Impact of QnrB19, a pentapeptide repeat protein mimicking double stranded DNA, on the quinolone resistance in *Salmonella* Typhimurium

（二重鎖DNAを模倣するペンタペプチド繰返し蛋白QnrB19の
ネズミチフス菌キノロン耐性化への影響）

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LIST OF PAPER

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CONTENTS

ABBREVIATIONS

NTS	Non-typhoidal *Salmonella*
iNTS	Invasive non-typhoidal salmonellosis
MIC	Minimum inhibitory concentration
EUCAST	European Committee on Antimicrobial Susceptibility Testing
WHO	The World Health Organization
Salmonella spp.	*Salmonella* species
S. Typhimurium	*Salmonella* Typhimurium
QRDR	Quinolone-resistant determining regions
PMQR	Plasmid-mediated quinolone resistance
Qnr	Plasmid-encoded quinolone resistance protein
PRP	Plasmid-encoded pentapeptide repeat protein
AhQnr	Pentapeptide repeat protein of *Aeromonas hydrophila*
MfpA	Pentapeptide repeat protein of *Mycobacterium tuberculosis*
AlbG	Pentapeptide repeat protein of *Xanthomonas albilineans*
EfsQnr	Plasmid-encoded quinolone resistance protein of *Enterococcus faecalis*
NAL	Nalidixic acid
CIP	Ciprofloxacin
NOR	Norfloxacin
Ni-NTA	Ni-nitrilotriacetic acid
LB	Luria-Bertani

DTT	Dithiothreitol
BSA	Bovine serum albumin
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
R	Relaxed pBR322 DNA
S	Supercoiled pBR322 DNA
N	Nicked pBR322 DNA
L	Linear pBR322 DNA
IC_{50}	Drug concentrations required to inhibit the activity by 50%

PREFACE

Salmonella are Gram-negative bacteria and facultative anaerobic bacilli that divided into two groups; typhoidal *Salmonella* and non-typhoidal *Salmonella* (NTS). Typhoidal *Salmonella* composed of *Salmonella* enterica subspecies enterica serovars Typhi and Paratyphi A, B and C causing enteric fever [1, 2]. NTS is the cause of approximately 153 million gastroenteritis cases in humans resulting in 57,000 deaths each year worldwide [3]. The various group of NTS comprises more than 2,500 serovars with *Salmonella enterica* subspecies *enterica* serovar Enteritidis and *S.* Typhimurium to be the major serotypes causing foodborne diseases [4]. These serovars are widely disseminated in domestic or wild animals and transmitted to humans by direct contact or consuming infected meat and food product such as pork, beef, chicken, raw eggs, seafood, fruits or vegetables [5-8]. The symptoms of *Salmonella* infections marked by nausea, possibly vomiting, abdominal pain, diarrhea and acute fever [4].

Remarkably, some NTS can cause invasive non-typhoidal salmonellosis (iNTS) with specific animal hosts such as *S*. Choleraesuis in pigs and *S*. Dublin in cattle but these serovars are rarely found infected in other mammalian hosts [9]. NTS, especially, *S.* Enteritidis and *S.* Typhimurium cause bloodstream infections with invasion of other organs in humans. The global yearly burden of iNTS is approximately 3.4 million infections and 681,316 deaths in 2010 [10]. Moreover, the Global Burden of Diseases, Injuries, and Risk Factors Study (GBD) 2017 [11] reported 535,000 cases of iNTS with the highest incidence in sub-Saharan Africa (34.5 cases per 100,000 person-years) and with a low number in Southeast Asia, East Asia and Oceania (1.2 cases per 100,000 person-years) as shown in Figure 1 [11].

In Thailand, NTS was found among 3.8% of blood culture isolates. The major serovars causing bacteremia in Thailand were *S*. Enteritidis and *S*. Choleraesuis with an estimated 51.6% of all iNTS cases during over 9 years [12]. These two serovars have been more commonly isolated

from blood than stool in Thailand. This is different from Africa where *S*. Enteritidis and *S*. Typhimurium are predominant in iNTS [13-15]. In contrast, Laos and Vietnam reported typhoidal *Salmonella* predominately until present [12].

Although NTS infections are self-limiting, the use of antimicrobials is advised for severe cases and that in immune compromised patients [4, 16, 17]. Multidrug resistance has been reported for traditional first-line antimicrobials, for example, trimethoprim/sulfamethoxazole, ampicillin, and chloramphenicol. Fluoroquinolones such as ciprofloxacin have also been better drug of choice for treating NTS in both humans and animals following resistance to first line drugs. However, increasing ciprofloxacin resistance has also been reported in China (5.5%) and the US (2.7%) [18, 19]. In 2009, NTS showed resistance to ciprofloxacin with the high prevalence from Taiwan (48.1%), Thailand (46.2%), Korea (36.5%), Singapore (24.5%), Philippines (14.9%), Hong Kong (7.1%), and Sri Lanka (8.0%) [20]. Recently, the World Health Organization (WHO) published a list of bacteria-antimicrobials combinations that should be taken care. Fluoroquinolone-resistant *Salmonella* spp. is categorized into that with high-priority for which new drugs are urgently required [21].

Fluoroquinolones target DNA gyrase, an important enzyme responsible for DNA replication and transcription, by binding to target sites in the enzyme-DNA complexes, which cause bacterial cell death [22]. DNA gyrase is a member of type IIA topoisomerase family consisted of Topo II, topo IV and DNA gyrase (Figure 2) as described by Klostermeier [23]. The function of DNA gyrase is to cleave DNA strands (G-and T-segments) and allow strand passage, which converts the DNA bound from a positive node into a negative node as shown in Figure 3 [23]. (1) G-segment DNA (orange) is wrapped around the C-terminal domain (CTD), (2) N-gate fixes the T-segment DNA (green) above the G-segment DNA, (3) G-segment DNA is cleaved to form DNA gate, (4) T-segment DNA passes through DNA gate and convert the DNA bound from a positive node into a negative node, (5) G-segment DNA is relegated, (6) ATP hydrolysis

induces re-opening the N-gate. This catalytic cycle is repeated.

Fluoroquinolone resistance is usually developed by chromosomally mediated mutations, which cause amino acid substitutions at the quinolone-resistant determining regions (QRDR) of the DNA gyrase subunits (GyrA and GyrB) in Gram-negative bacteria, and the DNA topoisomerase IV subunits (ParC and ParE) in Gram-positive bacteria. Plasmid-mediated quinolone resistance (PMQR) is also an important mechanism in quinolone resistance [24, 25].

PMQR has indeed become a threat to the effective therapeutic use of quinolones for the treatment of infections in humans and animals, because it is believed that the plasmid can rapidly spread among Enterobacteriaceae through horizontal gene transfer [25]. Plasmid-encoded quinolone resistance protein Qnr among PMQR includes seven Qnr families: QnrA, QnrB, QnrC, QnrD, QnrS, QnrE and QnrVC pentapeptide repeat proteins, which interact with DNA gyrases and reduce susceptibility to quinolones. In addition, efflux systems (OqxAB, QepA and QaqBIII) and an aminoglycoside-, ciprofloxacin- and norfloxacin-inactivating acetyl-transferase (Aac(6')-Ib-cr) are also reported to be responsible for quinolone resistance as summarized by Correia *et al* (Figure 4) [25-27].

Qnr belongs to the pentapeptide repeat protein (PRP) that have tandem copies of a 5-amino acid motif [28]. Qnr protects DNA gyrase and topoisomerase IV from quinolones, obviously by competing with bind the enzyme prior to bind the DNA [29, 30]. The models of DNA gyrase and PRP was constructed by Shah and Heddle [31] as in Figure 5, in which PRP acts as a DNA mimic. In this model, PRP mimics T-segment DNA and is trapped by the enzyme-DNA complex to which G-segment DNA is already bound [31]. This model showed interaction of AhQnr (Qnr from *Aeromonas hydrophila* (pdb 3PSS) [32] with *Staphylococcus aureus* DNA gyrase and ciprofloxacin (pdb2XCT) [33]. The crystal structure of *S.* aureus DNA gyrase bound to G-segment DNA and two ciprofloxacin molecules is demonstrated. AhQnr is put above of the G-segment DNA in position speculation to mimic that of the T-segment DNA as it entered the

DNA gate. Loop 2 of AhQnr monomer positions compete to the bound ciprofloxacin [31].

Allelic variants of *qnr* differing by 10% or less are classified in each family, including 7 alleles for *qnrA*, 71 for *qnrB*, 5 for *qnrVC* and 9 for *qnrS* [34]. Despite recent research advances, little information is known about the predominant Qnr types in *Salmonella* spp. Therefore, I examined the existing information of the prevalence of Qnr in 692 reports published from 2008 to 2017 to show evidences that 4,459 Enterobacteriaceae isolates had Qnrs (1,917 QnrB, 1,545 QnrS, 498 QnrA, 459 QnrD, 27 QnrVC, 12 QnrC and 1 QnrE; Figure 6) and QnrB19 in the highest number of isolates, 223, among Qnr reported in *Salmonella* spp., followed by 24 QnrB10, 17 QnrB2, 3 QnrB1 and 3 QnrB4 (Figure 7).

QnrB is also unique for being controlled by the SOS system. A DNA break produced by ciprofloxacin induces the expression of QnrB via the binding of LexA to LexA recognition site upstream of *qnrB*. In contrast, no LexA binding site is found upstream of *qnrA1* and *qnrS1* [35, 36]. Structure of QnrB1 was published by Vetting *et al* [37] as in Figure 8, based on the plasmid-encoded pentapeptide repeat protein (PRP) sequence diagram. The sequence of QnrB1 is divided into four columns showing the four faces of the right-handed quadrilateral *β*-helix (Figure 8A). Similar monomer structures of MfpA (*Mycobacterium tuberculosis*), AlbG (*Xanthomonas albilineans*), and EfsQnr (*Enterococcus faecalis*) were also shown as the marked color of each face (Figure 8B). Figure 8C shows the dimeric structure of QnrB1 consisted of β-helix structure with two loop structures known to play an important role in quinolone resistance. QnrB19 studied in my thesis carries a similar structure to QnrA1, QnrB1, QnrC, QnrD1, QnrVC3, QnrS1 and QnrE (Figure 9).

Previous studies indicated that QnrB1 and QnrB4 protein inhibited *Escherichia coli* DNA gyrase supercoiling activity, which in turn conferred reduced susceptibility to ciprofloxacin [38, 39]. It has also been reported that wildtype and mutant QnrS1 confers to *E. coli* DNA gyrase a low-level resistance to ciprofloxacin [40]. These studies showed Qnr can protect DNA gyrase

from quinolone inhibition.

