Characterization of *Salmonella* Typhimurium DNA Gyrase as a Target of Quinolones

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Running title: *Salmonella* Typhimurium DNA Gyrase and Quinolones
ABSTRACT

Quinolones exhibit good antibacterial activity against *Salmonella* spp. isolates and are often the choice of treatment for life-threatening salmonellosis due to multi-drug resistant strains. To assess the properties of quinolones, we performed an *in vitro* assay to study the antibacterial activities of quinolones against recombinant DNA gyrase. We expressed the *S.* Typhimurium DNA gyrase A (GyrA) and B (GyrB) subunits in *Escherichia coli*. GyrA and GyrB were obtained at high purity (>95%) by nickel-nitrilotriacetic acid agarose resin column chromatography as His-tagged 97-kDa and 89-kDa proteins, respectively. Both subunits were shown to reconstitute an ATP-dependent DNA supercoiling activity. Drug concentrations that suppressed DNA supercoiling by 50% (IC$_{50}$s) or generated DNA cleavage by 25% (CC$_{25}$s) demonstrated that quinolones highly active against *S.* Typhimurium DNA gyrase share a fluorine atom at C-6. The relationships between the minimum inhibitory concentrations (MICs), IC$_{50}$s and CC$_{25}$s were assessed by estimating a linear regression between two components. MICs measured against *S.* Typhimurium NBRC 13245 correlated better with IC$_{50}$s (R = 0.9988) than CC$_{25}$s (R = 0.9685). These findings suggest that the DNA supercoiling inhibition assay may be a useful screening test to identify quinolones with promising activity against *S.* Typhimurium. The quinolone structure-activity relationship demonstrated here shows that C-8, the C-7 ring, the C-6 fluorine, and N-1 cyclopropyl substituents are desirable structural features in targeting *S.* Typhimurium gyrase.

Key words: DNA gyrase/Quinolones/Salmonella Typhimurium
INTRODUCTION

Non-typhoidal \textit{Salmonella} is the primary foodborne zoonotic agent of salmonellosis in many countries. The global impact of non-typhoidal \textit{Salmonella} on human health is high, with an estimated 94 million cases and 155,000 deaths each year, of which 80 million are believed to be foodborne [1]. According to the World Health Organization, in most countries participating in the Global Foodborne Infections Network, \textit{Salmonella serovar Enteritidis} and \textit{Salmonella serovar Typhimurium} are the most frequently isolated serotypes of \textit{Salmonella} [2]. Gastroenteritis caused by \textit{Salmonella} is generally a self-limited illness. However, antimicrobials may be required to treat invasive cases and susceptible groups such as young children, the elderly and immunocompromised patients [3]. In general, chloramphenicol, ampicillin, and trimethoprim-sulfamethoxazole are used to treat salmonellosis, but resistance to these drugs has increased significantly in recent years [4–6]. In the case of invasive and systemic salmonellosis in humans and animals, quinolones are the preferred drugs for treatment.

DNA gyrases and DNA topoisomerase IV are essential enzymes for the maintenance of chromosomal metabolism in bacteria, and DNA gyrase is known to be the primary target of quinolones in salmonellae. This is due to most amino acid substitutions conferring quinolone resistance being found in DNA gyrase, but not in topoisomerase IV [7–9]. Mutations conferring quinolones resistance are mostly located in a region of the \textit{gyrA} gene specifying the N-terminal domain portion, known as the quinolone resistance-determining region (QRDR) [10]. It has been proposed that the quinolone-binding pocket (QBP) is a site surrounded by surfaces involving the QRDR of both GyrA and GyrB proteins [11,12]. The mechanisms of the interaction between quinolones, drugs, DNA and gyrases remain unclear. Elucidating this information would help to improve existing and design new drugs for the treatment of salmonellosis.
The aim of this work was to further investigate the \textit{in vitro} antibacterial activity of quinolones against \textit{Salmonella} DNA gyrases and to establish a more complete model of the wild-type (WT) gyrase-quinolone interaction. To that end, we assessed the potency of 10 quinolones with quinolone-inhibited supercoiling and quinolone-mediated DNA cleavage assays.

