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

3 Running title: Ser83Ile confers high quinolone resistance

4

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24

25 **Abstract**

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

29 **Materials and Methods:** Using 50% of the maximal inhibitory concentrations (IC₅₀s) of quinolones,
30 recombinant wild type (WT) and seven mutant DNA gyrases having amino acid substitutions, including
31 Ser83Ile, were screened for enzymatic activity that causes supercoils in relaxed plasmid DNA and resistance
32 to quinolones.

33 **Results:** Little differences in supercoiling activity were observed between WT and mutant DNA gyrases. By
34 contrast, the IC₅₀s of ciprofloxacin and norfloxacin against GyrA-Ser83Ile/WT-GyrB were 11.6 and 73.3
35 µg/mL, respectively, which were the highest used against the DNA gyrases examined in the present study.

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

41

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

43

44

45 **Introduction**

46 Food illness caused by nontyphoidal *Salmonella* is a great public health threat worldwide¹.
47 Currently, more than 2,500 *Salmonella* serovars have been identified, with about half of them belonging to
48 *Salmonella enterica subsp. enterica*, which cause the majority of *Salmonella* infections²⁻⁴. Among
49 *Salmonella enterica*, *S. Typhimurium* and *S. Enteritidis* are the two epidemiologically most important
50 serovars that infect humans⁵. In 2017 alone, global incidence of invasive nontyphoidal *Salmonella* was
51 estimated at 535,000 cases⁶. Although most nontyphoidal *Salmonella* infections cause only uncomplicated
52 gastroenteritis that hardly requires antimicrobial treatment, for cases presenting serious symptoms such as
53 bacteremia and sepsis in immunocompromised individuals, ampicillin and trimethoprim-sulfamethoxazole
54 are used as treatments^{5,7}. When nontyphoidal *Salmonella* resistant to these drugs are identified, quinolones
55 are used as the last resort medication⁸. As they are important antibacterial agents to treat both people and
56 animals, many quinolones have been developed thus far. For example, ciprofloxacin has been used to treat
57 *Salmonella* infections in humans and is listed as an essential medicine by the World Health Organization⁹.
58 For animals, norfloxacin has been used not only as antibiotic but also as growth promoter, especially in Asian
59 poultry farms¹⁰⁻¹². In epidemiological studies, nalidixic acid is used as the standard antibiotic for screening
60 quinolone-resistant bacteria such as *Salmonella* spp¹³. Unfortunately, in recent years, resistance to quinolones
61 has been observed in *Salmonella* strains. In the last 20 years, a number of quinolone-resistant *Salmonella*
62 isolates as well as the mechanisms by which quinolone resistance is acquired have been reported^{14,15}.

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

68 from the bacterial chromosomes and maintains the structure of supercoiled DNA¹⁸. Quinolones inhibit DNA
69 gyrase activity and exert bactericidal effects. In contrast, quinolone resistance is caused by occurrence of
70 amino acid substitutions in DNA gyrases, which lowers the affinity with quinolones¹⁹. These mutations have
71 been reported mostly at codons 83 and 87 in the amino acid sequence of GyrA in quinolone-resistant
72 *Salmonella*. Amino acids at these positions are considered to be strongly involved in the binding to
73 quinolones²⁰⁻²². Although quinolone-resistant *Salmonella* isolates with amino acid substitutions at both
74 positions 83 and 87 have been identified, these amino acid substitutions were not observed in the majority of
75 the isolates²³. This existing evidence seems to imply that not only occurrence of a single amino acid
76 substitution may not compromise the survival of *Salmonella* in the environment, but also it may be enough
77 to cause quinolone resistance.

78 Nontyphoidal *Salmonella* isolates with serine at the 83rd. amino acid of GyrA replaced by
79 isoleucine (GyrA-Ser83Ile) has been found in Asian countries. For example, in China, Thailand and Malaysia,
80 high-level, quinolone-resistant *Salmonella* Enteritidis strains with GyrA-Ser83Ile have been isolated from
81 humans and animals²³⁻²⁵. Moreover, in China, Ser83Ile was found in *Salmonella* Derby, one of the most
82 prevalent serovars in pork and poultry²⁶. In addition, a possible clonal expansion of a *Salmonella* Enteritidis
83 strain with Ser83Ile was also reported in Thailand²⁶. However, no direct evidence of the contribution of amino
84 acid substitutions to high-level quinolone resistance has been reported. Therefore, in the present study, we
85 examined the effect of Ser83Ile in nontyphoidal *Salmonella* DNA gyrases on enzyme activity and quinolone
86 resistance and compared it with those of other amino acid substitutions in GyrA.