However, the role of QnrB19, the predominant Qnr in *Salmonella* spp., in the acquisition of quinolone resistance has not yet been understood. Hence, in Chapter I, I have examined the role of QnrB19 in quinolone resistance acquisition *in vitro* utilizing recombinant *S.* Typhimurium DNA gyrases, QnrB19 and conventional quinolones (nalidixic acid, ciprofloxacin and norfloxacin).

Fluoroquinolones which are effective even for *Salmonella* strains carrying QnrB19 seemed to be important. Hence, in Chapter II, I tried to identify fluoroquinolones with such activities. According to the previous studies, WQ-3810, showed strong inhibitory activity against several pathogenic bacterial species such as *S.* Typhimurium, *E. coli*, *Acinetobacter baumannii*, *Mycobacterium tuberculosis* and *M. leprae* [41-44]. This compound has a unique substituent at the R1 groups (6-amino-3,5-difluoropyridine-2-yl) which enhance the binding affinity to DNA gyrase [41, 45]. Furthermore, WQ-3334 and WQ-4065 have a similar molecular structure with WQ-3810 on R1 (WQ-3334) and R8 (WQ-4065) groups. These novel fluoroquinolones are expected to show strong activity against fluoroquinolone resistant bacteria same as WQ-3810. Therefore, I have conducted *in vitro* assay to observe the inhibitory activity of three novel fluoroquinolones (WQ-3810, WQ-3334 and WQ-4065) as candidate drugs for *S.* Typhimurium. In addition, I have further elucidated the abilities of those fluoroquinolones against *S.* Typhimurium with QnrB19 by *in vitro* DNA supercoiling assay.

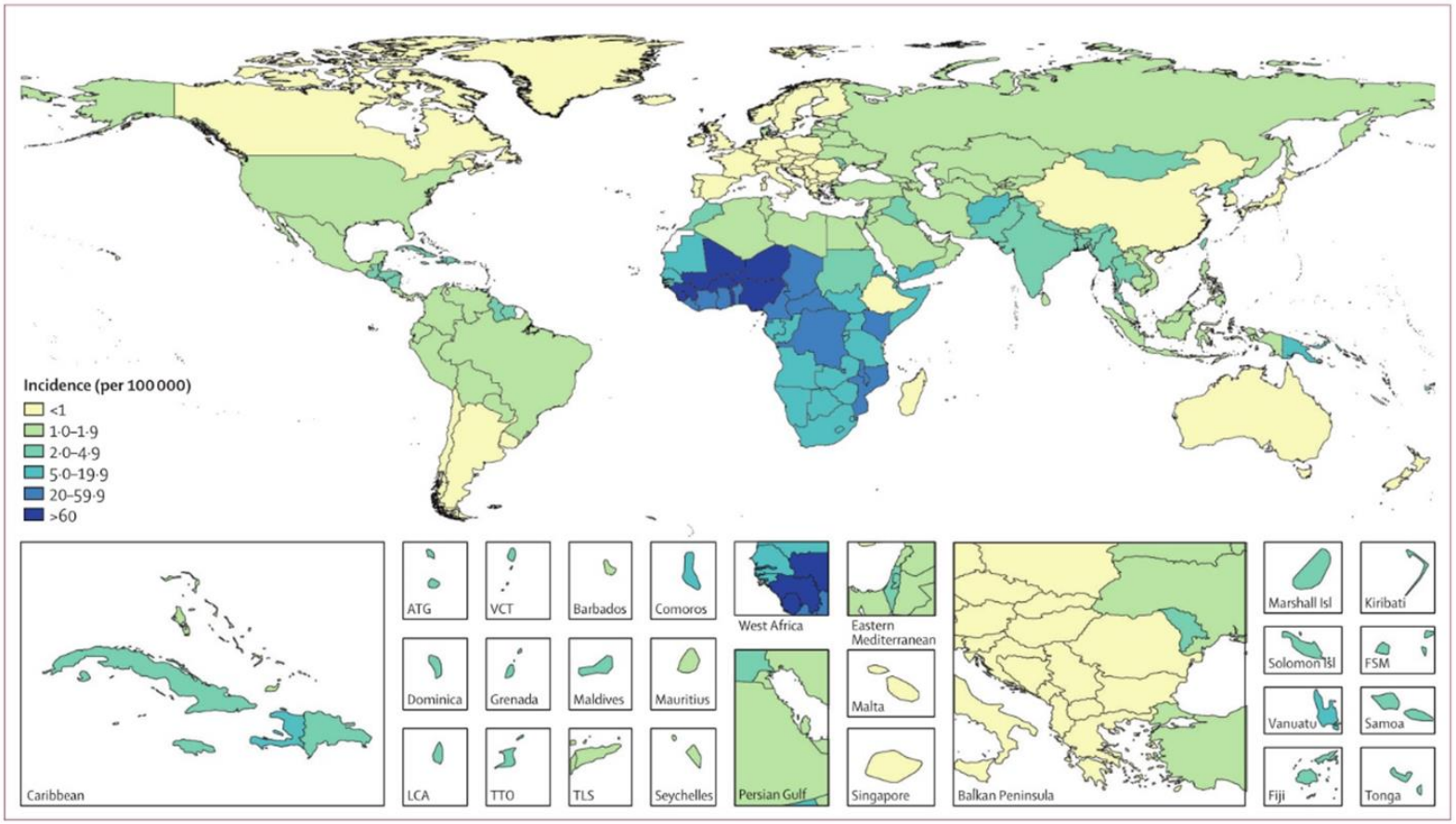

Figure 1. Incidence rates (per 100,000) of non-typhoidal *salmonella* invasive disease in 2017 [11].

White locations were not included to estimate by the Global Burden of Disease Study (GDB). The insert maps smaller locations, ATG; Antigua and Barbuda, VCT; Saint Vincent and the Grenadines, Isl; Island, FSM; Federated States of Micronesia, LCA; Saint Lucia, TTO; Trinidad and Tobago, TLS; Timor-Leste.

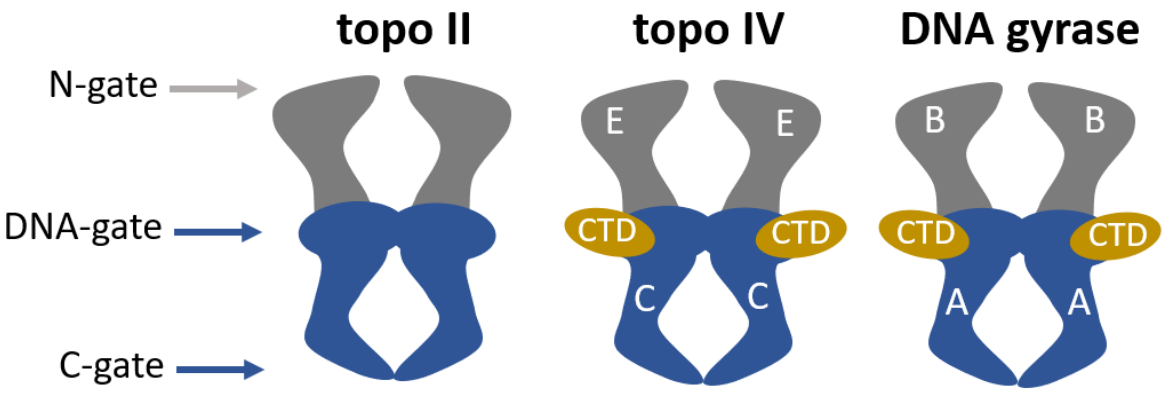

Figure 2. Structure of type IIA toposomerase as summarized by Klostermeier [23].

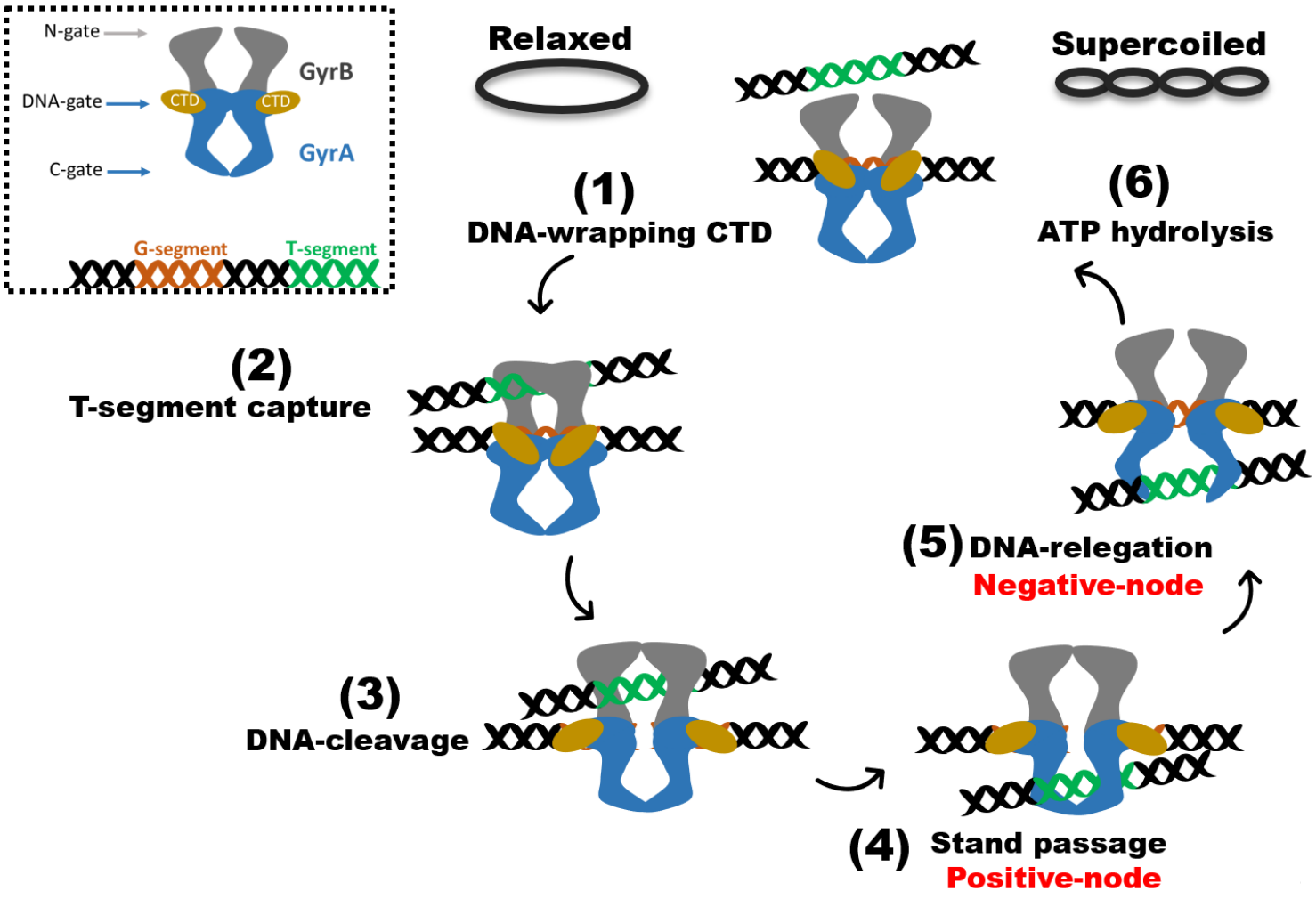

Figure 3. Strand DNA-passage mechanism of negative supercoiling with DNA gyrase and conformational changes as analyzed by Klostermeier [23].