\textbf{MATERIALS AND METHODS}

\textbf{Reagents and kits.} Ciprofloxacin (CIP), enrofloxacin (ENR), gatifloxacin (GAT), levofloxacin (LVX), ofloxacin (OFX) andsparfloxacin (SPX) were purchased from LKT Laboratories, Inc. (St. Paul, MN, USA). Oxolinic acid (OXO) and nalidixic acid (NAL) were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Moxifloxacin (MXF) was obtained from Toronto Research Chemical Inc. (Toronto, Ontario, Canada). Sitafloxacin (SIT) was a gift from Daiichi-Sankyo Pharmaceutical, Co., Ltd. (Tokyo, Japan). Oligonucleotide primers were synthesized by Life Technologies (Carlsbad, CA, USA). TaKaRa Mighty (Blunt End) Cloning Reagent Set (pUC118) and Ni-nitrilotriacetic acid protein purification kits were purchased from Life Technologies. Restriction enzymes were obtained from New England Biolabs, Inc. (Ipswich, MA, USA). Relaxed pBR322 DNA was purchased from John Innes Enterprises Ltd. (Norwich, United Kingdom). Protease inhibitor cocktail (Complete Mini, EDTA-free) was purchased from Roche Applied Science (Mannheim, Germany).

\textbf{Bacterial strains and plasmids.} \textit{S. Typhimurium} (strain LT2 / NBRC 13245) was purchased from NITE Biological Resource Center (Chiba, Japan). \textit{E. coli} strain TOP-10 (Life Technologies) was used as the host for cloning purposes. \textit{E. coli} strain BL21(DE3)/pLysS was purchased from Merck KGaA (Darmstadt, Germany) and used for protein expression. The vector plasmid pET-20b (+) was used to construct expression plasmids for the \textit{S. Typhimurium} proteins, GyrA and GyrB.
Determination of minimum inhibitory concentration (MICs). *S. Typhimurium* NBRC 13245 was analyzed for antimicrobial resistance patterns using the broth microdilution method based on guidelines established by the US National Committee on Clinical Laboratory Standards (NCCLS) [13]. An adjusted bacterial inoculum (10⁶ CFU/ml/10 μl/well) was added to each well of a sterile U-based microtitre plate containing the test concentrations of quinolones (90 μl/well). Consequently, a 5×10⁵ CFU/ml concentration from the last inoculum was obtained in each well, and this plate was incubated for 18 hours at 37ºC. The MIC was defined as the lowest antibiotic concentrations that inhibited visible bacterial growth after incubation. The panel of antimicrobial agents was CIP, ENR, GAT, LVX, OFX, SPX, OXO, NAL, MXF and SIT.

Construction of wild-type (WT) DNA gyrase expression vectors. DNA fragments, including *gyrA* and *gyrB*, were amplified from *S. Typhimurium* NBRC 13245 DNA by polymerase chain reaction (PCR) using the primers listed in Table 1. Two pairs of primers, ST1/ST2 and ST8/ST9, were used to amplify the complete *gyrA* and *gyrB* genes, respectively. *Nde*I restriction sites (CATATG) were included as overlaps of the ATG initiation codons for *gyrA* (ST1) and *gyrB* (ST8) primers, and the *Xho*I site (CTCGAG) was included after the stop codons for the *gyrA* (ST2) and *gyrB* (ST9) primers. PCR products corresponding to the 2.6-kb *gyrA* and 2.4-kb *gyrB* genes were ligated into the blunt-ended cloning plasmid, transformed into *E. coli* TOP-10, and plated on Luria-Bertani (LB) agar containing ampicillin (100 μg ml⁻¹). The *gyrA* and *gyrB* cassettes were digested with *Nde*I and *Xho*I, ligated into pET-20b (+) digested with the same restriction endonucleases, and transformed into *E. coli* TOP-10 to obtain GyrA and GyrB expression plasmids. Recombinant clones were selected from the resistant colonies on LB agar plates containing ampicillin (100 μg ml⁻¹).

