学位論文要約

Studies on DNA gyrases of *Campylobacter jejuni* as the target of quinolones

（キノロン剤の標的としての*Campylobacter jejuni* DNAジャイレースの性状）

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CONTENTS

ABBREVIATIONS

SIT	Sitafloxacin
CIP	Ciprofloxacin
MXF	Moxifloxacin
LVX	Levofloxacin
ENR	Enrofloxacin
GAT	Gatifloxacin
OFX	Ofloxacin
SPX	Sparfloxacin
OXO	Oxolinix acid
NAL	Nalidixic acid
GyrA	A subunit of DNA gyrase
GyrB	B subunit of DNA gyrase
ATP	Adenosine triphosphate
QRDRs	Quinolone resistance-determining regions
WT	Wild type
Thr86Ile	An amino acid substitution from Threonine to Isoleucine at position 86
Thr86Ala	An amino acid substitution from Threonine to Alanine at position 86
Thr86Lys	An amino acid substitution from Threonine

	to Lysine at position 86
Asp90Asn	An amino acid substitution from Aspartic acid to Asparagine at position 90
Asp90Tyr	An amino acid substitution from Aspartic acid to Tyrosine at position 90
PCR	Polymerase chain reaction
IPTG	Isopropyl-β-D-thiogalactopyranoside
LB	Luria-Bertani
MHB	Mueller Hinton broth
MHA	Mueller Hinton agar
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel Electrophoresis
R	Relaxed form pBR322 plasmid
SC	Supercoiled form pBR322 plasmid
IC_{50}	Half of maximal inhibitory concentration
MIC	Minimum inhibitory concentration
MEC	Minimum effective concentration
Ni-NTA	Nickel-nitrilotriacetic acid

PREFACE

Campylobacter is a bacterium causes intestinal infections and is estimated to affect 400-500 million people globally each year (1, 31). *Campylobacter jejuni* is considered one of the most important human pathogenic bacteria, commonly found in the gastrointestinal tract of many bird species. Generally, most patients infected with *C. jejuni* are not required treatment, except those with severe disease or in immune-compromised patients (11). Quinolones (Figure 1) belong to a family of broad-spectrum synthetic antimicrobials, particularly ciprofloxacin, have been used for the treatment of *Campylobacter* infections in patients with invasive disease (1, 35). However, the excessive use of quinolones in veterinary medicine has been reported to cause an emergence of quinolone-resistant *Campylobacter* (10, 22, 32). In fact, quinolone resistance in *Campylobacter* from food animals is now recognized as an emerging public health problem (11, 34). Testing for quinolone susceptibility is essential to provide guidance to physicians and veterinarians on the appropriate treatment for *Campylobacter* infections. *C. jejuni* is a slow-growing bacterium that requires microaerophilic conditions and supplemented growth media. Variations in the culture media and the incubation conditions, namely, atmosphere, temperature, and time of incubation, could affect the results of antimicrobial susceptibility tests.

In bacteria, the target of quinolones are the essential enzymes, DNA gyrase and DNA topoisomerase IV, belonging to the bacterial type II topoisomerase. DNA gyrase is

unique in that it catalyzes the negative supercoiling of DNA and is essential for DNA replication, transcription and recombination. On the contrary, topoisomerase IV has a specialized role in chromosome segregation. Complete genome sequencing of *C. jejuni* revealed the lack of genes encoding topoisomerase IV (5, 19, 31) and thus DNA gyrase turned out to be the sole target of quinolones in *Campylobacters*. Quinolone inhibits DNA supercoiling by stabilizing the complex between gyrase and the cleaved DNA, interrupting the propagation of the replication fork. When the DNA was cleaved by gyrase, the antibiotics prevent relegation of the broken strand, resulting in a quinolone-enzyme-DNA complex that leads to inhibition of DNA replication (9, 14).

Recently, *C. jejuni* has shown increasing resistance to quinolone, which has become a major concern for public health (9, 12, 36). The resistance is mediated by a point mutation in the quinolone resistance-determining region (QRDR) of *gyr*A. The amino acid substitution from Thr to Ile at codon 86 (Thr86Ile) have been identified dominantly and shown to associate with high-level quinolone resistance whereas other substitutions Thr86Ala, Thr86Lys, Asp90Asn, and Asp90Tyr were found in minor population (Figure 2) (11, 14, 29, 33). Because of increased quinolone resistance in *C. jejuni*, an *in vitro* method that would accelerate the identification of more potent quinolones against *C. jejuni* is needed for its treatment and effective control.

This present thesis consists of two chapters; in the chapter I, I have investigated the interaction of *C. jejuni* DNA gyrase with a number of quinolones using recombinant GyrA and GyrB subunits by *in vitro* DNA supercoiling assay. In chapter II, I conducted

assay same as chapter I and revealed the contribution of amino acid substitutions at position 86 and 90 in GyrA to quinolone resistance.