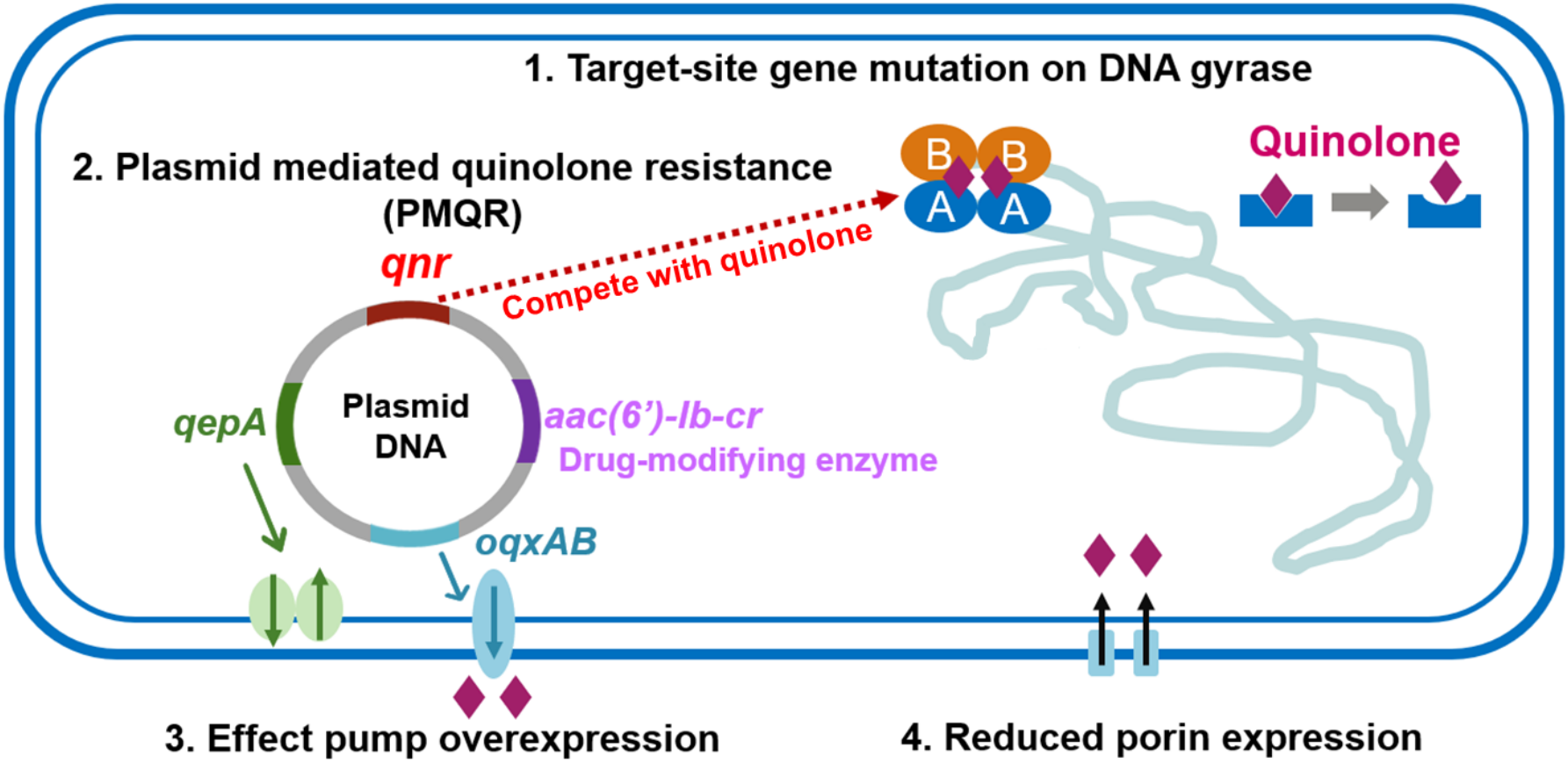

Figure 4. Quinolone resistance acquisitive mechanism as adapted from Correia *et al* [27].

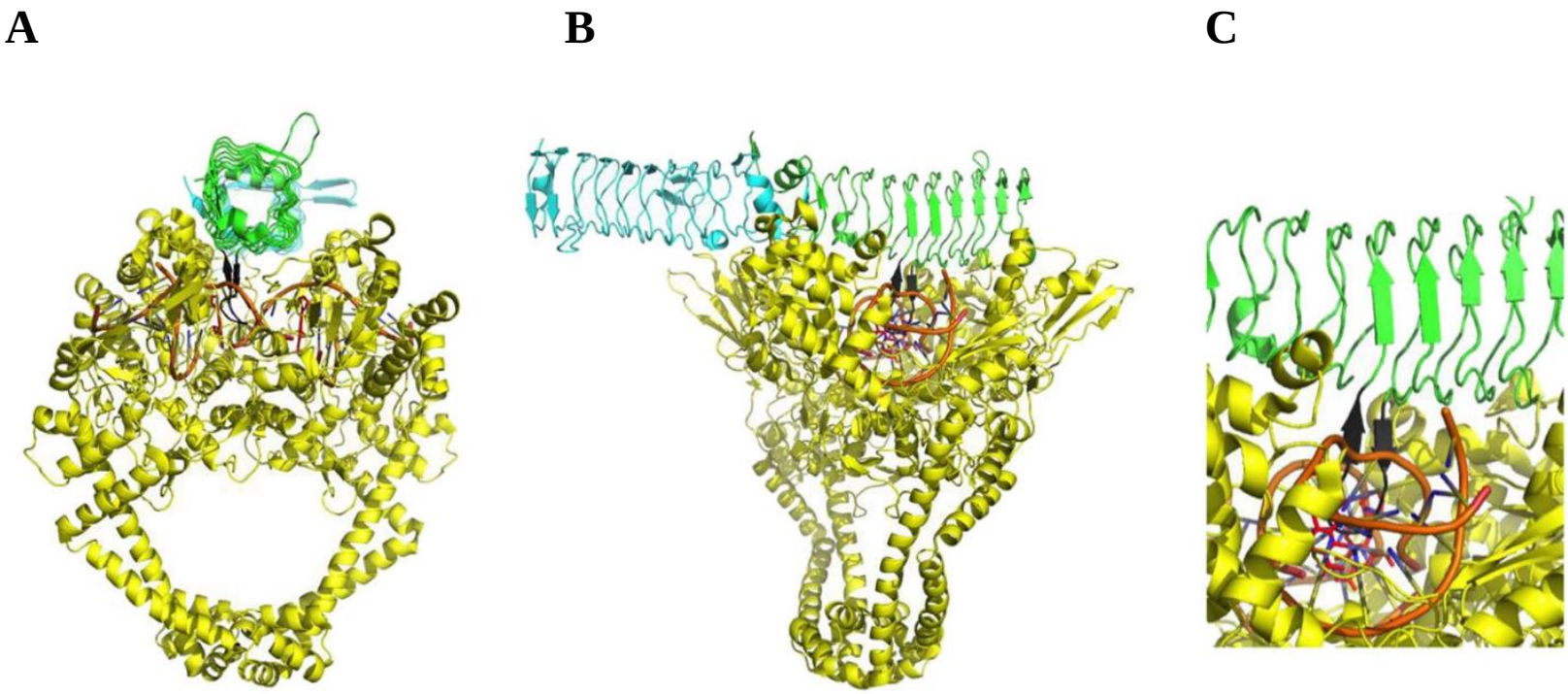

Figure 5. Model of the interaction of AhQnr with *S. aureus* DNA gyrase against ciprofloxacin [31]. (A) the crystal structure of *S.* aureus DNA gyrase (yellow) bound to G-segment DNA (orange) and two ciprofloxacin molecules (red) is demonstrated. AhQnr (dimer, is presented with one monomer colored green, the other cyan). (B) Loop 2 of AhQnr monomer positions (black) compete to the bound ciprofloxacin. (C) a zoomed image of (B).

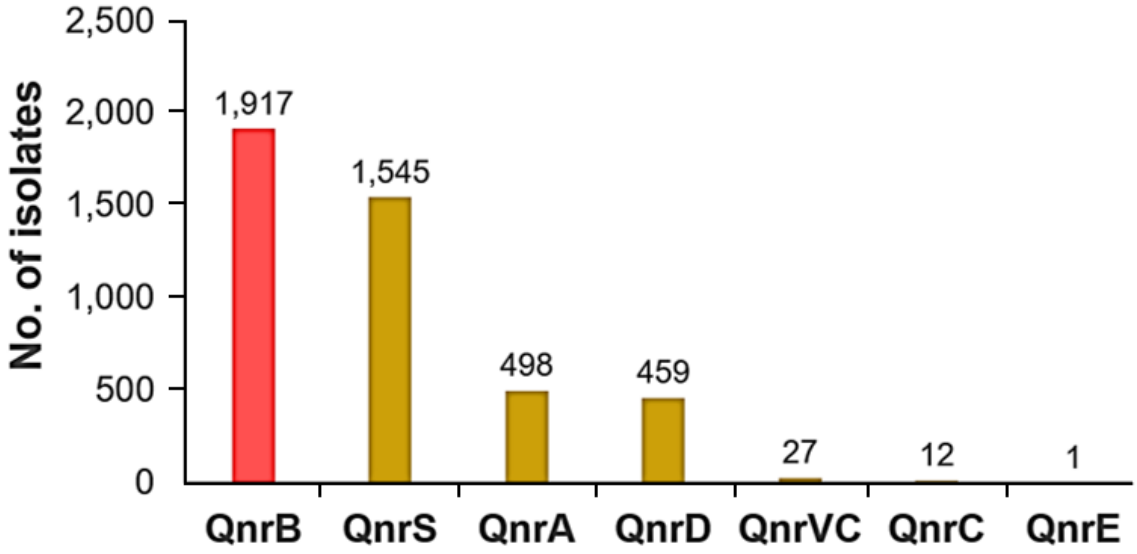

Figure 6. Qnr families were reported from 692 publications (2008 - 2017) with the distribution of QnrB (1,917), QnrS (1,545), QnrA (498), QnrD (459), QnrVC (27), QnrC (12) and QnrE (1) isolates.