Expression and purification of recombinant DNA gyrase. DNA gyrase subunits were purified as previously described [14–16]. Expression plasmids carrying the WT *gyrA* and WT
gyrB genes of S. Typhimurium were transformed into E. coli BL21(DE3)/pLysS. GyrA and GyrB expression was induced with the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (Wako Pure Chemical Industries Ltd., Tokyo, Japan), followed by further incubation at 18°C for 13 h. Recombinant DNA gyrase subunits in the supernatant of the sonicated lysate (by Sonifier 250; Branson, Danbury, CT, USA) were purified by nickel-nitrilotriacetic acid (Ni-NTA) agarose resin column chromatography (Life Technologies Corp.). Protein fractions were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After purification, the protein fractions were added by glycerol to yield 50% (wt/vol) and stored at -80°C until use.

**DNA supercoiling activities and inhibition by quinolones.** ATP-dependent and quinolone-inhibited DNA supercoiling assays were carried out as previously described [14–16] with the following modifications. The reaction mixture (total volume, 30 μl) consisted of DNA gyrase assay buffer, relaxed pBR322 DNA (0.3 μg), and GyrA and GyrB proteins (3 μM each). Reactions were run at 35°C for 20 min and stopped by the addition of 30 μl of chloroform-isoamyl alcohol (24:1 mixture, v/v) and 3 μl of 10X DNA loading dye. The total reaction mixtures were subjected to electrophoresis using a 1% agarose gel in 0.5X Tris-borate-EDTA (TBE) buffer. The gels were run for 60 min at 80 mA and stained with ethidium bromide (0.7 μg ml⁻¹). Supercoiling activity was evaluated by tracing the brightness of the bands with the software ImageJ (http://rsbweb.nih.gov/ij). The inhibitory effect of FQs on DNA gyrase was assessed by determining the drug concentrations required to inhibit the supercoiling activity of the enzyme by 50% (IC₅₀) in the presence or absence of 2-fold serial dilutions in the concentrations of 10 quinolones. 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 performed at least three times to confirm reproducibility.

**Quinolone-mediated DNA cleavage assay.** Quinolone-mediated DNA cleavage assays were
carried out as previously described [14–16]. Supercoiled, rather than relaxed, pBR322 DNA was used as the substrate for cleavage assays. The reaction mixture (total volume, 30 μl) contained DNA gyrase assay buffer, purified GyrA and GyrB (3 μM each), supercoiled pBR322 DNA (0.3 μg) and 2-fold serially diluted concentrations of ten quinolones. After incubation for 20 min at 35°C, 3 μl of 2% SDS and 3 μl of proteinase K (1 mg ml⁻¹) were added to the reaction mixture. After additional incubation for 30 min at 35°C, reactions were stopped to allow a relaxation activity by the addition of 3 μl of 0.5 mM EDTA, 30 μl chloroform-isooamyl alcohol (24:1 mixture, v/v) and 3 μl of 10×DNA loading dye. The plasmid pBR322 linearized by BamHI digestion was used as a marker for cleaved DNA. The total reaction mixtures were subjected to electrophoresis using 0.8% agarose gels in 0.5×TBE buffer. The gels were run for 60 min at 80 mA, stained with ethidium bromide (0.7 μg ml⁻¹) and photographed under UV transillumination. The extent of DNA cleavage was quantified with the Molecular Analyst software ImageJ (http://rsbweb.nih.gov/ij). The quinolone concentrations required to induce 25% of the maximum DNA cleavage (CC₂₅s) were determined for the 10 quinolones.

**Correlation between MICs, IC₅₀s and CC₂₅s against S. Typhimurium gyrases.** The relationships between the MICs, IC₅₀s and CC₂₅s were converted to log10 and assessed by estimating a linear regression between two components,. The strength of this relationship was quantified by the R coefficient and displayed graphically by the regression line and the two curves defining the 95% confidence interval for this regression.

**RESULTS**

**Quinolone susceptibility patterns.** The MICs of quinolones and fluoroquinolones were determined against S. Typhimurium NBRC 13245 (Table 2). The MICs of fluoroquinolones GAT, SPX, LVX, MXF, ENR, OFX, CIP and SIT were 64- to 512-fold lower than those of...
Expression and purification of recombinant GyrA and B proteins. Ni-nitrotriacetic acid affinity purification from 200 ml cultures resulted in 16 and 1.6 mg of soluble His-tagged 97-kDa and 89-kDa proteins, respectively, corresponding to GyrA and GyrB (Figure 1). Both recombinant subunits were obtained at high purity (>95%).

