acid. The effect of amino acid substitution at position 83 is also similar. The result that the IC_{50} for DNA gyrase with Ser83Thr was closer to that of WT than other mutants may be due to the fact that serine and threonine are similar properties (both neutral amino acids with similar molecular weights). On the other hand, since isoleucine is a nonpolar amino acid, the increase in IC_{50} for DNA gyrase with Ser83Ile was significant.

Ser83Ile showed the greatest effect of the amino acid substitutions on quinolone resistance (Table 2). The IC_{50}s of ciprofloxacin and norfloxacin against DNA gyrases with Ser83Ile were the highest of all used against DNA gyrases examined in the present study. These results showed that Ser83Ile was able to confer the highest quinolone resistance to *Salmonella*. These observations are in agreement with our previous report on the minimum concentrations of quinolones needed against clinical strains of *Salmonella* Enteritidis with Ser83Ile in GyrA, which were compared with those needed against isolates with different single amino acid substitutions[25].

In the present work, the amino acid substitution Ser83Phe was also proved to confer quinolone resistance to DNA gyrases, although the resistance to ciprofloxacin was lower than those conferred by other amino acid substitutions, except for Ser83Thr. Indeed, the DNA gyrase with Ser83Thr showed a lower resistance to norfloxacin than other amino acid substitutions, and susceptibility to ciprofloxacin. Furthermore, DNA gyrases with Ser83Thr were more susceptible to nalidixic acid than WT. Highly quinolone-resistant *Salmonella* isolates with GyrA-Ser83Ile have likely derived from WT *Salmonella* through at least two mutations, via either the pathway TCC-TTC-ATC or TCC-ACC-ATC. The first mutation pathway bears Ser83Phe, not Ser83Ile, which confers to *Salmonella* DNA gyrase a relatively lower resistance to quinolones, as shown in the present study. The second mutation pathway bears Ser83Thr, which does not confer quinolone

252 resistance. Elsewhere, *Salmonella* isolates with both Ser83Phe and Ser83Ile have been reported[24,26]. A
253 plausible explanation for this phenomenon may be that *Salmonella* strains carrying GyrA-Ser83Phe with low
254 resistance to quinolones were exposed to high concentrations of the drugs and as a result, only strains carrying
255 GyrA-Ser83Ile got selected.

256 Even if the use of quinolones is abruptly halted worldwide, it is likely that high-level quinolone
257 resistant *Salmonella* with Ser83Ile will remain in the environment, as it has happened with *Salmonella* with
258 other amino acid substitutions. The results from the present study showed that Ser83Ile in GyrA had no
259 adverse effect on the supercoiling activity of DNA gyrase. In addition, a previous study showed that
260 *Salmonella* Enteritidis with Ser83Ile had been isolated from human patients over several years[26]. Amino acid
261 substitution Ser83Ile in GyrA has also been detected in *E. coli* and *Klebsiella pneumonia*[34,35]. The nucleotide
262 sequence of serine at the codon 83 in GyrA of these bacteria is TCC, which is the same as that of *Salmonella*.
263 Thus, *E. coli* and *Klebsiella pneumoniae* also require a two-base substitution to change to ATC, as *Salmonella*
264 does. As mentioned above, it is likely that high-level quinolone resistant bacteria with Ser83Ile will remain
265 in the environment. Although it has been reported that *Salmonella* Enteritidis and Derby with Ser83Ile have
266 been observed only in limited areas[23–25], due to the presence of the amino acid substitution Ser83Ile, every
267 nontyphoidal *Salmonella* has the potential to become a high-level quinolone resistant phenotype. Therefore,
268 it should be only a matter of time before *Salmonella* with amino acid substitution Ser83Ile is isolated in
269 broader areas worldwide.

270 In conclusion, recently reported amino acid substitution Ser83Ile in GyrA was shown to confer
271 high-level quinolone resistance to DNA gyrases of nontyphoidal *Salmonella*, with no loss of supercoiling
272 activity. *Salmonella* strains carrying GyrA with Ser83Ile may emerge under a high concentration pressure of
273 quinolones and spread easily even with no selection bias by quinolones. Hence, avoiding the overuse of
274 quinolones is needed to prevent the spread of *Salmonella* with Ser83Ile in GyrA.

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Author Disclosure Statement

The authors declare that there are no conflicts of interest.