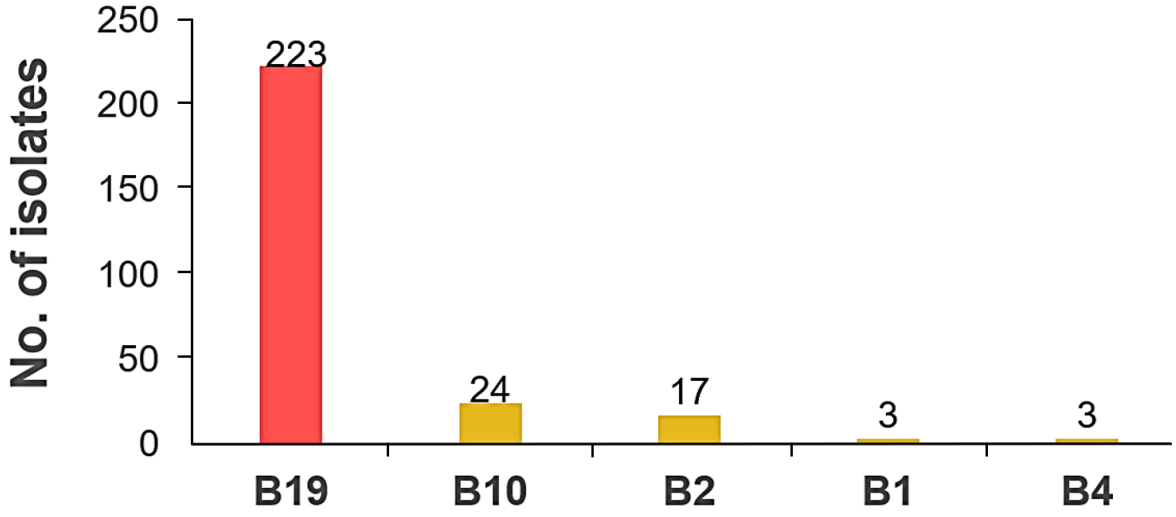

Figure 7. QnrB variants in *Salmonella* spp. were reported from 47 publications (2008 - 2017) with the distribution of QnrB19 (223), QnrB10 (24), QnrB2 (17), QnrB1 (3) and QnrB4 (3) isolates.

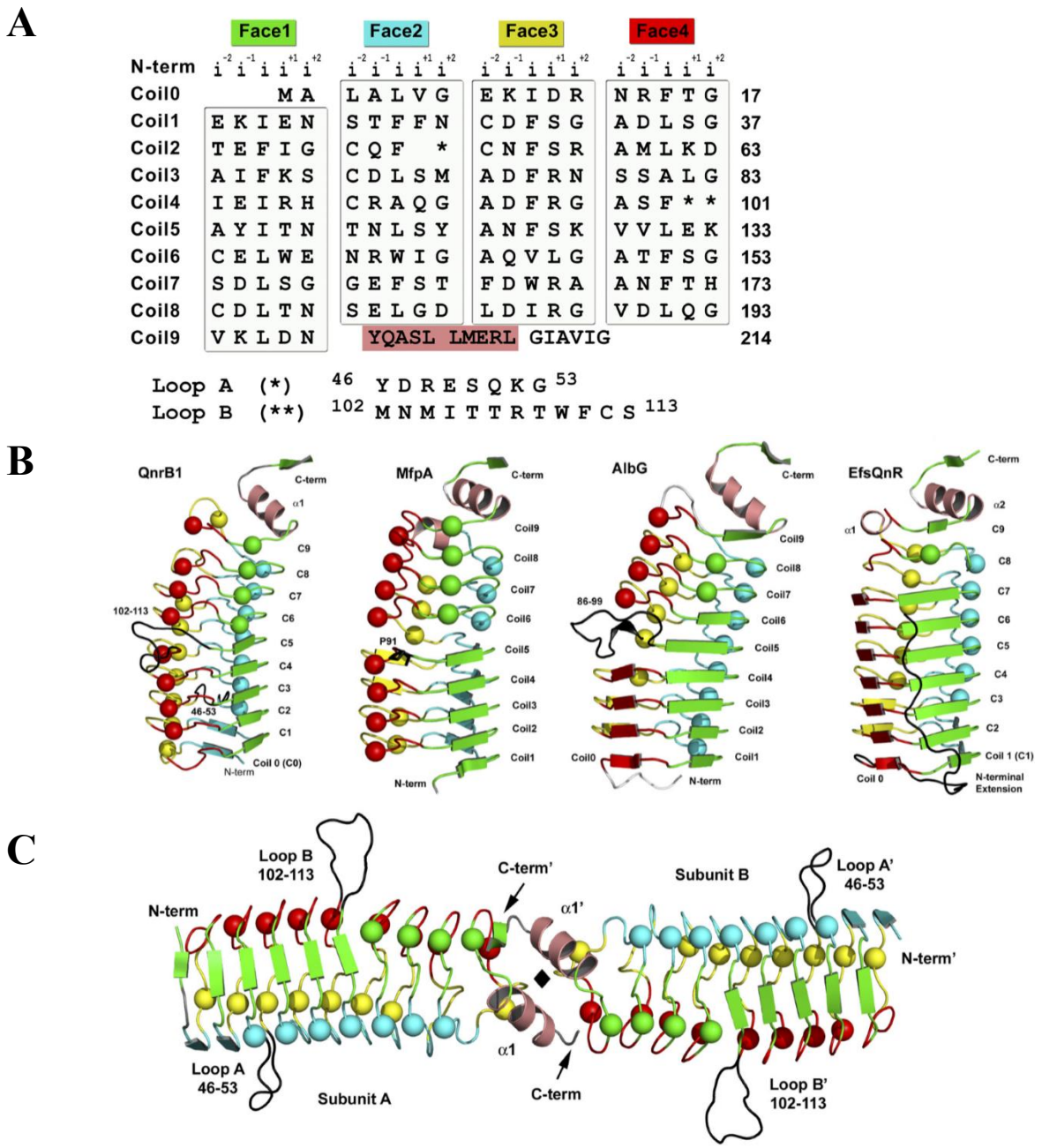

Figure 8. Structure of QnrB1 was analyzed by Vetting *et al* [37]. (A) Structure feature of QnrB1. (B) Similar monomer structures of QnrB1, MfpA, AlbG, and EfsQnr. (C) Dimeric structure of QnrB1.

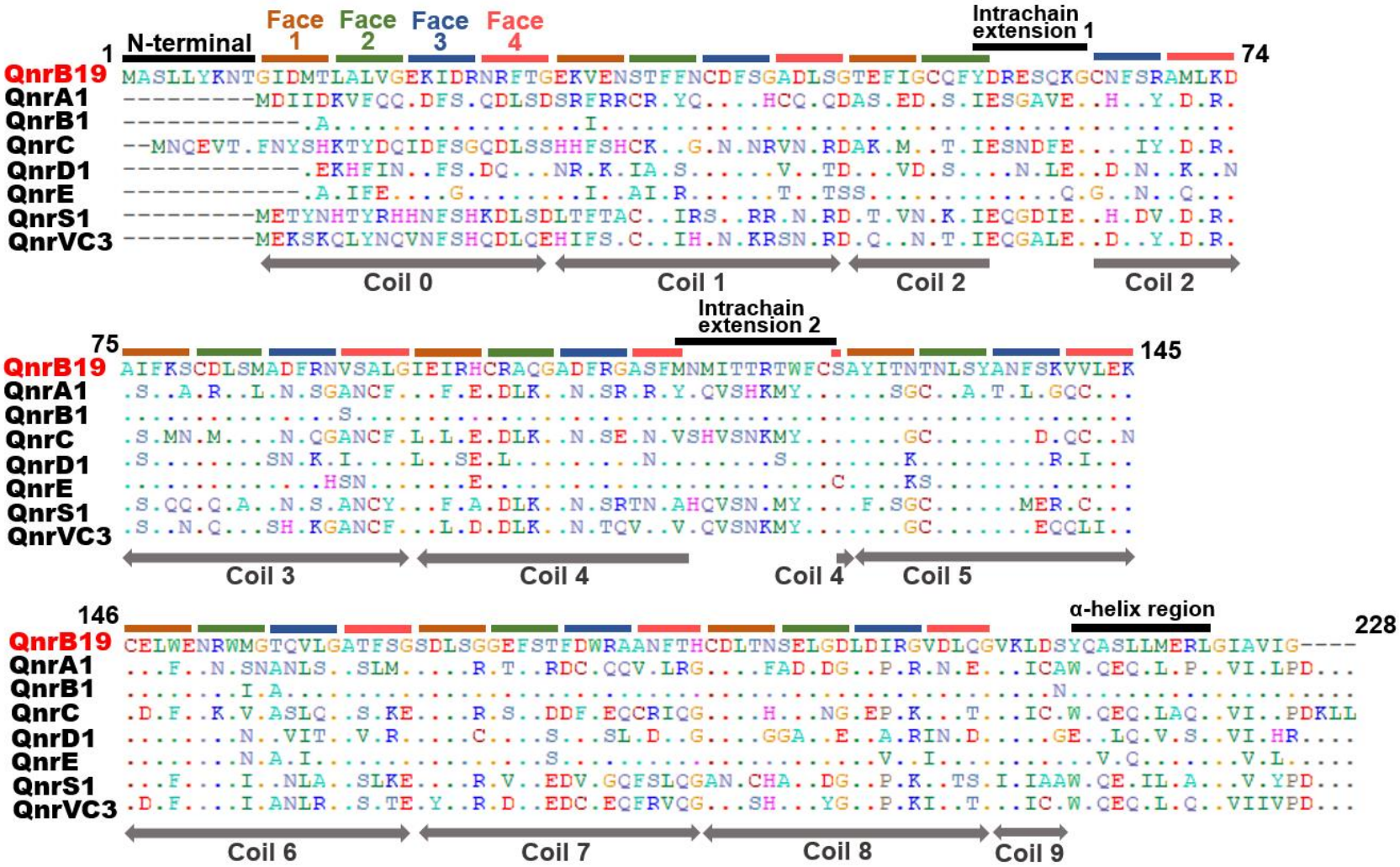

Figure 9. Comparation of amino acid sequences of Qnrs.