DNA supercoiling activity of recombinant DNA gyrase. Combinations of GyrA and GyrB were examined for DNA supercoiling activity. A combination of GyrA and GyrB at 3 µM each in the presence of ATP was sufficient for the conversion of 100% of 0.3 µg of relaxed plasmid pBR322 DNA to its supercoiled form and was used for all DNA supercoiling experiments. No subunit alone exhibited DNA supercoiling activity in the presence of 1 mM ATP, and no supercoiling activity was observed when ATP was absent from the reaction mixture, which indicated the lack of or low concentration of E. coli-derived DNA gyrase subunits (Figure 2).

IC_{50}s of quinolones. The inhibitory effects of quinolones including CIP, ENR, GAT, LVX, OFX, SPX, MXF, SIT, OXO and NAL on the S. Typhimurium gyrase were elucidated by the quinolone-inhibited DNA supercoiling assay. A set of representative results showing the inhibitory effect of CIP and NAL is shown in Figure 3, and the results for the other quinolones are presented in Figure S1. Each quinolone showed dose-dependent inhibition, with IC_{50}s ranging from 0.22 to 65.1 µg ml^{-1}. The inhibitory effects of quinolones against the recombinant gyrase are presented as IC_{50}s in ascending order in Table 2. A good correlation was found between the IC_{50} values and the corresponding MICs, as shown in Figure 5 (correlation coefficient values, R = 0.9988).

CC_{25}s of quinolones. To examine the effects of quinolones on the cleavable-complex formation by the recombinant DNA gyrase, quinolone-mediated DNA cleavage assays were carried out in which supercoiled pBR322 was incubated with recombinant GyrA and GyrB in
the presence or absence of increasing concentrations of quinolones. Figure 4 shows the
results of a representative quinolone-mediated DNA cleavage assay using CIP and NAL, and
those for the other quinolones are presented in Figure S2. Table 2 presents the CC\textsubscript{25}s of the
ten quinolones, in which each quinolone showed dose-dependent inhibition, with CC\textsubscript{25}s
ranging from 0.24 to 8.64 µg ml\textsuperscript{-1} (Table 2). A good correlation was found between the CC\textsubscript{25}
values and the corresponding MICs, as shown in Figure 6 (correlation coefficient values, R =
0.9685).

DISCUSSION

The incidence of human non-typhoidal \textit{Salmonella} infections has been increasing in
many countries, and the emergence of quinolone-resistant \textit{Salmonella} strains is a serious
concern because this class of antibacterial agents constitutes the treatment of choice in cases
of acute salmonellosis caused by multidrug-resistant strains. Although the main target of
quinolones is known to be DNA gyrase, the molecular details of quinolone-DNA gyrase
interactions have not been elucidated in \textit{Salmonella}. Hence, based on previous studies [14–
16], we produced the His-tagged GyrA and GyrB of \textit{S}. Typhimurium and obtained an
adequate amount of functional \textit{S}. Typhimurium DNA gyrase after reconstitution. The ability
of reconstituted enzymes to convert the relaxed form pBR322 plasmid DNA to the
supercoiled form allowed us to examine and compare the inhibitory effects of 10 quinolones
using the quinolone-inhibited DNA supercoiling assay and quinolone-mediated DNA
cleavage assay. The DNA gyrase supercoiling inhibition assay and quinolone-mediated DNA
cleavage assay are distinct in that the former is a measure of catalytic inhibition, whereas the
latter probes an established equilibrium between the ternary DNA gyrase-drug complexes in
which the DNA is either broken or intact [17,18].