87

88 **Material and Methods**

89 *Reagents and quinolones used in the present study*

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

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

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

99

100 *Expression and purification of recombinant subunits of Salmonella Typhimurium*

101 To evaluate the direct effect of amino acid substitutions on DNA gyrase activity, recombinant DNA
102 gyrases were created using plasmid vectors. Plasmid vector pET-20b (Merck KGaA, Darmstadt, Germany)
103 carrying wild type (WT) *gyrA* and *gyrB* of *S. Typhimurium* have been used in a previous study²⁷. In addition
104 to WT GyrA and GyrB, seven mutant GyrA were also produced by using the same expression vectors. As per
105 a previously described method²⁸, the desired mutations were introduced by PCR using complementary primer
106 sets containing the nucleotide substitutions and primers with restriction enzyme recognition sites. The
107 nucleotide sequences of the primers are shown in Table 1. Single-base substitutions corresponding to amino
108 acid substitutions Ser83Phe, Ser83Thr, Ser83Tyr, Asp87Asn, Asp87Gly and Asp87Tyr, were introduced into
109 the WT *gyrA* in the plasmid vectors. To construct the plasmid vectors coding *gyrA* with two-base substitution
110 causing mutation Ser83Ile, a second base substitution was introduced into the *gyrA* of Ser83Phe in the
111 plasmid vectors. The nucleotide sequences of the gene were confirmed using a BigDye Terminator cycle
112 sequencing kit version 3.1. An ABI Prism 3130xl Gene Analyzer (Thermo Fisher Scientific Inc., Waltham,
113 Mass.) was used to confirm that no misconstruction occurred.

114 Recombinant GyrA and GyrB were separately produced using the constructed plasmids as
115 previously described²⁹. Briefly, plasmids were introduced into *E. coli* BL21 (DE3) (Merck KGaA) and the
116 transformed *E. coli* was incubated at 37 °C in Luria-Bertani (LB) broth containing 100 µg/mL of ampicillin
117 up to the log phase. The expression of the recombinant subunits was then induced by adding 1 mM IPTG,
118 followed by further incubation. Different incubation periods and temperatures for the expression of GyrA
119 and GyrB were used to improve the purity and yield. The recombinant *E. coli* carrying the GyrA expression
120 plasmid was incubated for 40 h, and the temperature after the addition of IPTG was 16 °C. By contrast,
121 recombinant *E. coli* containing GyrB was incubated at 18 °C for 13 h. Afterwards, the *E. coli* was centrifuged
122 and sonicated to separate the expressed protein from cells. The recombinant DNA gyrase subunit was purified
123 by Ni-NTA agarose column chromatography and dialyzed against a DNA gyrase dilution buffer (50 mM Tris-
124 HCl, pH 7.5; 100 mM KCl, 2 mM DTT, 1 mM EDTA). Glycerol was added to the obtained protein and the
125 mixture was stored at -80 °C until further use. The purity of the protein was confirmed by sodium dodecyl
126 sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

127

128 *DNA gyrase supercoiling activity assay*

129 A time-course assay was carried out to differentiate the supercoiling activity caused by the amino
130 acid substitutions. Purified GyrA and GyrB were mixed with relaxed pBR322 (Inspiralis Ltd., Norwich, UK)
131 in gyrase assay solution [35 mM Tris-HCl, 6 mM MgCl₂, 1.8 mM spermidine, 24 mM KCl, 5 mM DTT, 0.36
132 mg/mL of BSA and 6.5% glycerol (w/v)]. The molar concentration of DNA gyrase and relaxed pBR322 was
133 set at 18 nM and 1.5 nM, respectively (ratio of DNA gyrase to DNA: 12:1). The reaction mixture was
134 incubated at 35 °C and the reaction was stopped by adding 8 µL of a stop solution (5% SDS, 25% glycerol
135 and 0.25mg/mL of bromophenol blue) after 0, 15, 30, 45, 60, 75, 90, 105 and 120 min. Next, 10 µL from
136 each time-point reaction mixture was loaded onto 1% agarose gels. Ten µL of Lambda HindIII was also