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390 Table 1. Oligonucleotide sequences of primer sets

391

Primer		Sequence (nucleotide position on *gyrA*)	Reference
Amino acid substitution			
Ser83Ile	Fw	5'-acggcgat**atc**gcagtgt-3' (239-256)	This study
	Rv	5'-acactgc**gat**atcgccgt-3' (239-256)	This study
Ser83Phe	Fw	5'-acggcgat**ttc**gcagtgt-3' (239-256)	28
	Rv	5'-acactgc**gaa**atcgccgt-3' (239-256)	28
Ser83Thr	Fw	5'-acggcgat**acc**gcagtgt-3' (239-256)	This study
	Rv	5'-acactgc**ggt**atcgccgt-3' (239-256)	This study
Ser83Tyr	Fw	5'-acggcgat**tac**gcagtgt-3' (239-256)	This study
	Rv	5'-acactgc**gta**atcgccgt-3' (239-256)	This study
Asp87Asn	Fw	5'-cgcagtgtat**aac**accatcg-3' (249-268)	28
	Rv	5'-cgatggt**gtt**atacactgcg-3' (249-268)	28
Asp87Gly	Fw	5'-cgcagtgtat**ggc**accatcg-3' (249-268)	28
	Rv	5'-cgatggt**gcc**atacactgcg-3' (249-268)	28
Asp87Tyr	Fw	5'-cgcagtgtat**tac**accatcg-3' (249-268)	28
	Rv	5'-cgatggt**gta**atacactgcg-3' (249-268)	28
Restriction enzyme region			
NdeI	Fw	5'-gg**catatg**agcgaccttgcgagaga-3' (1-20)	28
XhoI	Rv	5'-gg**ctcgag**ctcgtcagcgtcatccgc-3' (2617-2634)	28
AatII	Rv	5'-ggtcggcat**gacgtc**cgg-3' (466-483)	28

392 Mutated codons and restriction enzyme sites are shown in bold.

393 All oligonucleotide primers were synthesized by Life Technologies.

394

Table 2. IC_{50}s of ciprofloxacin, norfloxacin and nalidixic acid against WT and mutant DNA gyrases

GyrA	IC_{50} [95% Confidence Interval] (μg/ml)					
	Ciprofloxacin		Norfloxacin		Nalidixic acid	
WT	0.185	[0.167 – 0.212]	0.639	[0.538 – 0.741]	66.3	[54.3 – 78.4]
Ser83Ile	11.6	[9.29 – 13.8]	73.3	[57.6 – 89.1]	1680	[1430 - 1930]
Ser83Phe	0.917	[0.748 – 1.09]	7.90	[6.59 – 9.20]	2860	[1740 - 3990]
Ser83Thr	0.219	[0.187 – 0.250]	1.17	[0.989 – 1.35]	28.0	[22.3 – 33.7]
Ser83Tyr	5.29	[4.50 – 6.08]	50.1	[41.1 – 59.2]	1190	[1000 - 1380]
Asp87Asn	4.99	[4.23 – 5.74]	43.1	[34.5 – 51.6]	1150	[942 - 1360]
Asp87Gly	1.91	[1.66 – 2.15]	8.93	[7.62 – 10.2]	3040	[1790 - 4280]
Asp87Tyr	3.02	[2.58 – 3.46]	5.50	[4.54 – 6.46]	1260	[923 - 1610]

Purified GyrA (wild type and mutants), GyrB (wild type) and relaxed pBR322 were mixed in gyrase assay solution [35 mM Tris-HCl, 6 mM MgCl2, 1.8 mM spermidine, 24 mM KCl, 5 mM DTT, 0.36 mg/mL of BSA and 6.5% glycerol (w/v)] at the concentration of 18, 18 and 1.5 nM, respectively, with different concentrations of quinolones and incubated for 1 hour. DNAs in the reaction mixture were analyzed with agarose gel electrophoresis and the brightness of supercoiled DNA was measured with imageJ to access half maximal inhibitory concentrations (IC_{50}) of each quinolone.

Figure legends

Figure 1. SDS-PAGE analysis of purified DNA gyrase subunits.