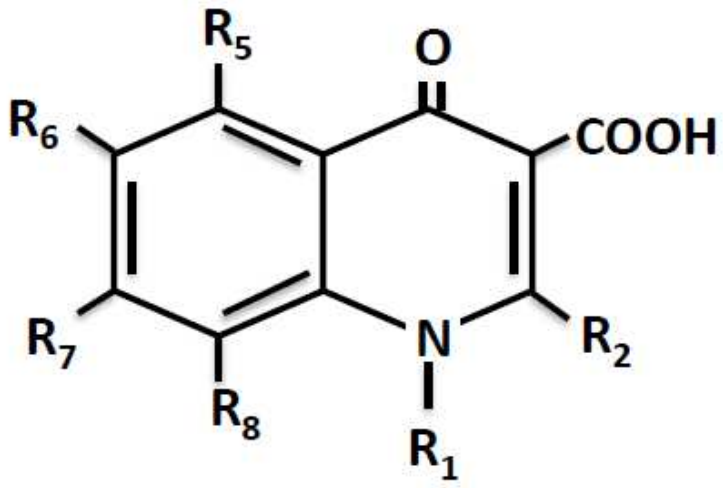


Figure 1. Structure of the quinolone molecule, using the accepted numbering scheme for positions on the molecule.

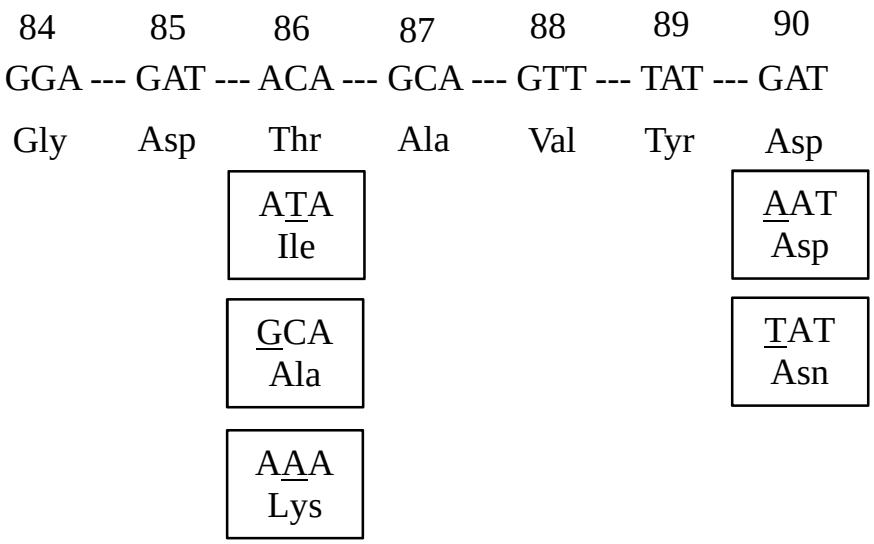


Figure 2. Amino acid substitutions found within the QRDR of GyrA in quinolone-resistant *C. jejuni*. The amino acid substitutions are shown below the nucleotide sequence.

CHAPTER I

Characterization of *Campylobacter jejuni* DNA gyrase as the target of quinolones

Introduction

Quinolones, particularly ciprofloxacin, have long been used as the first-line treatment for *Campylobacter* infections. However, an increased resistance to quinolones has raised public health concerns. The development of new quinolone-based antibiotics with high activity is critical for effective, as DNA gyrase, the target of quinolones, is an essential enzyme for bacterial growth in several mechanisms. The evaluation of antibiotic activity against *C. jejuni* largely relies on drug susceptibility tests, which require at least 2 days to getting the results. It would be likeable to develop an *in vitro* method for studying the activity of quinolones against the DNA gyrase of *C. jejuni*. To identify potent quinolones, I conducted the quinolone-mediated supercoiling activity inhibition assay using recombinant DNA gyrase of wild-type (WT) *C. jejuni* with 10 quinolones.

MATERIALS AND METHODS

Reagents

Ciprofloxacin (CIP), gatifloxacin (GAT), levofloxacin (LVX), sparfloxacin (SPX), enrofloxacin (ENR) and ofloxacin (OFX) were purchased from LKT Laboratories, Inc. (St. Paul, MN). Oxolinic acid (OXO) and nalidixic acid (NAL) were purchased from Wako Pure Chemicals Ltd. (Tokyo, Japan). Moxifloxacin (MXF) was obtained from Toronto Research Chemicals 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). A Ni-nitrolotriacetic acid (Ni-NTA) protein purification kit was purchased from Life Technologies. Restriction enzymes were obtained from New England BioLabs, Inc. (Ipswich, MA). Supercoiled and relaxed pBR322 DNA were purchased from John Innes Enterprises Ltd. (Norwich, United Kingdom). A protease inhibitor cocktail (Complete Mini, EDTA free) was purchased from Roche Applied Science (Mannheim, Germany).

Bacterial strains and plasmids

Escherichia coli strain TOP-10 (Life Technologies Corp., Carlsbad, CA) was used as the cloning host. The pUC118-*Hinc*II/BAP plasmid (Takara Bio, Kyoto, Japan) was used to clone amplified DNA fragments. *E. coli* strains BL-21 (DE3)/pLysS (Merck KGaA, Darmstadt, Germany) were used for protein expression. Vector plasmid pET-20b

(+) (Merck KGaA) was used to construct expression plasmids for *C. jejuni* proteins GyrA and GyrB.

Quinolones susceptibility testing

C. jejuni ATCC33560 was grown on Mueller-Hinton agar (MHA; Oxoid Ltd., Basingstoke, UK), and the minimum inhibitory concentrations (MICs) for this strain were determined by a broth dilution method. Briefly, 1 µl of suspension was inoculated into Mueller-Hinton broth (MHB; Oxoid Ltd.) supplemented with 5% defibrinated sheep blood and containing 2-fold serial dilutions of the quinolone. The microdilution tray was incubated at 37 ºC under microaerophilic conditions (10% CO_2, 5% O_2, and 85% N_2) for 48 hours. The MIC was predetermined as the lowest concentration of quinolone to cause a complete growth inhibition.