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

146

147 *Supercoiling inhibitory assay using quinolones*

148 The quinolone resistance of WT and mutant DNA gyrases was evaluated using the minimum
149 concentration of quinolone needed to inhibit supercoiling activity. Quinolone inhibition of DNA gyrases was
150 confirmed with gel electrophoresis and assessed by 50% of the maximal inhibitory concentrations (IC₅₀) of
151 ciprofloxacin, norfloxacin and nalidixic acid. The procedure for this inhibition assay was same as that
152 described above for the supercoiling activity assay, except that the quinolones were in the gyrase assay
153 solutions. The concentrations of the quinolones in the mixtures ranged from 0.032 ($\approx 10^{-1.5}$) to 3,200 ($\approx 10^{3.5}$)
154 µg/mL. Reaction mixtures containing different concentrations of quinolones were incubated for 1 hour and
155 afterwards, analyzed with agarose gel electrophoresis. The brightness of supercoiled DNA was measured
156 with imageJ and quantified by comparing it with that of pure quinolones, which were defined as 100% control.
157 The supercoiling inhibitory assay was carried out in triplicate and the obtained data were plotted with the
158 quinolone concentrations. Dose-response patterns from the supercoiling inhibitory assay were described by
159 fitting a four-parameter logistic model. Thus, IC₅₀ was determined after fitting the patterns into the model.

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

161

162

163 **Results**

164 *Supercoiling activity of recombinant DNA gyrases*

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

171

172 *Quinolone resistance of WT and mutant DNA gyrases*

173 Unlike supercoiling activity, quinolone resistance of DNA gyrase was considerably affected by
174 amino acid substitutions. The electrophoresis images obtained from the supercoiling inhibitory assay are
175 shown in Figure 3A. When compared with mutant DNA gyrases, generation of supercoiled DNA bands by
176 the WT DNA gyrase, which is quinolone susceptible, was inhibited at lower quinolone concentrations. In
177 addition, supercoiled DNA bands of mutant DNA gyrases tended to differ from each other. The dose-response
178 curves corresponding to the electrophoretic patterns are shown in Figure 3B, and the IC₅₀s calculated from
179 the results are summarized in Table 2. For the dose-response curves of ciprofloxacin, the results showed that
180 DNA gyrases consisting of GyrA with Ser83Ile and WT GyrB (GyrA-Ser83Ile/GyrB-WT) were on the right
181 end (higher concentration) whereas WT and DNA gyrases with serine at position 83 substituted with
182 threonine (GyrA-Ser83Thr/GyrB-WT) were on the left end (lower concentration). The IC₅₀ of ciprofloxacin

183 against GyrA-Ser83Ile/GyrB-WT was 11.6 µg/mL (95% confidence interval (95%CI: 9.29 - 13.8), which
184 was the highest ciprofloxacin concentration used against the DNA gyrases examined. In contrast, the lowest
185 IC₅₀ of ciprofloxacin was 0.185 µg/mL (95%CI: 0.167 – 0.212 µg/ml) against WT followed by 0.219 µg/mL
186 (95%CI: 0.187 – 0.250 µg/ml) against GyrA-Ser83Thr/GyrB-WT. The third lowest IC₅₀ of ciprofloxacin was
187 that against DNA gyrase with serine at position 83 substituted with phenylalanine (GyrA-Ser83Phe/GyrB-
188 WT; 0.917 µg/mL; 95%CI: 0.748 – 1.09 µg/mL). In the inhibitory assay using norfloxacin, the results were
189 similar to those obtained for ciprofloxacin. For example, the highest and lowest IC₅₀ of norfloxacin were 73.3
190 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-
191 Ser83Ile/GyrB-WT and WT DNA gyrases, respectively. The IC₅₀ of norfloxacin against GyrA-Ser83Thr
192 (1.17 µg/mL, 95%CI: 0.989 – 1.35 µg/mL) was the second lowest. Most dose-response curves overlapped at
193 higher concentrations at which two curves of WT and GyrA-Ser83Thr/GyrB-WT were farther left from other
194 curves. The lowest IC₅₀ of nalidixic acid was against GyrA-Ser83Thr/GyrB-WT (28.0 µg/mL, 95%CI: 22.3
195 – 33.7 µg/ml) followed by that against WT (66.3 µg/mL; 95%CI: 54.3 – 78.4 µg/mL). The IC₅₀s of nalidixic
196 acid against the other mutants were higher than 1,000 µg/mL (Table 2).