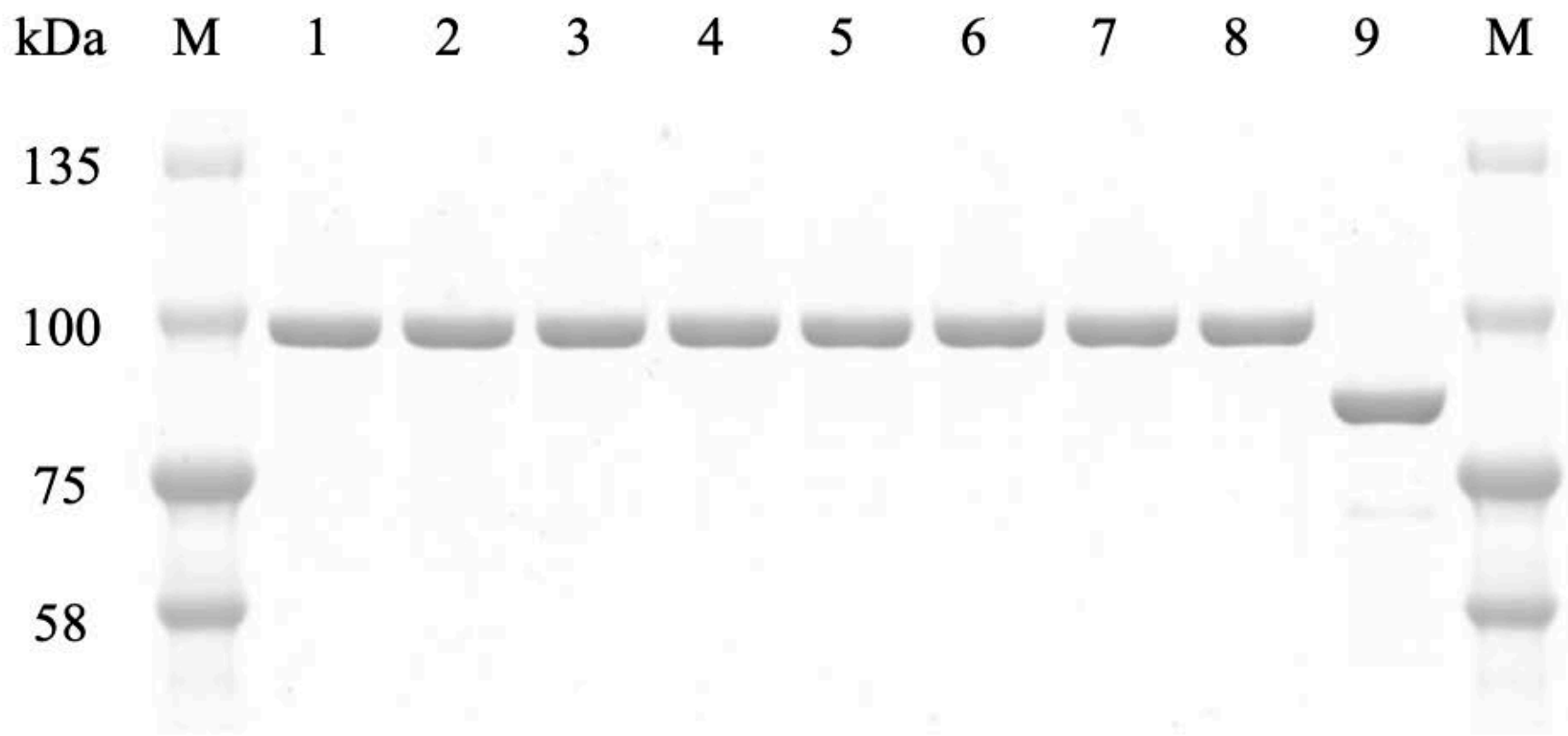
Three hundred ng of each purified protein was loaded onto a 5-20% gradient polyacrylamide gel. High-degree purification of GyrA and GyrB at 97 and 89 kDa, respectively, was confirmed. Lanes: M, protein marker; 1, WT-GyrA; 2, Ser83Ile-GyrA; 3, Ser83Phe-GyrA; 4, Ser83Thr-GyrA; 5, Ser83Tyr-GyrA; 6, Asp87Asn-GyrA; 7, Asp87Gly-GyrA; 8, Asp87Tyr-GyrA; 9, WT-GyrB.