Quinolones inhibited the DNA supercoiling activity of \textit{S}. Typhimurium DNA gyrase
in a dose-dependent manner (Fig. 3 and Supplementary Fig. 1), as it has been reported for other bacteria [19–23]. Among the ten quinolones examined, eight fluoroquinolones exhibited high inhibitory activity against *S. Typhimurium* DNA gyrase with IC$_{50}$s below 1 μg ml$^{-1}$, in contrast to two quinolones OXO and NAL whose IC$_{50}$s were 4.93 and 65.1 μg ml$^{-1}$, respectively (Table 2). Similar results were observed in the quinolone-mediated DNA cleavage assay. Eight fluoroquinolones exhibited high activity to mediate DNA cleavage with CC$_{25}$s below 1 μg ml$^{-1}$, while the two quinolones without fluorine at position 6 had lower activities (Table 2). Analysis of the quinolone structure-activity relationship showed that the eight fluoroquinolones shared certain structural features. Position 1 is the part of the enzyme-DNA binding complex, and has a hydrophobic interaction with the major groove of DNA [24]. A cyclopropyl substituent is now considered the most potent modification here. Another structure at this position is found in OFX and LVX which has a fused ring between position 1 and 8. Position 7 is one of the most influential points on the molecule, and the presence of a five- or six-membered nitrogen heterocycle at this position has been reported to improve the molecule’s activity and pharmacokinetic profile [25]. The most common heterocycles employed at position 7 are aminopyrrolidines and piparazines. The addition of azabicyclo to position 7 has resulted in MXF with significant anti-Gram-positive activity and marked lipophilicity [26].

DNA gyrase is a type II topoisomerase and an essential enzyme for DNA supercoiling, which is required for DNA replication and gene transcription. A domain of the N-terminal part of the A subunit of DNA gyrase is highly conserved among prokaryotes. In addition, the amino acid residues from the positions 67 to 106 of the A subunit in the numbering system used in *E. coli* was defined as the QRDR [27], which is supposed to be the site of interaction between the A subunit of gyrase and quinolones. The *S. Typhimurium* QRDR sequence of GyrA was compared with 4 bacterial species (Fig. 7). The results of the quinolone structure-
activity relationship analysis based on the *S. Typhimurium* WT QRDR sequence were highly concordant with those based on the *E. coli* WT QRDR sequence, which has a 100% identical QRDR sequence with *S. Typhimurium* (Fig. 7). As summarized in Table 3, IC$_{50}$s of quinolones against *S. Typhimurium* and *E. coli* [28] WT DNA gyrase were lower than those against *Mycobacterium tuberculosis* [22], *Streptococcus pneumoniae* [29] and *Mycoplasma pneumoniae* [23]. This result suggested that the interaction of quinolones with *S. Typhimurium* and *E. coli* WT DNA gyrase are stronger than those from other bacterial species. An early study by Guillemin *et al.* [30] proposed that the amino acid residue at position 83 in GyrA played a key role in the intrinsic susceptibility of DNA gyrases to quinolones in mycobacteria. In their study, the serine residue and alanine residue correlated with low and high MICs, respectively. As shown in Figure 7, the amino acid at position 83 in *S. Typhimurium* and *E. coli* DNA gyrase (exhibited by arrowhead) was serine, while those at equivalent positions in DNA gyrase of *M. tuberculosis*, *S. pneumoniae* and *M. pneumoniae* were alanine, serine and methionine, respectively. The findings by Guillemin *et al.* agree in part with our current observation. The amino acid residue at position 83 may be the cause of the intrinsic susceptibility of WT DNA gyrases to quinolones in *M. tuberculosis* and *M. pneumonia* but not in *S. pneumoniae*. ClastalW analysis demonstrated a strong similarity in the QRDR amino acid sequence QRDR of WT GyrA beyond the genus (Fig. 7), a similarity that was a critical point for the wide spectrum of quinolones. However, the amino acids in the QRDR that are distinct between bacterial species may contribute to various intrinsic susceptibilities; nevertheless, amino acid residues outside of QRDR also need to be considered.