Construction of DNA gyrase expression vectors

DNA fragments encoding *gyr*A and *gyr*B were amplified by polymerase chain reaction PCR using the primers listed in Table 1. The reaction mixture (25 µl) consisted of primeSTAR GXL buffer (Mg^{2+} plus); 200 µM each of dATP, dCTP, dGTP, and dTTP; 10 ng DNA from *C. jejuni* ATCC33560; 2.0 units of PrimeSTAR GXL DNA polymerase (Takara Bio Inc., Kyoto, Japan); and 0.4 µM of each primer. PCR was carried out in an iCycle Thermal Cycler (Bio-Rad Laboratories GmbH, California, US) under the following amplification conditions: pre-denaturation at 98 ºC for 2 min, 35 cycles of denaturation at 98 ºC for 10 sec, annealing at 50 ºC for 10 sec, extension at 72 ºC for 3

min, and a final extension step at 72 ºC for 2 min. The PCR products corresponding to the 2.5-kb *gyr*A and 2.3-kb *gyr*B fragments were ligated into the pUC118-*Hinc*II/BAP plasmid and introduced into *E. coli* Top10 competent cells, according to the manufacturer's instructions. Recombinant plasmids were recovered from white colonies and digested with *Nde* I and *Xho* I, and the obtained DNA fragments were ligated into *Nde* I-*Xho* I-digested pET-20b and transformed into *E. coli* Top10. Recombinant clones were selected from the resistant colonies on Luria-Bertani (LB) agar containing ampicillin (100 μg/mL). The nucleotide sequences of the DNA gyrase genes in the plasmids were analyzed using the ABI Prism BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). Cycle sequencing products were subsequently analyzed in an ABI PRISM 3130x automated genetic analyzer (Applied Biosystems). The sequences were compared with their respective wild-type sequences using BioEdit software (http://www.mbio.ncsu.edu/bioedit/bioedit.html).

Recombinant expression and purification of DNA gyrase

DNA gyrase subunits were expressed and purified as previously described (18, 24, 38), with modifications. Briefly, expression vectors carrying *C. jejuni gyr*A and *gyr*B were introduced to *E. coli* BL 21(DE3)/pLysS. The transformants were grown in LB medium in the presence of 100 μg/mL ampicillin to the log phase. Expression of GyrA and GyrB was induced with the addition of 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) (Wako Pure Chemicals Industries Ltd.), and further incubation was conducted for 16 h at 18 °C. Harvested *E. coli* was lysed by sonication at 30% duty cycle, 10 cycles of

40 sec on and 40 sec off using Sonifier 250 (Branson, Danbury, CT). After supernatants were centrifuged (10000×g) for 30 min, recombinant DNA gyrase subunits were purified by Ni-NTA resin column chromatography and dialyzed against DNA gyrase dilution buffer (50 mM Tris-HCl pH 7.5, 100 mM KCl, 2 mM DTT, 1 mM EDTA). Protein fractions were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Wako Pure Chemicals Industries Ltd.) with a protein molecular weight marker (New England Biolab).

DNA supercoiling assay and inhibition by quinolones

The DNA supercoiling activity of *C. jejuni* DNA gyrase was assayed by monitoring the conversion of relaxed pBR322 to its supercoiled form. The DNA supercoiling activity was tested with a combination of purified *C. jejuni* GyrA and GyrB. The reaction mixture consisted of DNA gyrase assay buffer (35 mM Tris-HCl pH 7.5, 24 mM KCl, 4 mM $MgCl_2$, 2 mM DTT, 1.8 mM spermidine, 1 mM ATP, 6.5% glycerol, 0.1 mg/mL of BSA), relaxed pBR322 DNA (0.3 µg) with 12.5 nM each of GyrA and GyrB for a total volume of 30 µl. Reactions were run for 30 min at 37 °C and stopped by the addition of 30 µl of chloroform-isoamyl alcohol (24:1 mixture, v/v) and 3 µl of 10× DNA loading dye. The total reaction mixtures were subjected to electrophoresis in 1% agarose gel in Tris-borate-EDTA (TBE) buffer. The gels were run for 1 h at 80 mA and stained with ethidium bromide (0.7 µg/mL). The extent of supercoiled DNA was quantified with ImageJ software (http://rsbweb.nih.gov/ij) by comparing with the titration curve drawn using various concentration of supercoiled pBR322 on the same agarose gel. The

inhibitory effect of 10 quinolones on DNA gyrase was assessed by determining the drug concentrations required to inhibit the supercoiling activity of the enzyme by 50% (IC_{50}s).

RESULTS

Construction and purification of recombinant WT DNA gyrase subunits

Full-length *gyr*A and *gyr*B of *C. jejuni* ATCC33560 were inserted downstream of the T7 promoter in expression vector pET-20b (+) for expression as His-tagged recombinant protein, as His-tag has been previously shown not to interfere with the catalytic function of GyrA and GyrB (3). The expression of GyrA and GyrB in *E. coli* BL21 (λDE3)pLysS by induction with IPTG and subsequent purification by Ni-NTA resin resulted in 2.8 mg and 1.3 mg of soluble His-tagged 97-kDa protein of GyrA and 87-kDa protein of GyrB, respectively, from 500 mL cultures. The high purity (>95%) of recombinant protein was confirmed by SDS-PAGE (Figure 3).