197

198 **Discussion**

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

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

206 DNA gyrases play a key role in bacterial replication and transcription^{18,19}. In the present work, none of the
207 wild-type and mutant recombinant DNA gyrases showed significant differences in supercoiling activity,
208 regardless of the amino acid substitution. The enzyme activity of DNA gyrases can be estimated not only by
209 the quantity but also by the quality of the supercoiling activity. The DNA gyrase binds to bacterial
210 chromosomes to exert the supercoiling activity. The closer the position of the amino acid substitution that
211 confers quinolone resistance is to the active site where DNA is bound³³, the higher is the binding affinity of
212 the DNA gyrase with the bacterial chromosome³⁴⁻³⁶. Thus, the gene expression is differentially affected by
213 the binding affinity with the chromosome³⁷. The typical size of the nontyphoidal *Salmonella* chromosome is
214 about 4.8 Mbp, which was 1,000-fold larger than the relaxed plasmid pBR322 (4,361 bp) used in the present
215 study as the enzyme substrate. Hence, the topology of the nontyphoidal *Salmonella* chromosome is much
216 more complex than pBR322, and therefore, it was difficult to estimate differences in the effect of the amino
217 acid substitutions on the chromosome DNA topology using only the results obtained from the present work.
218 Thus, further investigation is deemed necessary to elucidate the effect of the amino acid substitutions on the
219 binding of DNA gyrases to nontyphoidal *Salmonella* chromosomes. In future studies, we plan to analyze the
220 gene expression in the bacterial cells.

221 Although the supercoiling activity of DNA gyrases was not affected by the amino acid substitutions,
222 quinolone resistance was greatly changed (Fig. 3). Increased IC₅₀s of quinolones against mutant DNA gyrases
223 compared with those used against WT gyrases indicated that all tested amino acid substitutions on GyrA
224 conferred quinolone resistance to DNA gyrase³⁸. Quinolones bind to DNA gyrase and inhibit its enzymatic
225 activity. Previous studies have shown that serine and acidic amino acids at positions 83 and 87 of GyrA,
226 respectively, work as anchor points for DNA gyrase at this binding mechanism, forming a metal-water ion-
227 mediated bridge between the quinolone and DNA gyrase^{22,39,41}. Thus, amino acid substitutions at these
228 positions weaken the binding of quinolones to DNA gyrase. The 87th amino acid of GyrA was aspartic acid,

229 which is an acidic amino acid, and the mutated amino acid was neutral or non-polar; the increase in IC₅₀ for
230 the mutant DNA gyrase with the amino acid substitution at position 87 was most likely due to a change in
231 the property of amino acid. The effect of amino acid substitution at position 83 is also similar. The result that
232 the IC₅₀ for DNA gyrase with Ser83Thr was closer to that of WT than other mutants may be due to the fact
233 that serine and threonine are similar properties (both neutral amino acids with similar molecular weights).
234 On the other hand, since isoleucine is a nonpolar amino acid, the increase in IC₅₀ for DNA gyrase with
235 Ser83Ile was significant.

236 Ser83Ile showed the greatest effect of the amino acid substitutions on quinolone resistance (Table
237 2). The IC₅₀s of ciprofloxacin and norfloxacin against DNA gyrases with Ser83Ile were the highest of all used
238 against DNA gyrases examined in the present study. These results showed that Ser83Ile was able to confer
239 the highest quinolone resistance to *Salmonella*. These observations are in agreement with our previous report
240 on the minimum concentrations of quinolones needed against clinical strains of *Salmonella* Enteritidis with
241 Ser83Ile in GyrA, which were compared with those needed against isolates with different single amino acid
242 substitutions²⁵.

243 In the present work, the amino acid substitution Ser83Phe was also proved to confer quinolone
244 resistance to DNA gyrases, although the resistance to ciprofloxacin was lower than those conferred by other
245 amino acid substitutions, except for Ser83Thr. Indeed, the DNA gyrase with Ser83Thr showed a lower
246 resistance to norfloxacin than other amino acid substitutions, and susceptibility to ciprofloxacin. Furthermore,
247 DNA gyrases with Ser83Thr were more susceptible to nalidixic acid than WT. Highly quinolone-resistant
248 *Salmonella* isolates with GyrA-Ser83Ile have likely derived from WT *Salmonella* through at least two
249 mutations, via either the pathway TCC-TTC-ATC or TCC-ACC-ATC. The first mutation pathway bears
250 Ser83Phe, not Ser83Ile, which confers to *Salmonella* DNA gyrase a relatively lower resistance to quinolones,
251 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 years²⁶. Amino acid
261 substitution Ser83Ile in GyrA has also been detected in *E. coli* and *Klebsiella pneumoniae*^{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 areas²³⁻²⁵, 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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283