The IC$_{50}$s of the *S. Typhimurium* DNA gyrase correlated well with MICs, confirming their ability to inhibit the growth of *S. Typhimurium* (R = 0.999). However, the IC$_{50}$s and MICs were not proportional; for example, sitafloxacin and ciprofloxacin MICs were about
30-fold higher than in the gyrase assay. This nonproportionality has been noted by others [31] and presumably reflects basic differences in the cell-permeability properties and accumulation of the different quinolones [32]. In the quinolone-mediated DNA cleavage assay, the effective quinolone concentrations were slightly different from those inhibiting supercoiling and less correlated with those inhibiting S. Typhimurium growth (R = 0.969). The strong correlation between IC$_{50}$s and MICs observed in other bacterial species (Table 3 and Fig. 7) corroborated this notion. Although the properties of bacteria to incorporate drugs may have some influence on MICs, the quinolone-inhibited DNA supercoiling assay on recombinant S. Typhimurium DNA gyrase could be used as a quick test to screen drugs with promising antibacterial activities. Quinolones with IC$_{50}$s below 1 μg/ml can potentially be active against S. Typhimurium. In contrast, quinolones with high IC$_{50}$s in the enzyme assay may not be suitable for further evaluation as antibacterial drugs. Additionally, the high similarity of the amino acid sequences of both the A and B subunits of DNA gyrase between the quinolone-susceptible typhoidal and non-typhoidal Salmonella strains enables the usage of recombinant S. Typhimurium DNA gyrase for the in vitro selection of quinolones not only against S. Typhimurium, but also against other Salmonella species including highly pathogenic serovers, S. Typhi and S. Parayphi A.

In conclusion, we succeeded in expressing and purifying recombinant S. Typhimurium DNA gyrase, which is the primary target of quinolones in this particular microorganism. Based on measurements of the interaction between quinolones and purified S. Typhimurium DNA gyrase, we carried out two simple assays for the rapid investigation of the quinolone structure-activity relationship and screening of new quinolone derivatives for their anti-S. Typhimurium activities. The results of this study suggest that the in vitro quinolone-inhibited supercoiling assay may be a useful and predictive technique to monitor the antibacterial potency of quinolones.
Acknowledgements

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REFERENCES


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<th>Sequence (nucleotide position)</th>
<th>Comment</th>
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<td>5'-ggcatatgcacccgagcctcgac-3' (1-20), NdeI site</td>
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<td>ST9</td>
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### TABLE 2 Structural features, minimum inhibitory concentration and concentration of quinolones inhibiting *S. Typhimurium* wildtype gyrase activity

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<th>Quinolone</th>
<th>R-1</th>
<th>R-6</th>
<th>R-7</th>
<th>R-8</th>
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<th>CC₂₅ (µg/ml)</th>
<th>MIC (µg/ml)</th>
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<td>SIT</td>
<td>fluorinated</td>
<td>F</td>
<td>pyrrolidine</td>
<td>Cl</td>
<td>0.22 ± 0.04</td>
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<td>CIP</td>
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<td>F</td>
<td>piperazine</td>
<td>H</td>
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<td>0.31 ± 0.10</td>
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<tr>
<td>GAT</td>
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<td>piperazine</td>
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<td>OFX</td>
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<tr>
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<td>65.1 ± 19.08</td>
<td>8.64 ± 0.38</td>
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TABLE 3 IC$_{50}$s (μ g/ml) and MIC$_{50}$s (μ g/ml) for different bacterial species

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<th>Quinolone</th>
<th>S. Typhimurium$^a$</th>
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<th>M. tuberculosis$^c$</th>
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$^a$This work
$^b$Reference 28
$^c$Reference 22
$^d$Reference 29
$^e$Reference 23
Figure 1. SDS-PAGE analysis of recombinant DNA gyrase subunits of *S. Typhimurium*. His-tagged proteins were overexpressed by an *E. coli* expression system and purified by nickel resin chromatography. Each protein sample (approximately 3 μM) was loaded in a SuperSep™Ace 5-20% gradient gel. Following electrophoresis, proteins were revealed by Quick CBB staining. Lane M, size markers (sizes are indicated to the left in kilodaltons); lane 1, wildtype GyrA subunit; lane 2, wildtype GyrB subunit.