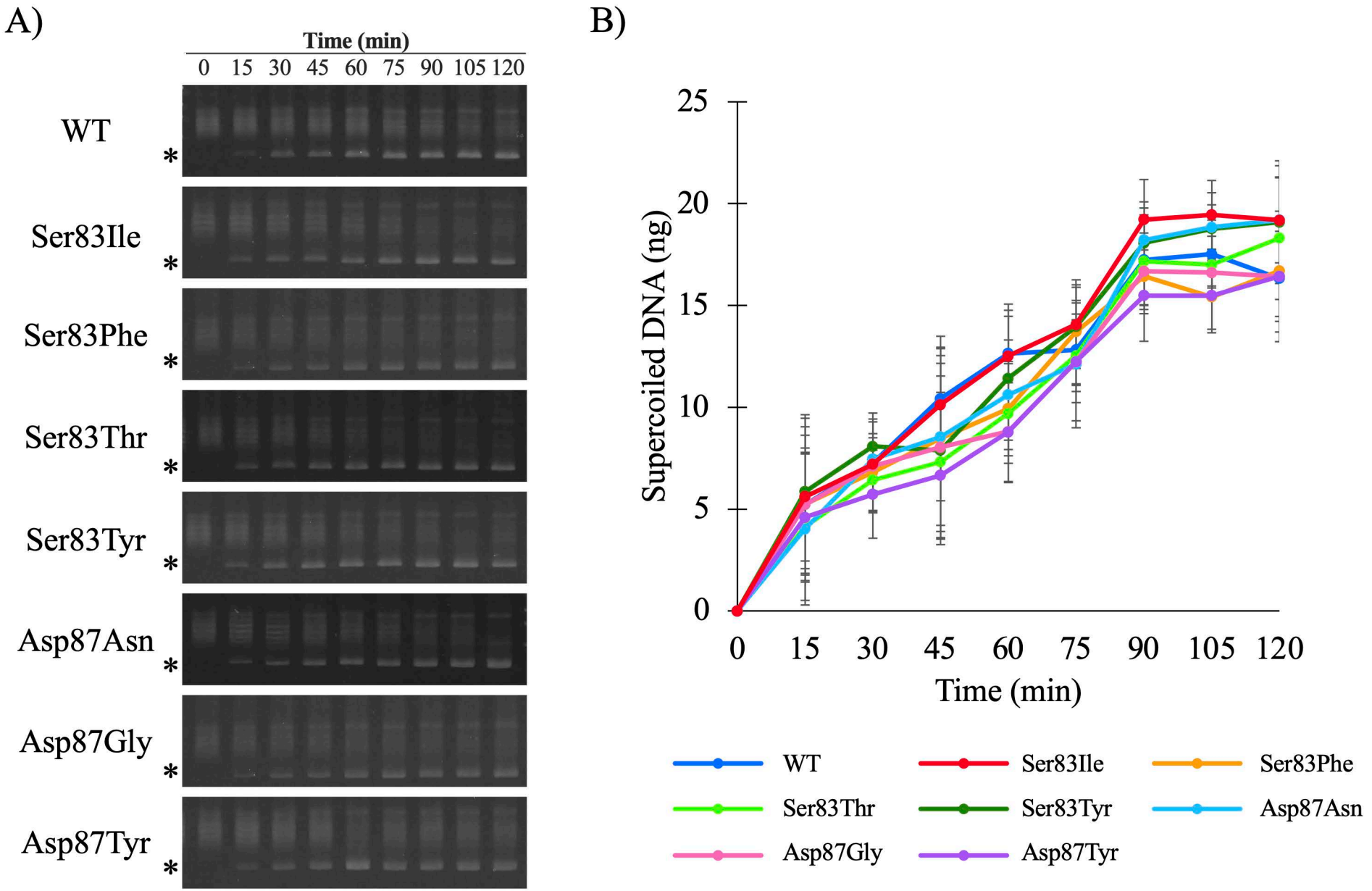
Figure 2. Supercoiling activity of WT and mutant DNA gyrases.

A) The electrophoretic images of supercoiled DNA bands by DNA gyrase. The asterisk (*) on the right of the electrophoresis image indicate the positions of supercoiled DNA. B) Increases in the amounts of supercoiled DNA were measured for 120 min.