CHAPTER I

Interaction of the plasmid-encoded quinolone resistance protein QnrB19 with *Salmonella* Typhimurium DNA gyrase

Introduction

Fluoroquinolones have been used as the drugs of choice for the treatment of non-typhoidal *Salmonella* in humans and animals. Plasmid-mediated quinolone resistance mechanism is a serious problem for the treatment of infections. Plasmid-encoded Qnr proteins have been reported to reduce susceptibility to quinolone. From my review of 692 publications showed that QnrB19 to be the predominant Qnr in *Salmonella* spp. isolates. However, there is no study on the role of QnrB19 in the acquisition of quinolone resistance. Hence, in chapter I, *in vitro* DNA gyrase assay was conducted to observe the impact of QnrB19 in quinolone resistance acquisition utilizing recombinant *S.* Typhimurium DNA gyrases, QnrB19 and three conventional quinolones, nalidixic acid, ciprofloxacin and norfloxacin.

Materials and Methods

Reagents

In the guideline "Expert Rules v 3.2" (https://www.eucast.org/fileadmin/src/media/ PDFs/ EUCAST_files/Expert_Rules/2019/Salmonella_ExpertRules_V3.2_20190613.pdf) of The European Committee on Antimicrobial Susceptibility Testing (EUCAST), ciprofloxacin is used as the indicator agents for MIC against *Salmonella* spp. Hence, I selected ciprofloxacin for one target. In addition, a structurally related new quinolone, norfloxacin, and a structurally related old quinolone, nalidixic acid, were used for comparison. Ciprofloxacin was purchased from LKT Laboratories, Inc. (St Paul, MN). Nalidixic acid and norfloxacin were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The chemical structures of quinolone Nalidixic acid and fluoroquinolones Norfloxacin and Ciprofloxacin are shown in Figure 10. Kanamycin was purchased from Wako Pure Chemical Industries, Ltd. Ni-nitrilotriacetic acid (Ni-NTA) protein purification kits were obtained from Thermo Fisher Scientific Inc. (Waltham, MA). Restriction enzymes were obtained from New England Biolabs, Inc. (Ipswich, MA). Relaxed pBR322 DNA and supercoiled pBR322 DNA were purchased from John Innes Enterprises Ltd (Norwich, UK), and complete™, Mini, EDTA-free protease inhibitor cocktail tablets were purchased from Roche Applied Science (Mannheim, Germany).

Bacterial strains and plasmids

Synthetic target gene *qnrB19* of *S.* Typhimurium, accession No. NC_012916.1, in pUC57 plasmid was purchased from Genscript (Piscataway, NJ). Vector plasmid pET-28b(+) was purchased from Merck KGaA (Darmstadt, Germany) and used to construct expression plasmids for GyrA, GyrB and QnrB19. *E. coli* DH5 α (Takara Bio Inc., Shiga, Japan) was used as the cloning host. *E. coli* BL-21 (DE3) was purchased from Merck KGaA and used for expression of

GyrB. *E. coli* BL-21 (DE3)/pLysS was purchased from Merck KGaA and used for expression of GyrA and QnrB19.

Construction of *Salmonella* Typhimurium QnrB19 expression vector

Target gene *qnrB19* of *S.* Typhimurium in pUC57 was cut out with *Nco* I and *Xho* I and ligated into pET-28b(+) plasmid, which was the digested by these enzymes, and transformed into *E. coli* DH5α, as per the manufacturer's instructions. The sequence of the *qnrB19* gene in pET-28b(+) was confirmed using a BigDye Terminator (version 3.1) cycle sequencing kit and an ABI PRISM 3130*x* automated genetic analyzer (Applied Biosystems).

Expression and purification of QnrB19

Purification of the QnrB19 protein [40] was conducted as follows. *E. coli* BL21 (DE3)/pLysS carrying *qnrB19* in plasmid vector pET-28b(+) was cultured at 37 °C in 500 mL of Luria-Bertani (LB) broth (Wako Pure Chemical Industries Ltd.) with kanamycin (50 mg/L), and within an OD_{590} range of 0.4 - 0.6. Expression of QnrB19 was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside (Wako Pure Chemical Industries Ltd.). The bacteria were further incubated for 24 h at 18 °C. *E. coli* cells were retrieved by centrifugation, washed with cold buffer A [50 mM Tris, pH 8.0; 200 mM $(NH_4)_2SO_4$; 10% glycerol; and 20 mM imidazole], resuspended in buffer A supplemented with 1 mg/mL of lysozyme and 0.5% Triton X-100, and kept overnight at 4 °C. Using a Sonifier 250 (Branson, Danbury, CT), the lyzed suspension was sonicated on ice for 4 min in 10 cycles as follows: 40 sec of sonication, 40 sec of cooling, duty cycle at 30% and output at 4%. To remove DNA and obtain a clean supernatant, DNase was added to the lysate at a final concentration of 0.1 mg/mL, further incubated on ice for 15 min and centrifuged at 10,000 rpm for 30 min at 4 °C. The supernatant containing soluble proteins was then filtered with a 0.2-μm pored membrane and loaded onto the column with Ni-NTA agarose resin to trap the histidine-

tagged protein. Trapped proteins were eluted with different imidazole concentrations (20, 30, 40, 50, 100, and 200 mM) in buffer A containing an EDTA-free protease-inhibiting cocktail and collected in 1 mL fractions. Eluted fractions in 100 mM and 200 mM imidazole-containing buffer A were loaded onto a PD-10 column and eluted with buffer B [20 mM Tris-HCl, pH 8.0, 10% glycerol and 50 mM arginine] as previously described [46], and concentrated using Millipore Amicon Ultra tubes (Merck Millipore Ltd., Burlington, MA). After purification, the eluted fractions were combined using glycerol to a concentration of 40% (wt/vol) and kept at -80 °C until further use. The quality and quantity of purified protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE: Wako Pure Chemical Industries Ltd.).

Expression and purification of *S.* Typhimurium DNA gyrase subunits

S. Typhimurium DNA gyrase subunits (GyrA and GyrB) were expressed and purified according to the previous procedures using *E. coli* BL21 (DE3)/pLysS carrying *gyrA* in plasmid vector pET-28b(+) and *E. coli* BL21 (DE3) carrying *gyrB* in the same plasmid [47-50]. The quality and quantity of purified proteins were analyzed by SDS-PAGE.

Inhibition of *S.* Typhimurium DNA gyrase by quinolones and protection by QnrB19

The DNA supercoiling activity of *S.* Typhimurium DNA gyrase was assessed as previously described [47-50]. Briefly, the reaction mixture (total volume, 30 μL) consisting of a 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; 6.5% glycerol; 0.1 mg/mL of BSA), 1 mM ATP, relaxed pBR322 DNA (0.3 μg), 18 nM DNA gyrase subunits (with/without quinolones), 50 μg/mL of nalidixic acid, 1 μg/mL of norfloxacin and 0.5 μg/mL of ciprofloxacin, and various concentrations of QnrB19 (6 to 4,374 nM; 3-fold serial increases). Reactions were run at 35 °C for 60 min and stopped by adding 8 μL of 5X dye mix (5% SDS, 25% glycerol and 0.25 mg/mL of bromophenol blue). Total reaction

mixtures were diluted 4 times with a 1× DNA sample buffer (0.1% SDS; 0.05% Bromo Phenol Blue; 10% glycerol; and 100 mM Tris-HCl, pH 8.0) and analyzed by electrophoresis [1% agarose gel in Tris-borate-EDTA (TBE) buffer]. The gels were run for 96 min at 50 mA and stained with 0.5 μg/mL of GelRed (Wako Pure Chemical Industries Ltd.). Supercoiling activity was evaluated by tracing the brightness of the bands with software ImageJ (http://rsbweb.nih.gov/ij). To confirm reproducibility, the supercoiling activity assay was conducted at least three times.

Impact of QnrB19 on IC_{50} of quinolones

To assess the ability of QnrB19 to confer quinolone resistance to DNA gyrase, the DNA supercoiling activity of *S.* Typhimurium DNA gyrase under the presence of QnrB19 and quinolones was examined as previously described [47-50]. The reaction mixture (total volume, 30 μL) consisted of a 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; 6.5% glycerol; and 0.1 mg/mL of BSA), 1 mM ATP, relaxed pBR322 DNA (0.3 mg) with 18 nM DNA gyrase subunit, quinolones (3-fold serial concentration increases); 3.2 - 1,000 μg/mL of nalidixic acid, 0.032 - 10 μg/mL of norfloxacin, and 0.01 - 3.2 μg/mL of ciprofloxacin, in the absence or presence of 18 nM QnrB19. After reaction and analysis of the reaction products by agarose gel electrophoresis as in above section, the supercoiling activity was evaluated as described above. And the inhibitory effects of nalidixic acid, norfloxacin and ciprofloxacin on DNA gyrase were assessed by determining the drug concentrations required to inhibit the supercoiling activity of the enzyme by 50% (IC_{50}s), using the calculator at AAT Bioquest (https://www.aatbio.com/tools/ic50-calculator). To confirm reproducibility, the supercoiling activity assay was carried out at least three times.

Results

Expression and purification of recombinant QnrB19

Purified recombinant QnrB19 protein was obtained from the supernatant with a His-tag at approximately 25-kDa, by eluting it from Ni-NTA agarose column with the buffer containing 200 mM imidazole (Figure 11). Approximately 1.4 mg of recombinant QnrB19 protein with high purity (>95%; Figure 12, lanes 3) was obtained from 500 mL *E. coli* culture.

Expression and purification of recombinant *S.* Typhimurium DNA gyrase

Purified recombinant *S.* Typhimurium DNA GyrA and GyrB were obtained from supernatants with His-tags at approximately 97-kDa and 89-kDa, respectively (Figure 12, lanes 1 and 2). Approximately 0.12 mg and 0.23 mg of GyrA and GyrB, respectively, with high purity (>95%) were obtained from 500 mL *E. coli* culture.

Impact of QnrB19 on *S.* Typhimurium DNA gyrase supercoiling activity in the presence or absence of quinolones

Figure 13 shows the interaction between QnrB19, *S.* Typhimurium DNA gyrase and quinolones. At concentrations of 6 - 18 nM, QnrB19 showed little or less impact on DNA supercoiling activity of DNA gyrase. QnrB19 exhibited a concentration-dependent inhibitory activity against DNA gyrase at concentrations higher than 54 nM, while it completely inhibited the DNA gyrase activity at the highest concentration of 4,374 nM. On the other hand, QnrB19 protected DNA gyrase from the impact of nalidixic acid, norfloxacin and ciprofloxacin. Nalidixic acid at the concentration of 50 μg/mL (215 μM), norfloxacin at 1 μg/mL (3 μM) and ciprofloxacin at 0.5 μg/mL (1.5 μM) reduced the DNA gyrase activity to 23.4, 17.6 and 8.7 %, respectively. QnrB19 at the concentration of 6 - 54 nM restored the DNA gyrase activity in dose dependent

manner. DNA gyrase activities reduced by nalidixic acid, norfloxacin and ciprofloxacin were restored by addition of 54 nM QnrB19 up to approximately 1.8 times (From 23.4% to 42.4% of original DNA gyrase activity; Figure 13A and D), 3.6 times (from 17.6 % to 62.8 %; Figure 13B and E) and 7.1 times (from 8.7% to 62.0%; Figure 13C and F), respectively.