284 **Author Disclosure Statement**

285 The authors declare that there are no conflicts of interest.

286

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- 389

390 Table 1. Oligonucleotide sequences of primer sets

391

Primer		Sequence (nucleotide position on <i>gyrA</i>)	Reference
Amino acid substitution			
Ser83Ile	Fw	5'-acggcgat at cgcagtgt-3' (239-256)	This study
	Rv	5'-acactgc gat atcgccgt-3' (239-256)	This study
Ser83Phe	Fw	5'-acggcgatt tc 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'-acggcgatt ac gcagtgt-3' (239-256)	This study
	Rv	5'-acactgc gta atcgccgt-3' (239-256)	This study
Asp87Asn	Fw	5'-cgcagtgtat aac caccatcg-3' (249-268)	28
	Rv	5'-cgatggt gtt atacactgcg-3' (249-268)	28
Asp87Gly	Fw	5'-cgcagtgtat ggc caccatcg-3' (249-268)	28
	Rv	5'-cgatggt gcc atacactgcg-3' (249-268)	28
Asp87Tyr	Fw	5'-cgcagtgtatt ac caccatcg-3' (249-268)	28
	Rv	5'-cgatggt gta atacactgcg-3' (249-268)	28
Restriction enzyme region			
NdeI	Fw	5'-gg catatg agcgacctgagaga-3' (1-20)	28
XhoI	Rv	5'-gg ctc gagctcgcagcgtcatccgc-3' (2617-2634)	28
AatII	Rv	5'-ggtcggcat gacgt ccgg-3' (466-483)	28

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

393 All oligonucleotide primers were synthesized by Life Technologies.

395 Table 2. IC₅₀s 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]

396 Purified GyrA (wild type and mutants), GyrB (wild type) and relaxed pBR322 were mixed in gyrase assay
397 solution [35 mM Tris-HCl, 6 mM MgCl₂, 1.8 mM spermidine, 24 mM KCl, 5 mM DTT, 0.36 mg/mL of BSA
398 and 6.5% glycerol (w/v)] at the concentration of 18, 18 and 1.5 nM, respectively, with different concentrations
399 of quinolones and incubated for 1 hour. DNAs in the reaction mixture were analyzed with agarose gel
400 electrophoresis and the brightness of supercoiled DNA was measured with imageJ to access half maximal
401 inhibitory concentrations (IC₅₀) of each quinolone.