Figure 2. Wildtype GyrA and wildtype GyrB proteins of *S. Typhimurium* generate ATP-dependent DNA supercoiling activity. Relaxed pBR322 DNA (0.3 μg) was incubated with mutant DNA gyrase reconstituted from wildtype (WT) GyrA (3 μM) and WT GyrB (3 μM) 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. Lanes 1, relaxed pBR322 DNA; lane 2, relaxed pBR322 DNA and both recombinant WT GyrA and WT GyrB proteins; lane 3, relaxed pBR322 DNA and only GyrA protein; lane 4, relaxed pBR322 DNA and mutants GyrB protein; lane 5, absence of ATP. R and SC denote relaxed and supercoiled pBR322 DNA, respectively.

Figure 3. Inhibitory activities of CIP and NAL on the supercoiling activities of *S. Typhimurium* DNA gyrase. Relaxed pBR322 DNA (0.3 μg) was incubated with each DNA gyrase subunit (3 μM) in the presence of the indicated amounts (μg ml⁻¹) of CIP and NAL. 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.

Figure 4. CIP- and NAL-mediated DNA cleavage assay by DNA gyrase of *S. Typhimurium*. Supercoiled pBR322 DNA (0.3 μg) was incubated with DNA gyrase (3 μM) in the presence of the indicated amounts (μg ml⁻¹) of CIP and NAL. After the addition of SDS
and protease K, the reactions were stopped and the mixtures were analyzed by electrophoresis in 0.8% agarose gels. R, L and SC denote relaxed, BamHI-linearized and supercoiled pBR322 DNA, respectively.

Figure 5. Correlation between the antibacterial activity (MICs) of quinolones and the corresponding concentration of quinolones inhibiting the supercoiling activity (IC_{50} values) of DNA gyrase from *S. Typhimurium* (*R* = correlation coefficient value).

Figure 6. Correlation between the antibacterial activity (MICs) of quinolones and the corresponding concentration of quinolones inducing cleavable complex formation (CC_{25} values) of DNA gyrase from *S. Typhimurium* (*R* = correlation coefficient value).

Figure 7. Alignment of amino acid sequences of GyrA QRDR for 5 bacterial species.
Supplementary Figure 1. Inhibitory activities of a) SIT, b) GAT, c) SPX, d) LVX, e) MXF, f) ENR, g) OFX, and h) OXO on the supercoiling activities of S. Typhimurium wildtype DNA gyrase. Relaxed pBR322 DNA (0.3 μg) was incubated with DNA gyrase (3 μM) in the presence of the indicated amounts (μg ml⁻¹) of the respective quinolones. 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.

Supplementary Figure 2. Quinolone-mediated DNA cleavage assay of a) SIT, b) GAT, c) SPX, d) LVX, e) MXF, f) ENR, g) OFX, and h) OXO on the DNA gyrase of S. Typhimurium. Supercoiled pBR322 DNA (0.3 μg) was incubated with each WT DNA gyrase (3 μM) in the presence of the indicated amounts (μg ml⁻¹) of the respective quinolones. After the addition of SDS and protease K, the reactions were stopped and the mixtures were analyzed by electrophoresis in 0.8% agarose gels. R, L and SC denote relaxed, BamHI-linearized and supercoiled pBR322 DNA, respectively.
Kongsoi et al. Figure 1

kDa

175

80

58

M  1  2
Figure 2

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Kongsoi et al. Figure 3
Kongsoi et al. Figure 4

**CIP (μg/ml)**

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**NAL (μg/ml)**

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Figure 6

Kongsoi et al.

\[ R = 0.969 \]

DIC CIP

GAT MXF

SPX ENR

LVX

NAL

OXO

OFX

\[ \log CC_{25} \ (\mu g/ml) \]

\[ \log \text{MIC} \ (\mu g/ml) \]
Kongsoi et al. Figure 7

S. Typhimurium

E. coli

M. tuberculosis

S. pneumoniae

M. pneumoniae

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** ..: ::::***** :::****: **** :* ** *:::*
Correlation between quinolone inhibition of *C. jejuni* gyrase (IC$_{50}$s for DNA supercoiling) and quinolone MICs for *C. jejuni*. $R$ is the correlation coefficient. SIT, sitafloxacin; GAT, gatifloxacin; MXF, moxifloxacin; SPX, sparfloxacin; ENR, enrofloxacin; CIP, ciprofloxacin; LVX, levofloxacin; OFX, ofloxacin; OXO, oxolinic acid; NAL, nalidixic acid.