ATP-dependent DNA supercoiling activities of WT DNA gyrase

GyrA and GyrB were examined for DNA supercoiling activity with relaxed pBR322 DNA as the substrate in the presence and absence of ATP (Figure 4). A combination of GyrA and GyrB at 50 ng each was sufficient for the conversion of 0.3 µg of relaxed plasmid pBR322 DNA to its supercoiled form and was used for all DNA supercoiling experiments in this study. The DNA supercoiling activities were observed in the presence of ATP and both recombinant DNA gyrase subunits, which confirmed the reconstitution of functional DNA gyrase (Figure 4, Lane 2). No subunit alone exhibited DNA supercoiling activity (Figure 4, Lane 3 and 4) and no supercoiling activity was observed when ATP was omitted (Figure 4, Lane 5).

Inhibition of DNA supercoiling by quinolones

The inhibitory effect of 10 quinolones on DNA gyrase was investigated by the quinolone-inhibited DNA supercoiling assay. Each quinolone showed a dose-dependent inhibition and their IC_{50}s ranged from 0.4 (SIT) to 106.8 μg/mL (NAL), as summarized in Table 2. Figure 5 showed the inhibitory activity of all quinolones on the supercoiling activities of *C. jejuni* DNA gyrase. The MICs of 10 quinolones ranged from 0.0078 to 4 μg/mL (Table 2). Furthermore, the IC_{50}s and the MICs correlated significantly (R = 0.9943), as shown in Figure 6.

DISCUSSION

Quinolones, a family of drugs for treating *C. jejuni* infection, are used in veterinary medicine for both prophylactic and therapeutic purposes (1, 35). However, there is concern about the use of quinolones in animal production, as their use selectively enriches quinolone-resistant *Campylobacter*, which can then be transmitted to humans via the food chain (21). Consequently, CIP- and ENR-resistant *Campylobacter* strains are widely found in humans and animals, respectively (32, 35). Hence, the development of new quinolone-based antibiotics with higher activity than CIP and ENR has become essential, as DNA gyrase, the target of quinolones, is a vital enzyme required for bacterial growth in several strains. Efforts have been made to find alternative quinolones for the treatment of *C. jejuni* infections. However, the complicated culturing of *C. jejuni* has been a serious impediment for the study of this pathogen, particularly the screening of new drugs. Studying the interaction of *C. jejuni* DNA gyrase with quinolones is an important step to understand the quinolone structure-activity relationship and, thereby lead to select those quinolones with good antibacterial activity.

Based on findings of previous studies (18, 38), His-tagged recombinant GyrA and GyrB of *C. jejuni* were expressed and purified to obtain a functional DNA gyrase after reconstitution. The reconstituted DNA gyrase allowed us to examine and compare the inhibitory effect of 10 quinolones. The inhibition of DNA supercoiling activities by quinolone was observed in a dose-dependent manner, as previously found in other

bacterial DNA gyrase (6, 16, 29). In the present study, eight quinolones (SIT, CIP, LVX, SPX, GAT, MXF, ENR, and OFX) showed a high activity against *C. jejuni* DNA gyrase, with IC_{50}s of 0.4 to 2.3 µg/mL, whereas the remaining two quinolones, OXO and NAL, showed a lower inhibitory activity, with IC_{50}s of 15.6 and 106.8 µg/mL, respectively (Table 2). Similar results were observed during the quinolone-inhibited DNA supercoiling assay with DNA gyrase from various bacterial species. Indeed, quinolones OXO and NAL weakly inhibited the DNA supercoiling activities of *Mycobacterium tuberculosis* (4), *M. leprae* (23) and *E. coli* (37). The addition of a fluorine atom at R-6 was the earliest common modification in old quinolones. This single substitution is reported to cause an increase in inhibitory activity of more than 10-fold against DNA gyrase and an improvement in MIC also up to 10-fold (2). The analysis of the quinolone structure-activity relationship showed that the other eight quinolones shared an additional structural feature: a substituent that consisted of three carbon atoms at R-1. Both of these structures exist in many quinolones.

In this study, SIT showed the highest inhibitory activity against *C. jejuni* DNA gyrase with an IC_{50} of 0.4 µg/mL. Analysis of the quinolone structure-activity relationship showed that SIT has a fluorinate cyclopropyl ring at R-1 while other fluoroquinolones have a cyclopropyl ring or a N1-C8 bridge. Furthermore, the addition of a chloride substituent at R-8 may potentially provide an active compound. A number of previous studies have focused on the *in vitro* efficacy of SIT on microbes other than campylobacters. SIT has better activity than other available fluoroquinolones against

several enterobacterial species, with CIP-resistant potentials (25). Yokoyama *et al*. conducted an *in vitro* assay using recombinant mutant DNA gyrases of *M. leprae* and showed that SIT has an improved activity against quinolone-resistant *M. leprae* bearing mutant DNA gyrase (38). Furthermore, SIT showed higher activity against the recombinant DNA gyrase of quinolone-resistant *Enterococcus faecalis* with alteration in GyrA and ParC than did other tested fluoroquinolones (28). SIT belongs to the 4th generation of quinolones that are more effective than CIP, 2nd generation of quinolones, against *C. jejuni* strains (20). Thus, SIT might be a promising candidate for the treatment of campylobacteriosis caused by CIP-resistant *C. jejuni*. Although SIT, now only available in Japan, has been reported to cause mild gastrointestinal disorders as an adverse reaction, our data might encourage its use for *C. jejuni* infections (38).