Effect of QnrB19 on IC_{50}s of quinolones

In a concentration-dependent manner, nalidixic acid, norfloxacin and ciprofloxacin inhibited *S.* Typhimurium DNA gyrase activity. Addition of 18 nM QnrB19, the same concentration to DNA gyrase, to the DNA supercoiling assay caused a little effect on the inhibitory properties of nalidixic acid (Figure 14A and D). In contrast, addition of same concentration of QnrB19 to those with norfloxacin and ciprofloxacin reduced the inhibitory effect of these quinolones on the DNA gyrase activity (norfloxacin; Figure 14B and E, ciprofloxacin; Figure 14C and F). Table 1 shows the effect of QnrB19 on IC_{50}s of quinolones against *S.* Typhimurium DNA gyrase. While the IC_{50} of nalidixic acid was not affected by QnrB19, the those of norfloxacin and ciprofloxacin had been increased to 2.7- and 3-fold, respectively.

Discussion

Fluoroquinolones are the drugs of choice for the treatment of NTS; however, resistance to fluoroquinolones has significantly increased in bacteria. In the present study, I focused on the QnrB19, the predominant Qnr among *Salmonella* spp. Recombinant QnrB19 and DNA gyrase subunits were expressed in *E. coli* host cells and purified by Ni-NTA agarose column chromatography with high purity. Utilizing recombinant proteins, DNA supercoiling assays were conducted with three quinolones in the presence or absence of QnrB19 to access the property (Figure 13A - C). Results showed that QnrB19 had little impact on DNA supercoiling activity of DNA gyrase (18 nM GyrA and 18 nM GyrB) at the concentration of 6 - 18 nM; however, increasing amount of QnrB19 exerted the ability to reduce the activity of DNA gyrase in concentration dependent manner. On the contrary, QnrB19 showed the ability to protect DNA gyrase from the effect of nalidixic acid (215 μM; 50 μg/mL), norfloxacin (3 μM; 1 μg/mL) and ciprofloxacin (1.5 μM; 0.5 μg/mL). At the concentration of 54 nM, QnrB19 exhibited the maximum restoring ability on the nalidixic acid, norfloxacin and ciprofloxacin inhibited DNA supercoiling activity of DNA gyrase by approximately 1.8-, 3.6- and 7.1-fold, respectively (Figure 13D - F). Indeed, the analyses showed that QnrB19 protected DNA gyrase from the impact of quinolones at certain extent depending on quinolones used. This agreed with the previous epidemiological information reported. It is noteworthy that QnrB19 totally abolished DNA supercoiling activity at the highest concentration of 4,374 nM even without quinolones. QnrB19 at very high concentration might compete with the substrates of DNA gyrase such as positively supercoiled or relaxed DNA and inhibit the supercoiling activity of DNA gyrase even in the absence of quinolones because of the similar three dimensional structure of QnrB19 and DNA double strand. My results are similar to those previously reported by *in vitro* studies [29, 38, 40, 51, 52]. For example, previous work showed that at 2.17, 0.725 and 0.242 μM, QnrA1

protected *E. coli* DNA gyrase from inhibition (96%, 75%, 31%, respectively) by ciprofloxacin (1.5 μM; 0.5 μg/mL) [29]. Nonetheless, at 81, 27, 9 and 3 nM, QnrA1 exerted no protection for ciprofloxacin (1.5 μM; 0.5 μg/mL) inhibited DNA gyrase. It is worth noting that a simple concentration of 2.01 μM QnrA1 seemed to be enough to fully restore the activity of DNA gyrase, which had been inhibited by 0.75 μM ciprofloxacin [29]. Nevertheless, 2.01 μM QnrA1 is progressively less effective against the inhibitory activity of 3.0 μM or 6.0 μM ciprofloxacin [29]. A separate study [38] reported that little protection of *E. coli* DNA gyrases occurred if concentrations of QnrB1 were low in relation to ciprofloxacin (2 μg/mL; 6 μM). However, the protection increased to 50% when ciprofloxacin was 0.5 nM and to 100% when ciprofloxacin was 5 pM [38]. Nonetheless, a concentration of 25 μM QnrB1 was observed to completely inhibit DNA gyrase activity [38]. Separately, purified QnrS1 (0.05 nM) was shown to fully protect *E. coli* DNA gyrase supercoiling activity from the effect of 0.6 μg/mL (1.8 mM) of ciprofloxacin, and a 100-fold lower concentration (0.5 pM) of QnrS1 could still provide half protection [40]. *Efs*Qnr, the other chromosomally encoded pentapeptide repeat protein in *Enterococcus faecalis*, was also shown to partially protect *E. coli* DNA gyrase from ciprofloxacin inhibition, even at concentrations as low as 20 nM [51]. Thus, from all the above evidence, it can be inferred that a very delicate interaction takes place between quinolones, Qnr and DNA gyrases, and that Qnr protection is inversely proportional to the concentrations of quinolones.

Figure 14 and Table 1 showed that IC_{50} nalidixic acid was not affected by the presence of 18 nM QnrB19. In contrast, to be effective, the IC_{50}s of norfloxacin and ciprofloxacin had been increased to 2- to 3-fold. The IC_{50} of ciprofloxacin for *E. coli* DNA gyrase in the absence of QnrB4 was reported to be 1 μM, but it had to be increased 5-fold if the concentration of QnrB4 was 0.5 μM [39]. Similarly, in the absence of *Efs*Qnr, the IC_{50} of ciprofloxacin for *E. faecalis* DNA gyrase was about 0.25 μM, but in the presence of 0.2 μM *Efs*Qnr, the IC_{50} had to be increased to 1.4 μM [51, 52]. Those studies seemed to suggest that DNA gyrase activity was

protected from quinolone inhibition by Qnr in Gram-negative bacteria (*E. coli*) and Gram-positive bacteria (*E. faecalis*). In the present work, QnrB19 was shown to protect *S.* Typhimurium DNA gyrase from the inhibitory effects of norfloxacin and ciprofloxacin. Other studies also showed that QnrA1, QnrB1, QnrB4, QnrS1 and *Efs*Qnr protected *E. coli* DNA gyrase from ciprofloxacin [29, 38, 39, 40, 51, 52].

To the best of my knowledge, this is the first report that compared the effect of QnrB19 on *S.* Typhimurium DNA gyrases by specific features of quinolones at positions R1, R6, R7 or R8 (Table 1). Position R1 forms part of the enzyme-DNA-quinolone complex and has a hydrophobic interaction with a major grove in DNA [53]. This position in quinolones is known to correlate with the inhibition of DNA supercoiling activity of DNA gyrase. Ciprofloxacin has cyclopropyl substituents at this position, however, norfloxacin and nalidixic acid have an ethyl group. QnrB19 at the concentration 54 nM restored norfloxacin inhibited DNA gyrase activity up to 3.6-fold which is two times lower than ciprofloxacin (restoration by QnrB19 was 7.1-fold) as shown in Figure 13. The IC_{50} of ciprofloxacin (0.16 µg/mL) was 1.88-fold lower than norfloxacin (0.3 µg/mL) in the absence of QnrB19 as shown in Table 1. This was similar to the difference of IC_{50}s between these two quinolones in the presence of 18 nM QnrB19 (0.48 and 0.81 µg/mL for ciprofloxacin and norfloxacin, respectively). These results showed that the contribution of QnrB19 did not associate with the difference between R1 group of these two quinolones.

Furthermore, other positions in norfloxacin and ciprofloxacin such as R6 with fluorine or R7 with piperazine directly interacted with DNA gyrases, and position R8 with hydrogen substituents alters drug access to the enzyme or DNA binding sites [53]. QnrB19 at the concentration of 54 nM, nalidixic acid (with hydrogen at R6, methyl group at R7 and nitrogen at R8) inhibited DNA supercoiling activity of DNA gyrase was restored up to 1.8-fold. The restoration activity of QnrB19 was 2- and 4-folds lower comparing to those against norfloxacin and ciprofloxacin inhibited DNA supercoiling activity of DNA gyrase, respectively. The IC_{50} of

nalidixic acid (17.7 µg/mL) was 59- and 110-folds higher than norfloxacin (0.3 µg/mL) and of ciprofloxacin (0.16 µg/mL) in the absence of QnrB19, respectively, and this was similar to the that in the presence of 18 nM QnrB19 (17.3 µg/mL) as shown in Table 1. These results showed that the fluorine at R6 and/or piperazine at R7 and/or hydrogen at R8 group might associate with the ability of QnrB19 to increase IC_{50}s.

In the present study, QnrB19 was shown to have ability to protect *S.* Typhimurium DNA gyrase from the inhibitory effects of norfloxacin and ciprofloxacin but have less protective ability from nalidixic acid. This is the first direct *in vitro* evidence of QnrB19 to be the cause of non-classical quinolone resistance.

Summary

Plasmid-encoded quinolone resistance protein Qnr is an important factor in bacterial resistance to quinolones. Qnr interacts with DNA gyrase and reduces susceptibility to quinolones. The gene *qnr* likely spreads rapidly among Enterobacteriaceae via horizontal gene transfer. Though the vast amounts of epidemiological data are available, molecular details of the contribution of QnrB19, the predominant Qnr in *Salmonella* spp., to the acquisition of quinolone resistance has not yet been understood well.

I aimed to examine the role of QnrB19 in quinolone resistance acquisition using recombinant *S.* Typhimurium DNA gyrases and QnrB19. Recombinant QnrB19 was expressed in *E. coli* and purified by Ni-NTA agarose column chromatography. DNA supercoiling activities of recombinant *S.* Typhimurium DNA gyrase were assessed with or without QnrB19 under the existence of three quinolones to measure IC_{50}s, the concentration of each quinolone required for 50% inhibition *in vitro*.