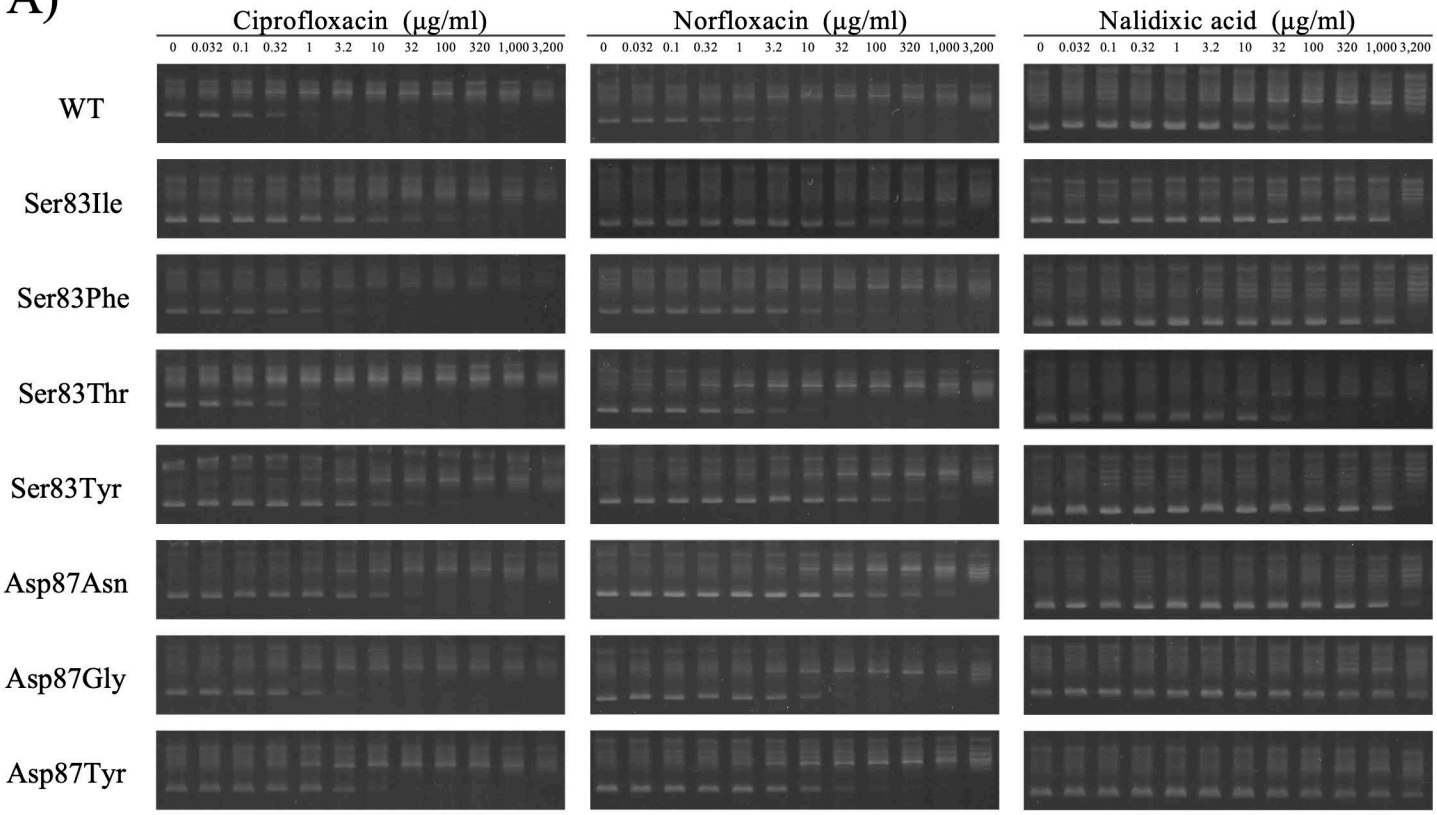
Figure 3. Supercoiling inhibitory assay.

A) Electrophoretic image measuring the quinolone resistance by amino acid substitution. B) Dose-response relationships of ciprofloxacin, norfloxacin and nalidixic acid are shown. One hundred percent of supercoiling activity was defined as the supercoiling activity in quinolone-free samples. Error bars indicate 95% confidence intervals.