A strong correlation (R = 0.9943) between MICs of quinolones against C. jejuni and IC_{50}s of its DNA gyrase was confirmed (Figure 6). This result strongly suggested that DNA gyrase inhibition is the major factor in quinolone resistance of *C. jejuni*. Based on MIC values, the quinolones were classified into the same categories as by IC_{50} values. However, the IC_{50}/MIC diversity ratio ranged from 8 to 51 among quinolones. This proportional diversity between the MICs and the IC_{50}s has also been found in *E. coli* (37), *Streptococcus pneumoniae* (26), *Mycoplasma pneumoniae* (27), *M. tuberculosis* (4) and *M. leprae* (23), and might be due to differences in cell-permeating properties and accumulation in each quinolones (17). As shown in Table 3, IC_{50}s of quinolones against *C. jejuni* and *E. coli* DNA gyrase were lower (37) than those against *S. pneumoniae* (26),

M. pneumoniae (27), *M. tuberculosis* (4), and *M. leprae* (23). These data suggested a stronger interaction of quinolones with *C. jejuni* and *E. coli* DNA gyrase than with DNA gyrase from the other 4 homogenous bacteria. Moreover, the analysis of QRDR sequences showed that *E. coli* has highly identical QRDR sequences to *C. jejuni*. In *E. coli*, substitutions of several amino acids in the GyrA have been shown to be important with regard to quinolone resistance. The most frequent substitution seen was the replacement of serine 83. Substitution of serine 83 with leucine or tryptophan has been shown to confer a high-level quinolone resistance in both clinical isolates and laboratory-derived *E. coli* resistant mutants (37). The alignment of 40 amino acid sequences of *C. jejuni* GyrA QRDR with analogous sequences from *E. coli*, *S. pneumoniae*, *M. pneumoniae*, *M. tuberculosis*, and *M. leprae* is shown in Figure 7, in which threonine 86 (indicated by arrow head) in *C. jejuni* is analogous to amino acid 83 in *E. coli* GyrA, while those at an equivalent position in GyrA of *S. pneumoniae*, *M. pneumoniae*, *M. tuberculosis*, and *M. leprae* were serine, methionine, alanine, and alanine, respectively. From these results it can be inferred that amino acid residues at an equivalent position to threonine 86 in *C. jejuni* may be the cause of the increased quinolone resistance of mycobacterial DNA gyrase. In contrast, *S. pneumoniae* DNA gyrase with serine at the same position showed intrinsic resistance to quinolone. The amino acids in QRDR that are distinct between bacteria species may contribute to various intrinsic susceptibilities.

An early study by Han *et al*. demonstrated the inhibitory activity of a fluoroquinolone, CIP, against *C. jejuni* DNA gyrase and showed the minimum effective

concentration (MEC) as 32 µg/mL (13). The extremely high concentration of DNA gyrase used in their study, 300 nM, comparing to our study (12.5 nM) might be the cause of the discrepancy with our result (IC_{50} = 1.0 µg/mL). Further, the quality or specific activity of the DNA gyrase used in our study might have been much higher than that used in theirs. These data clearly exhibited the advantage of the usage of recombinant DNA gyrase with high activity as produced in our study.

SUMMARY

C. jejuni is a foodborne bacterial pathogen that is common in developed world. Quinolones used as the drug of choice for treatment of *Campylobacter* infections. Quinolones inhibit bacterial DNA replication by the inhibition of enzyme DNA gyrase. However, *C. jejuni* is fastidious bacteria, multiplies more slowly than usual bacteria. It would be likeable to develop an *in vitro* method which would accelerate progress in identifying more potent quinolones for their activity against *C. jejuni.* The interaction of *C. jejuni* gyrase with a panel of quinolones was investigated, using recombinant GyrA and GyrB of *C. jejuni*. The combination of purified subunits exhibited DNA supercoiling activity in ATP dependent manner. The IC_{50} were determined for 10 different quinolones to range from 0.4 (SIT) to >100 µg/ml (NAL). SIT showed the highest inhibitory activity and the analysis of the quinolone structure-activity relationship demonstrated that the fluorine atom at R-6 may play the most important role for the inhibitory activity against *C. jejuni* gyrase. The measured concentration of quinolones inhibit 50% DNA supercoiling correlated well with MICs (R = 0.9943). The results of this study suggest that the *in vitro* supercoiling inhibition assay on purified *C. jejuni* DNA gyrase is useful and predictive technique to monitor the antibacterial potency of quinolones.