The IC_{50}s of norfloxacin, ciprofloxacin and nalidixic acid against DNA gyrases were measured to be 0.30, 0.16 and 17.7 μg/mL, respectively. The addition of QnrB19 increased the IC_{50}s of norfloxacin and ciprofloxacin to be 0.81 and 0.48 μg/mL, respectively, where no effect of QnrB19 was observed on the IC_{50} of nalidixic acid. The IC_{50} of ciprofloxacin was 1.88-fold lower than norfloxacin in the absence of. This was similar to the difference of IC_{50}s between these two quinolones in the presence of 18 nM. These results showed that the contribution of QnrB19 did not associate with the difference between R1 group of these two quinolones. Other positions in norfloxacin and ciprofloxacin such as R6 with fluorine or R7 with piperazine directly interacted with DNA gyrases, and position R8 with hydrogen substituents alters drug access to the enzyme or DNA binding sites. In the presence of QnrB19, nalidixic acid inhibited DNA supercoiling activity of DNA gyrase was restored up to 1.8-fold. The restoration activity of QnrB19 was 2-

and 4-folds lower comparing to those against norfloxacin and ciprofloxacin inhibited DNA supercoiling activity of DNA gyrase, respectively. The IC_{50} of nalidixic acid was 59- and 110-folds higher than norfloxacin and ciprofloxacin in the absence of QnrB19, respectively, and this was similar to the that in the presence of 18 nM QnrB19. These results showed that the fluorine at R6 and/or piperazine at R7 and/or hydrogen at R8 group might associate with the ability of QnrB19 to increase IC_{50}s. QnrB19 was shown for the first time *in vitro* to have ability to grant non-classical quinolone resistance to *S.* Typhimurium DNA gyrase in connection with the structure at R6 and/or R7 and/or R8.

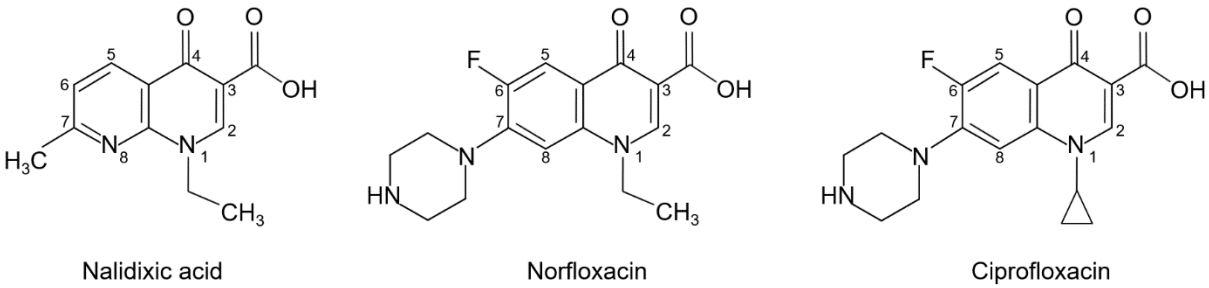

Figure 10. Chemical structures of quinolones used in the present study.

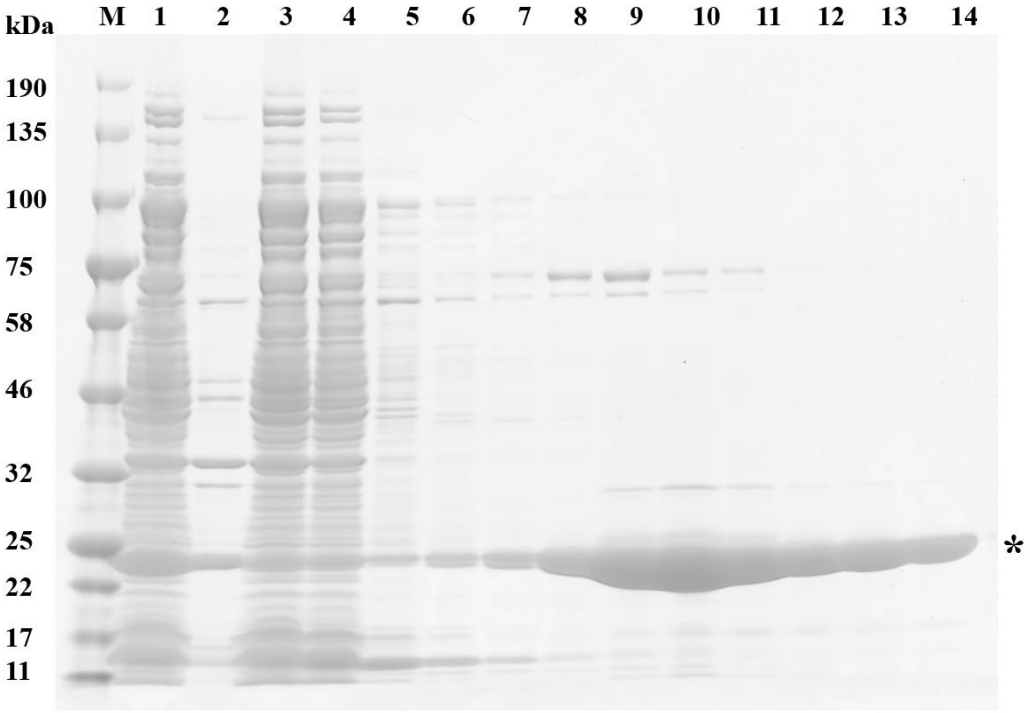

Figure 11. Expression and purification of recombinant QnrB19 by using SDS-PAGE.

The gene encoding QnrB19 was introduced into *Escherichia coli* expression vector pET28b. Recombinant QnrB19 was expressed in *E. coli* and purified by Ni-NTA agarose column chromatography. Two- μL of each sample was loaded on a SuperSep™ Ace 5-20% gradient gel. lane M: markers; lane 1: supernatant of *E. coli* lysate after centrifuge; lane 2: resuspended pellet of centrifuge in the original volume; lane 3: pass-through of Ni-NTA agarose column; lanes 4 - 14: eluted fraction by 20, 30, 40, 50, 100, 200 (fraction 1), 200 (fraction 2), 300 (fraction 1), 300 (fraction 2), 300 (fraction 3) and 300 (fraction 4) mM imidazole. "*" denotes recombinant QnrB19 with the MW of ~25 kDa.

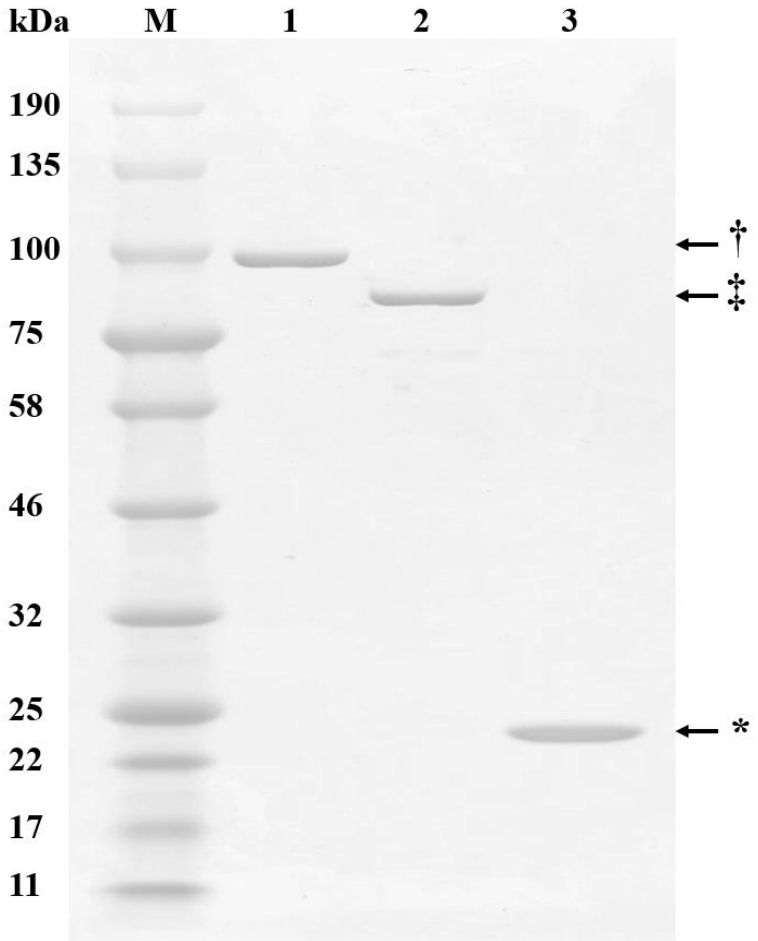

Figure 12. Purified recombinant DNA gyrase subunits of *S.* Typhimurium and QnrB19 used in this study.

Recombinant DNA gyrase of *S.* Typhimurium and QnrB19 expressed and purified as in Materials and Methods were examined their purity by SDS-PAGE. Each protein sample (500 ng) was loaded on a SuperSep™ Ace 5-20% gradient gel. lane M: protein size markers, lane 1: GyrA, lane 2: GyrB, lane 3: QnrB19. "†" denotes GyrA with the MW of ~97 kDa, "‡" denotes GyrB with the MW of ~89 kDa, "*" denotes recombinant QnrB19 with the MW of ~25 kDa.

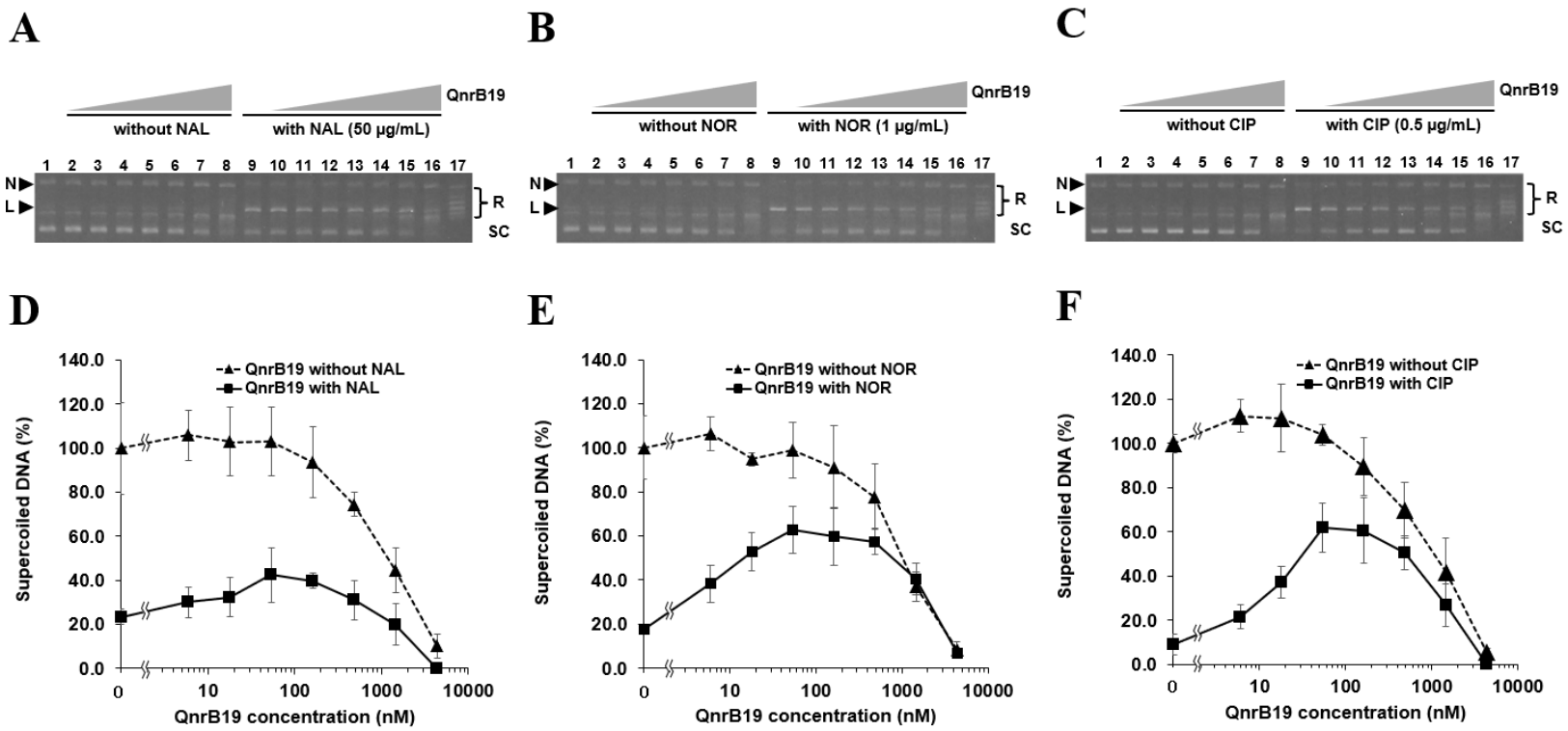

Figure 13. Inhibition of wild type *S*. Typhimurium DNA gyrase by quinolones and protection by QnrB19