402

403

404 Figure legends

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

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

410

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

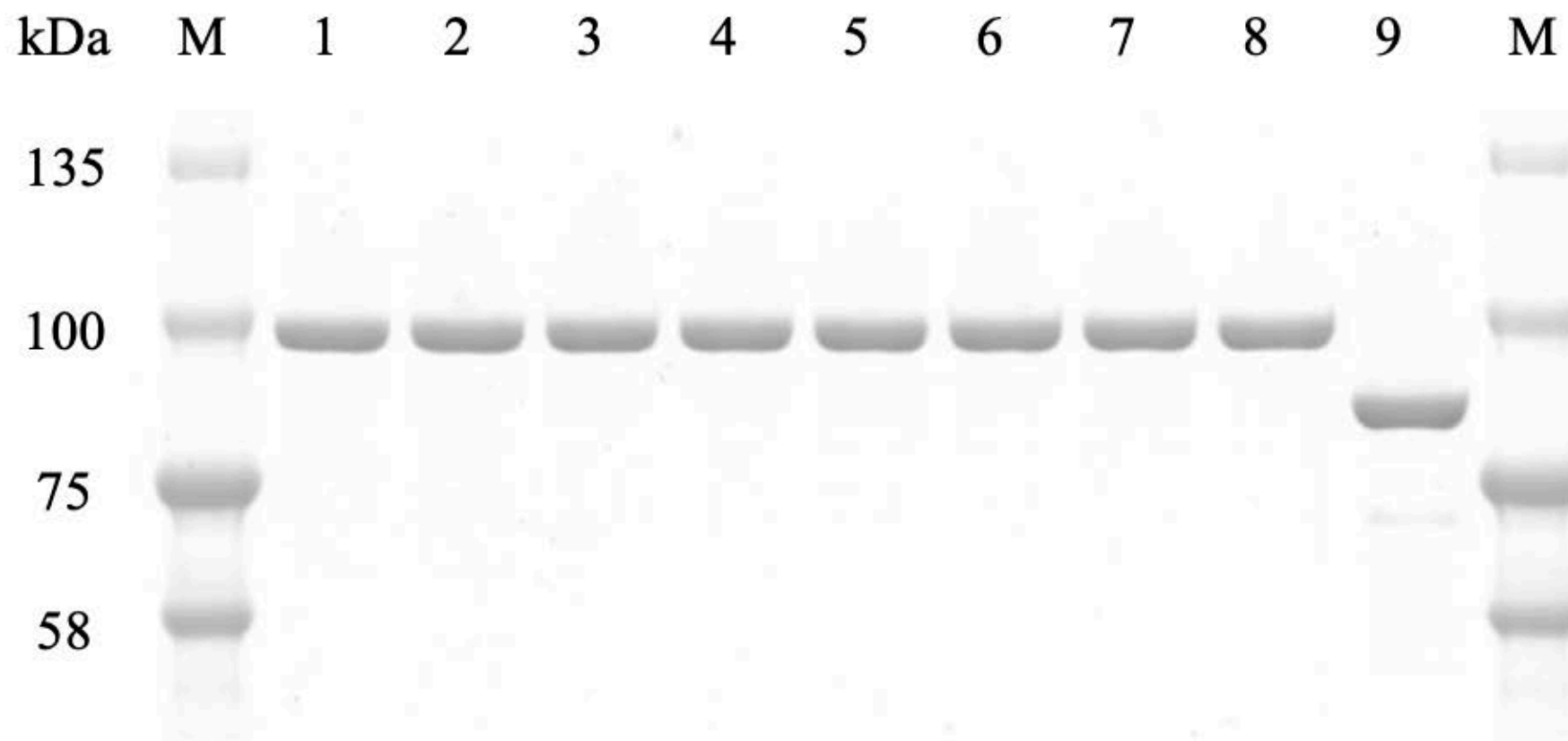
412 A) The electrophoretic images of supercoiled DNA bands by DNA gyrase. The asterisk (*) on the right of
413 the electrophoresis image indicate the positions of supercoiled DNA. B) Increases in the amounts of
414 supercoiled DNA were measured for 120 min.