Table 1 Oligonucleotide sequences of primers used in PCR

Primers	Sequences	Comments
R-5	5'-CCCATATGGAGAATATTTTTAGCAAAG-3' (1-22), *Nde*I site	WT *gyr*A
R-36	5'-GGCTCGAGTTAATGATGATGATGATGATGCAAATCTAAACCAAAGTTTTCATC-3' (1455-1510), *Xho*I site	WT *gyr*A
R-1	5'-CCCATATGCAAGAAAATTACGGTGC-3' (1-25), *Nde*I site	WT *gyr*B
R-47	5'-GGCTCGAGTTAATGATGATGATGATGATGCACATCCAAATGCTTTACATC-3' (2177-2230), *Xho*I site	WT *gyr*B
R-48	5'-GACTTCATCACATGATTTATGAAG-3' (101-124)	WT *gyr*B
R-49	5'-CTTCATAAATCATGTGATGAAGTC-3' (103-126)	WT *gyr*B
R-50	5'-TCAAAAATTTGATCATCAGGC-3' (1455-1474), *Bcl*I site	WT *gyr*B

* Underlines denote the restriction endonuclease recognition sequences.

** Double underline denotes the coding sequences for His hexamer.

Table 2. Structure features and IC_{50} and MIC of quinolones against *C. jejuni*

Drugs	Substituent					IC_{50} (μg/ml)	MIC (μg/ml)
	R-1	R-5	R-6	R-7	R-8		
SIT	Fluorinated cyclopropyl	H	F	Pyrrolidine	C-Cl	0.4	0.0078
CIP	Cyclopropyl	H	F	Piperazine	C-H	1.0	0.125
LVX	Bridge N1-C8	H	F	Bridge N1-C8	C-H	1.1	0.125
SPX	Cyclopropyl	NH_2	F	Piperazine	C-F	1.2	0.0312
GAT	Cyclopropyl	H	F	Piperazine	C-OCH_3	1.4	0.125
MXF	Cyclopropyl	H	F	Azabicyclo	C-OCH_3	1.5	0.0625
ENR	Cyclopropyl	H	F	Piperazine	C-OCH_3	2.1	0.0625
OFX	Bridge N1-C8	H	F	Bridge N1-C8	C-H	2.3	0.25
OXO	Ethyl	H	Bridge C6-C7	Bridge C6-C7	C-H	15.6	1.0
NAL	Ethyl	H	H	CH_3	N	106.8	4.0

Table 3. IC_{50} and MIC of quinolones inhibiting various DNA gyrases

Quinolone	*Campylobacter jejuni*		*Escherichia coli*[a]		*Streptococcus pneumoniae*[b]		*Mycoplasma pneumoniae*[c]		*Mycobacterium tuberculosis*[d]		*Mycobacterium leprae*[e]	
	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC[f]
SIT	0.4	0.0078	ND	ND	2	0.005	ND	ND	2.5	0.25	1	25
CIP	1.0	0.125	ND	ND	96	0.2	ND	ND	3.5	0.5	3.5	>150
LVX	1.1	0.125	ND	ND	54	0.1	47.5	0.5	5	0.5	7	ND
SPX	1.2	0.0312	0.39	0.0125	114	0.15	ND	ND	2	0.25	5	15
GAT	1.4	0.125	ND	ND	ND	ND	5.71	0.125	3	0.12	2	ND
MXF	1.5	0.0625	ND	ND	ND	ND	7.44	0.0625	4.5	0.5	2	150
ENR	2.1	0.0625	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
OFX	2.3	0.25	ND	ND	88	0.3	ND	ND	10	1.0	10	150
OXO	15.6	1.0	3.13	0.39	ND	ND	ND	ND	300	32	170	ND
NAL	106.8	4.0	50	3.13	ND	ND	ND	ND	1100	128	300	ND

[a] Data from Yoshida *et al*. (37)

[b] Data from Morrissey *et a*l. (26)

[c] Data from Nakatani *et a*l. (27)

[d] Data from Aubry *et al*. (4)

[e] Data from Matrat *et al*. (23)

[f] Data from animal experiment using mouse model (23)

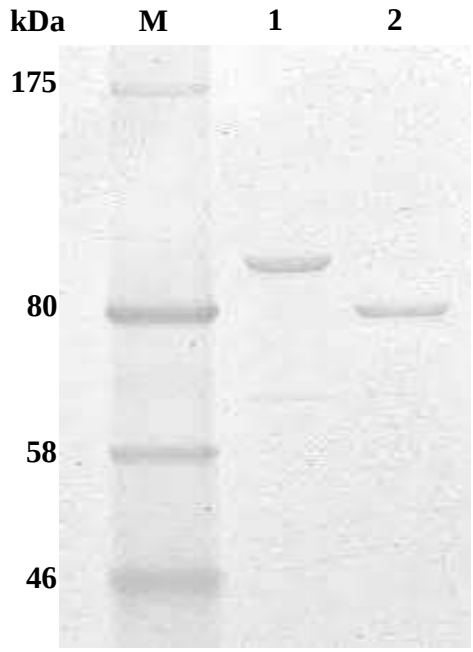
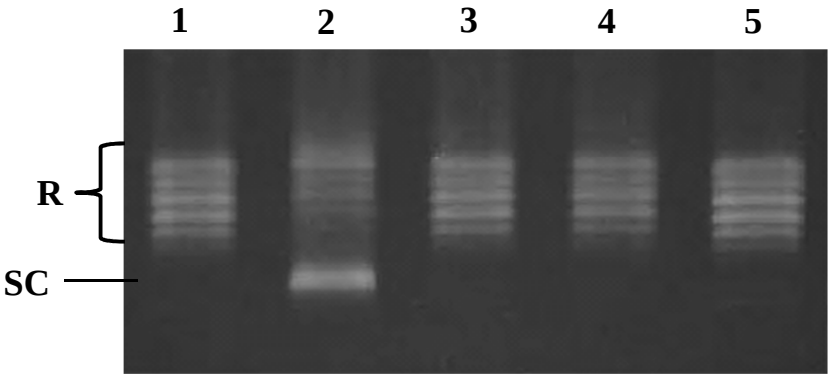