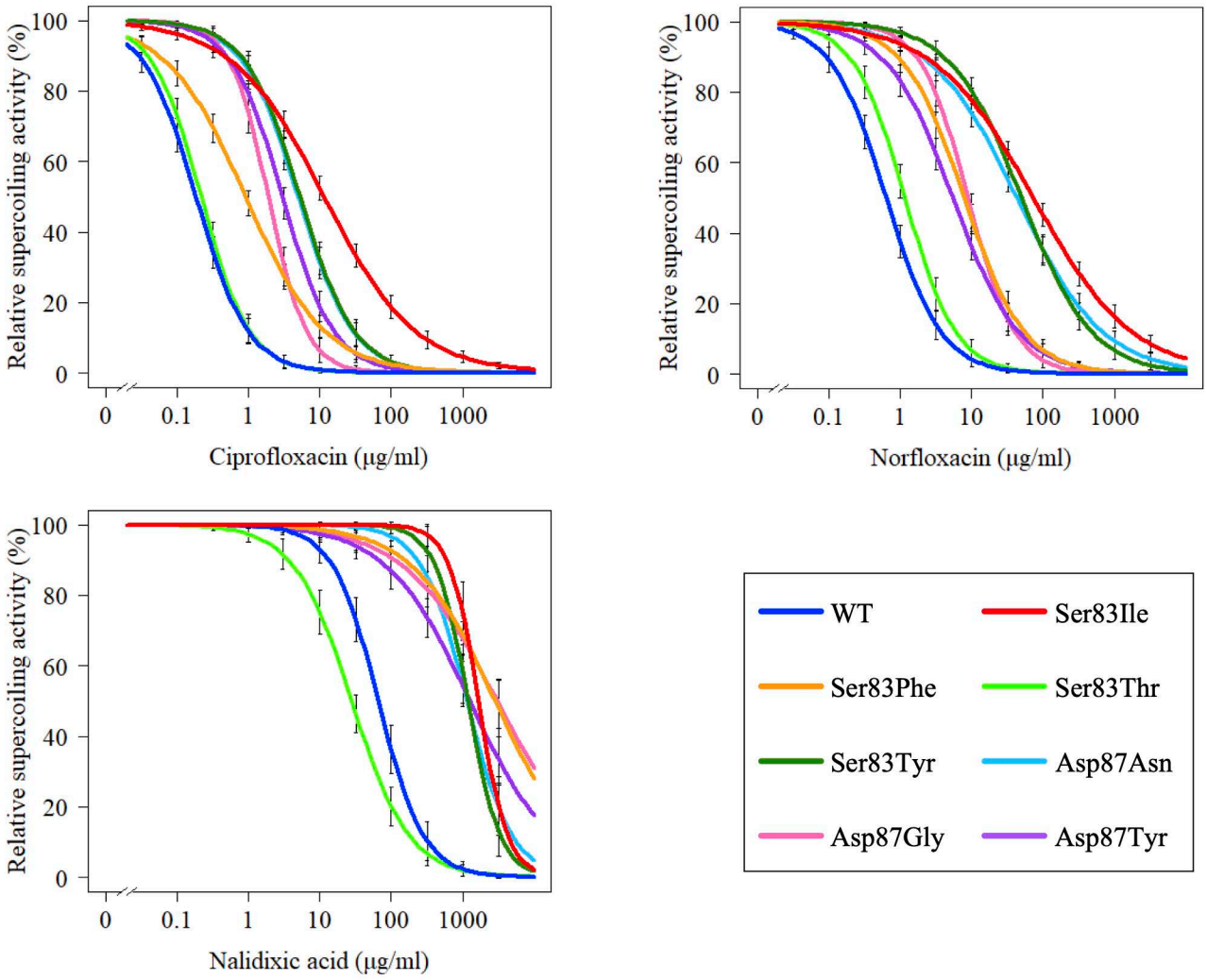

kDa
M
1
2
3
4
5
6
7
8
9
M
135
100
75
58


A)
Time (min)
0 15 30 45 60 75 90 105 120
WT
Ser83Ile
Ser83Phe
Ser83Thr
Ser83Tyr
Asp87Asn
Asp87Gly
Asp87Tyr
*
B)
Supercoiled DNA (ng)
0 5 10 15 20 25
0 15 30 45 60 75 90 105 120
Time (min)
WT
Ser83Ile
Ser83Phe
Ser83Thr
Ser83Tyr
Asp87Asn
Asp87Gly
Asp87Tyr

A)


Ciprofloxacin (μg/ml)
Norfloxacin (μg/ml)
Nalidixic acid (μg/ml)
0 0.032 0.1 0.32 1 3.2 10 32 100 320 1,000 3,200
WT
Ser83Ile
Ser83Phe
Ser83Thr
Ser83Tyr
Asp87Asn
Asp87Gly
Asp87Tyr

B)


Relative supercoiling activity (%)
Ciprofloxacin (μg/ml)
Norfloxacin (μg/ml)
Nalidixic acid (μg/ml)
0 0.1 1 10 100 1000
0 20 40 60 80 100
WT
Ser83Ile
Ser83Phe
Ser83Thr
Ser83Tyr
Asp87Asn
Asp87Gly
Asp87Tyr