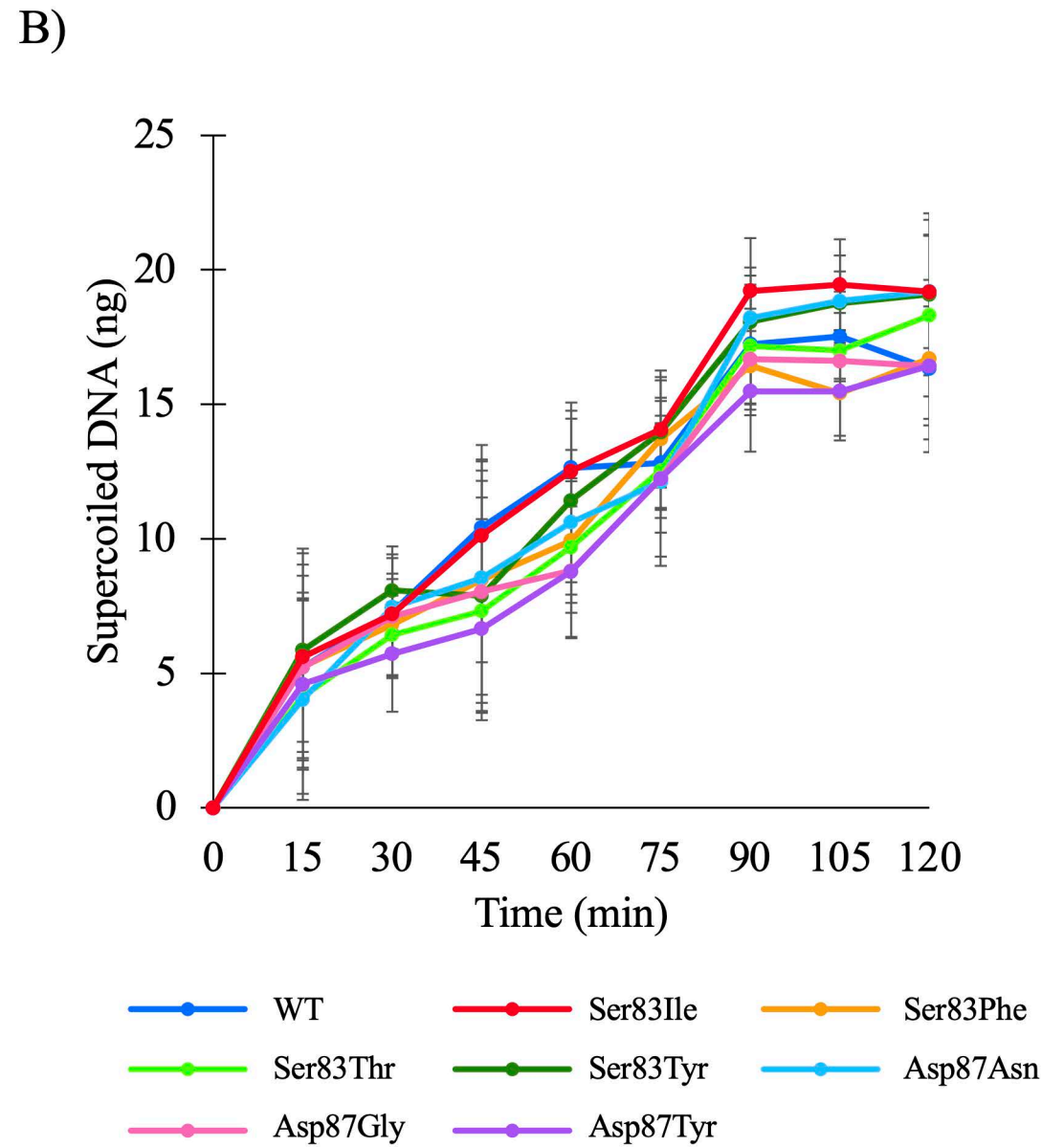
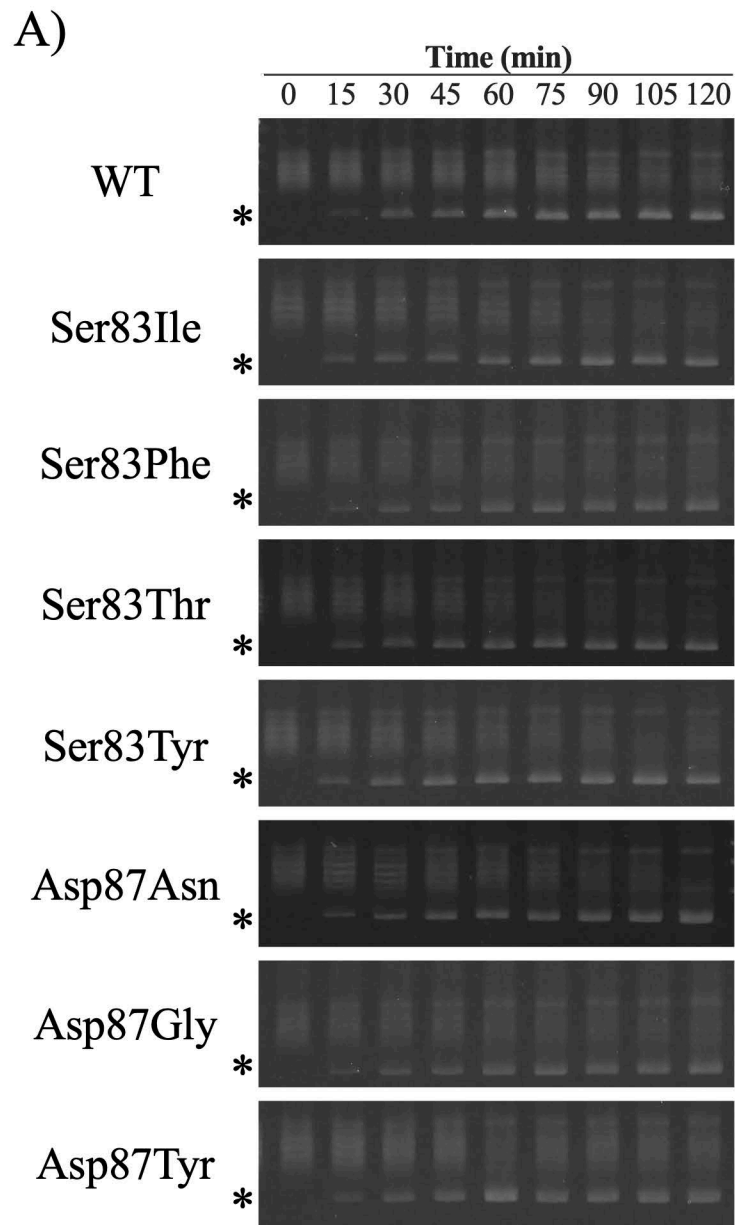
415