Figure 3. SDS-PAGE analysis of recombinant DNA gyrase subunits. The His-tagged proteins were overexpressed by *E. coli* expression system, and purified by Ni-NTA, and approximately 300 ng of each protein was loaded on a 5-20% gradient polyacrylamind gel. Following electrophoresis, proteins were stained with Quick CBB. Lanes: M, protein molecular marker (sizes are indicated at the left in kilodaltons; kDa); 1, GyrA; 2, GyrB



GyrA	-	+	-	+	+
GyrB	-	+	+	-	+
ATP	-	+	+	+	-
DNA	+	+	+	+	+

Figure 4. Reconstitution of ATP-dependent DNA supercoiling activity. Relaxed pBR322 DNA (0.3 µg) was incubated with DNA gyrase reconstituted with GyrA (0.5 µM) and GyrB (0.5 µM) in the presence and absence of 1 mM ATP. The reactions were stopped, and the DNA products were analysed by electrophoresis in 1% agarose gels. Lanes : 1, relaxed pBR322 DNA; 2, relaxed pBR322 and both recombinant GyrA and GyrB ; 3, relaxed pBR322 and only GyrB; 4, relaxed pBR322 and only GyrA; 5, absence of ATP. R and SC, relaxed and supercoiled pBR322 DNA, respectively.

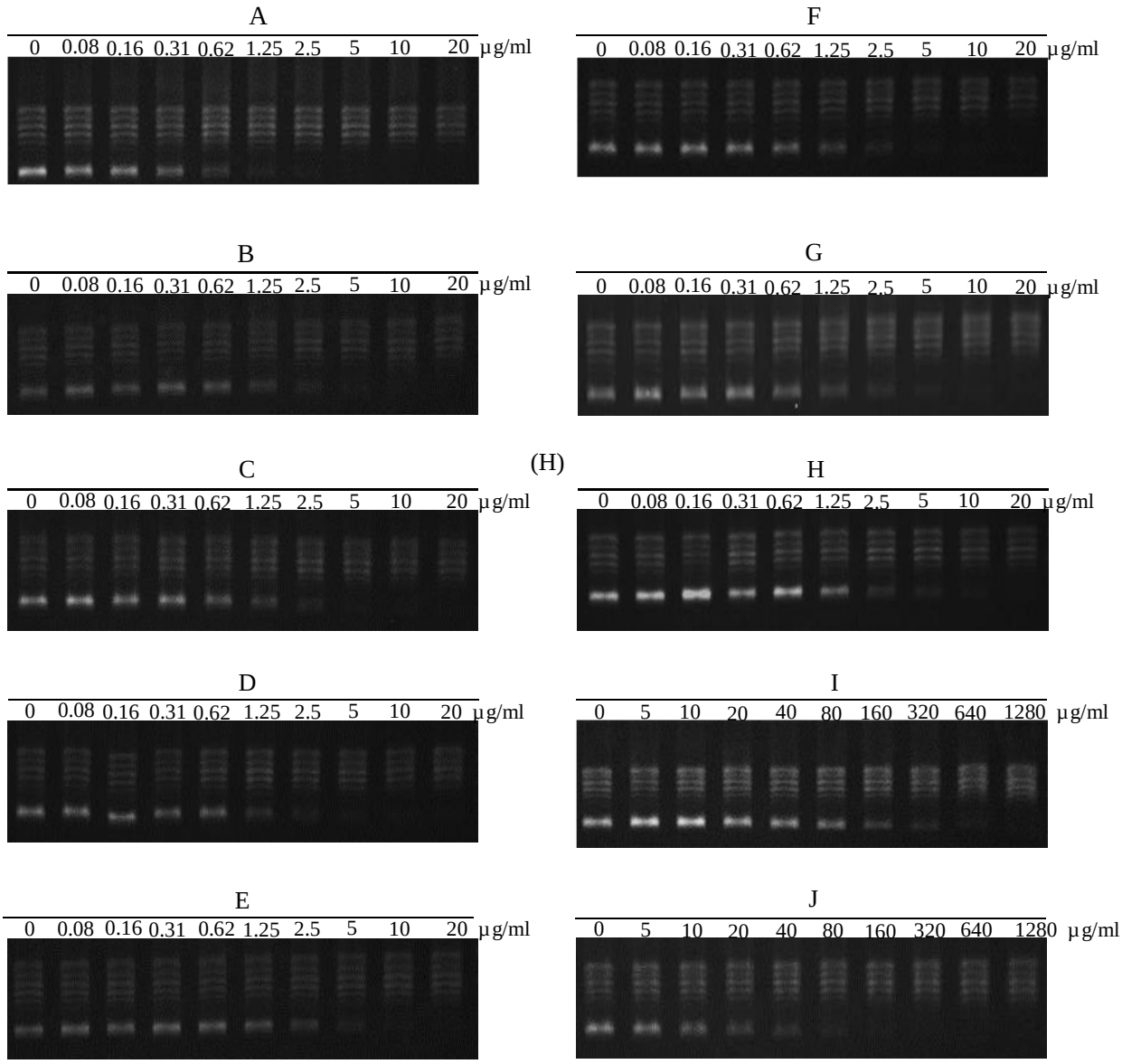


Figure 5. Inhibitory activity of (A) SIT, (B) GAT, (C) LVX, (D) SPX, (E) OFX, (F) MXF, (G) CIP, (H) ENR, (I) NAL and (J) OXO on the supercoiling activities of *C. jejuni* DNA gyrase. Relaxed pBR322 DNA (0.3 μg) was incubated with 50 ng of each GyrA and GyrB with the indicated amount (in μg/ml) of quinolones. The reactions were stopped and the DNA products were analyzed by electrophoresis in 1% agarose gels.