416 Figure 3. Supercoiling inhibitory assay.

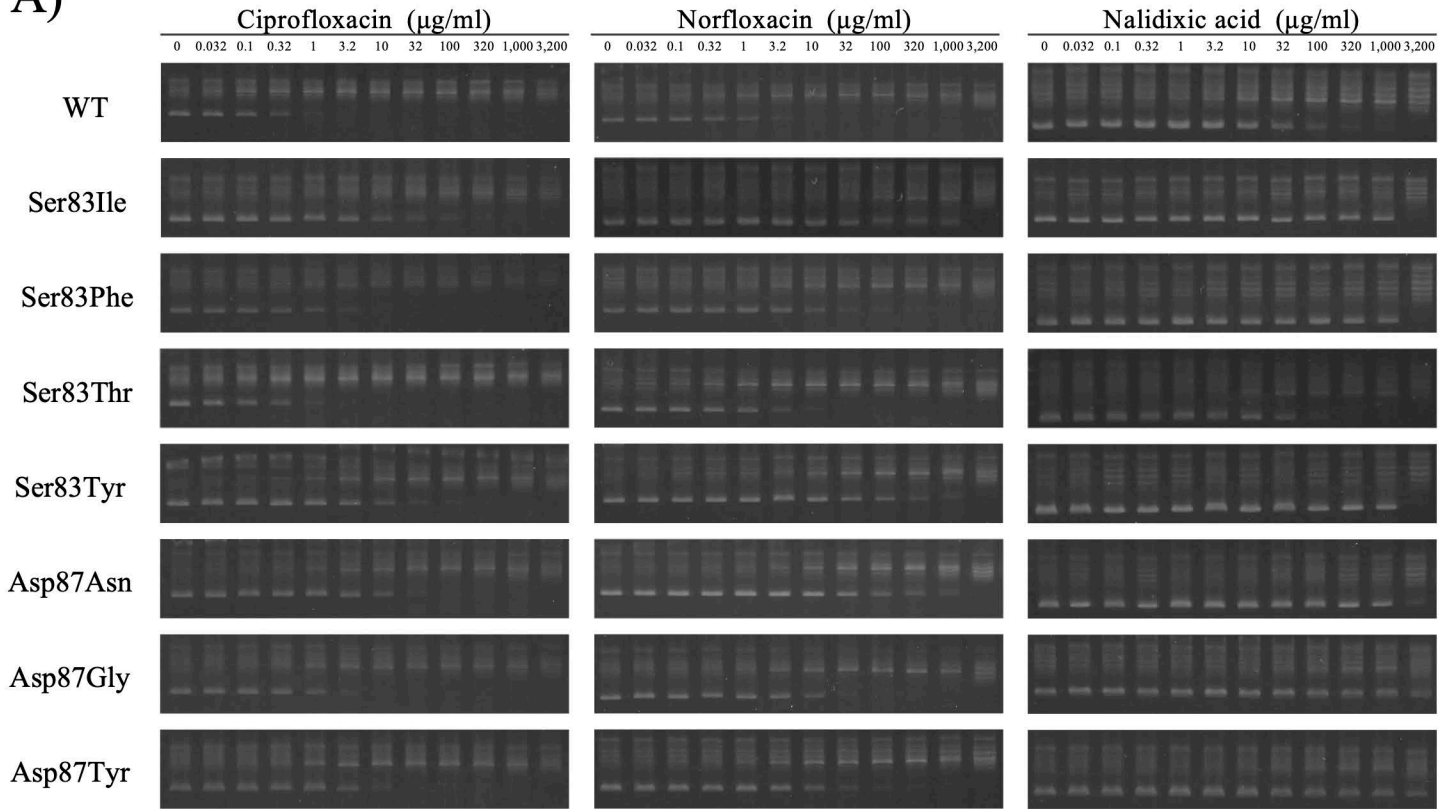
417 A) Electrophoretic image measuring the quinolone resistance by amino acid substitution. B) Dose-response
418 relationships of ciprofloxacin, norfloxacin and nalidixic acid are shown. One hundred percent of supercoiling
419 activity was defined as the supercoiling activity in quinolone-free samples.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

421





A)



B)