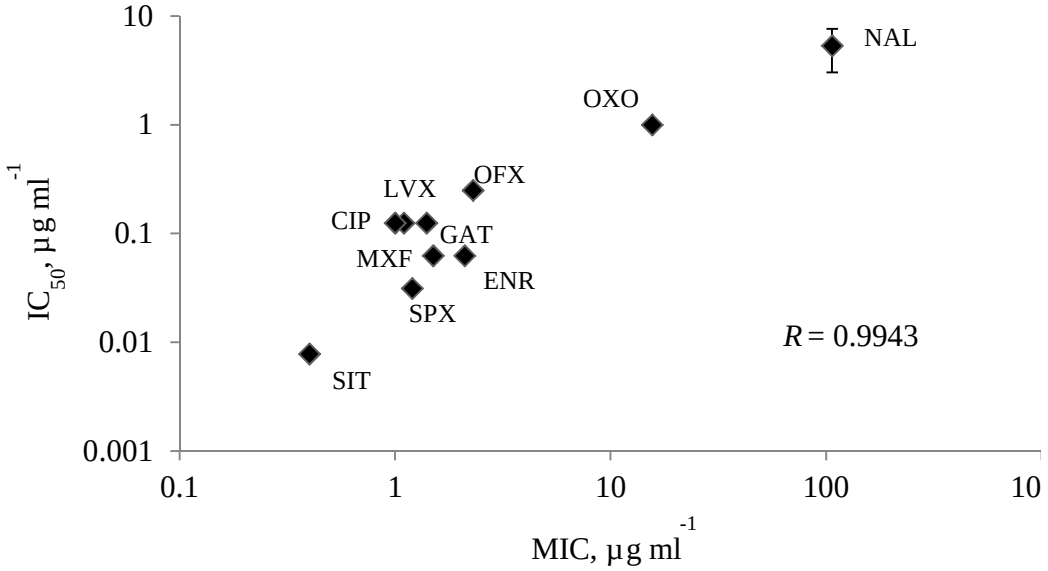


Figure 6. Correlation between logarithm of quinolone inhibition of *C. jejuni* gyrase (IC_{50} for DNA supercoiling) and quinolone MICs for *C. jejuni*. *R* is the correlation coefficient. SIT, sitafloxacin; GAT, gatifloxacin; MXF, moxifloxacin; SPX, sparfloxacin; ENR, enrofloxacin; CIP, ciprofloxacin; LVX, levofloxacin; OFX, ofloxacin; OXO, oxolinic acid; NAL, nalidixic acid.

GyrA

Figure 7. Alignment of amino acid sequences of GyrA QRDR. Identity with the sequences from *C. jejuni* represented by dashes. The numbering system used is that for *C. jejuni gyr*A. An arrow head indicated the position 86 in *C. jejuni*.

CHAPTER II

Comparison of mutations in DNA gyrase genes on quinolones resistance in *Campylobacter jejuni*

The original paper of chapter II had been accepted for publication by journal, however, have not been formally published, yet. Hence, I didn't include the contents of chapter II.

CONCLUSION

Quinolones have been used for the treatment of *C. jejuni* infection in human and animals. However, introduction of quinolones in veterinary medicine caused increase emergence of quinolone-resistant *C. jejuni.* Recently, quinolone resistance in *C. jejuni* from food animals is now recognized as an emerging public health problem. For appropriate treatment, the assessment of antimicrobial susceptibility is essential. However, *C. jejuni*, the microaerophilic fastidious bacteria, present problems in antimicrobial susceptibility testing related to particular cultural requirements and their slow growth. Hence, development of an *in vitro* method for studying the activity of quinolones against the DNA gyrase of *C. jejuni* seemed to be necessary.

In this regard, in Chapter I, I have succeeded in producing and purifying *C. jejuni* DNA gyrase, which is the sole target of quinolones. SIT showed the highest inhibitory activity, and the analysis of the quinolone structure-activity relationship demonstrated that a fluorine atom at R-6 might play the important role in the inhibitory activity against *C. jejuni* gyrase. Moreover, the results of this chapter suggested that the *in vitro* supercoiling inhibition assay may be a useful and predictive technique to monitor the antibacterial potency of quinolones.

In Chapter II, I elucidated the contribution of amino acid substitutions in GyrA of *C. jejuni* DNA gyrase; Thr86Ile, Thr86Ala, Thr86Lys, Asp90Asn, and Asp90Tyr to

quinolone resistance. The quinolone-inhibited supercoiling assay demonstrated the important role of these amino acid substitutions, especially Thr86Ile, in reduced sensitivity to quinolone. In addition, my results demonstrated that SIT might be a candidate for the treatment of campylobacteriosis caused by CIP-resistant *C. jejuni*.

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