Reaction mixtures were diluted 4 times with 1× DNA sample buffer and 10 μL of them were analyzed by electrophoresis in 1% agarose gels. lanes 2 - 16: contained 0.3 μg relaxed plasmid pBR322, reaction buffer and 18 nM DNA gyrase subunit. Lane 1 - 8: with the absence of nalidixic acid/norfloxacin/ciprofloxacin; lane 9 - 16: with the presence of 50 μg/mL nalidixic acid (A) 1 μg/mL norfloxacin (B) 0.5 μg/mL ciprofloxacin (C). Lanes 2 and 10: with 6 nM QnrB19; lanes 3 and 11: with 18 nM QnrB19; lanes 4 and 12: with 54 nM QnrB19; lanes 5 and 13: with 162 nM QnrB19; lanes 6 and 14: with 486 nM QnrB19; lanes 7 and 15: with 1458 nM QnrB19; lanes 8 and 16: with 4374 nM QnrB19; lane 17: relaxed plasmid pBR322. R, SC, L and N denote relaxed, supercoiled, linear and nicked form of pBR322 DNA, respectively. In panel D - F, dotted lines with filled triangles denote the effect of QnrB19 on the DNA supercoiling activity of DNA gyrase (expressed as % activity comparing to that without QnrB19). Solid lines with filled squares denote those with the presence of 50 μg/mL nalidixic acid (D) 1 μg/mL norfloxacin (E) 0.5 μg/mL ciprofloxacin (F).

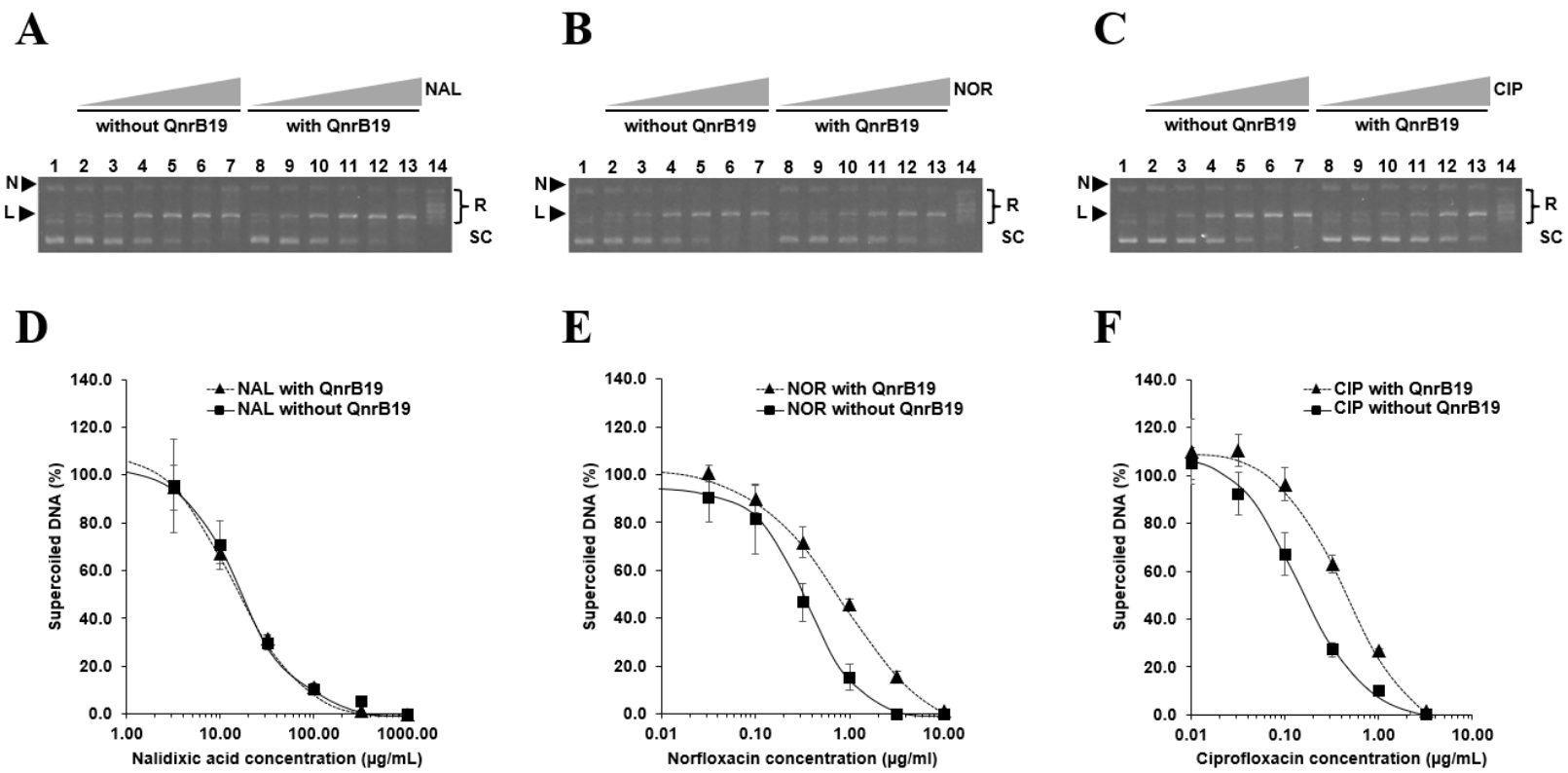

Figure 14. Effect of QnrB19 on IC_{50}s of quinolones

Reaction mixtures were diluted 4 times with 1× DNA sample buffer and 10 μL of them were analyzed by electrophoresis in 1% agarose gels. Lane 1 - 13: Reaction mixture contained 0.3 μg relaxed plasmid pBR322, reaction buffer and 18 nM DNA gyrase subunit; Lanes 2 - 7: with 3.2 - 1,000 μg/mL nalidixic acid (A), with 0.032 - 10 μg/mL norfloxacin (B), with 0.01 - 3.2 μg/mL ciprofloxacin (C) in the absence of QnrB19; lanes 8 - 13: with 3.2-1,000 μg/mL nalidixic acid (A), with 0.032-10 μg/mL norfloxacin (B), with 0.01-3.2 μg/mL ciprofloxacin (C) in the presence of 18 nM QnrB19; lane 14: relaxed plasmid pBR322. R, SC, L and N denote relaxed, supercoiled, liner and nicked form of pBR322 DNA, respectively. Solid lines with filled squares in panels D, E and F denote the impact of nalidixic acid, norfloxacin and ciprofloxacin on the DNA supercoiling activity of DNA gyrase (expressed as % activity comparing to that without quinolones), respectively. Dotted lines with filled triangles denote those in the presence of 18 nM QnrB19.

Table 1. Effect of the IC_{50}s of quinolones on wild type *S.* Typhimurium DNA gyrase in the presence or absence of QnrB19

Quinolones	R-1[a]	R-6[a]	R-7[a]	R-8[a]	IC_{50}[b] (μg/mL)		Ratio (Without : With QnrB19)
					Without QnrB19	With QnrB19	
Nalidixic acid	Ethyl	Hydrogen	Methyl	Nitrogen	17.7 ± 0.35	17.3 ± 0.18	-
Norfloxacin	Ethyl	Fluorine	Piperazine	Hydrogen	0.30 ± 0.24	0.81 ± 0.09	1 : 2.7
Ciprofloxacin	Cyclopropyl	Fluorine	Piperazine	Hydrogen	0.16 ± 0.20	0.48 ± 0.17	1 : 3.0

[a] Position of substituents in the structures of quinolones.

[b] IC_{50}: Concentrations of quinolones to inhibit the supercoiling activity of DNA gyrase by 50%.

CHAPTER II

Impact of QnrB19 on the inhibitory activity of fluoroquinolones carrying difluoropyridine derivatives as R1 groups

Introduction

Novel fluoroquinolone, WQ-3810 with a unique substituent at the R1 groups (6-amino-3,5-difluoropyridine-2-yl) showed potent inhibitory activity against several pathogenic bacterial species including *S.* Typhimurium [41-44]. Recently, WQ-3334 and WQ-4065 with a similar structure to WQ-3810 have also been developed and are expected to have a strong inhibitory effect on fluoroquinolone resistant bacteria like WQ-3810. However, there are limited reports on the inhibitory activities of these compounds against *S.* Typhimurium DNA gyrase in the absence and presence of QnrB19. Hence, in chapter II, I examined the inhibitory activities of these compounds against DNA gyrase of *S.* Typhimurium and elucidated the impact of QnrB19 in quinolone resistance by utilizing recombinant *S.* Typhimurium DNA gyrases, QnrB19 and these fluoroquinolones.

Materials and Methods

Antibacterial agents

The antibacterial agents, WQ-3810, WQ-3334 and WQ-4065 were provided by Wakunaga Pharmaceutical Co., Ltd. (Osaka, Japan) (Figure 15).

Expression and purification of recombinant *S.* Typhimurium DNA gyrase subunits and QnrB19

Recombinant *S.* Typhimurium DNA gyrase subunits and QnrB19 used in this study were prepared as in Chapter I.

Inhibition of *S.* Typhimurium DNA gyrase by quinolones and protection by QnrB19

The DNA supercoiling activity of *S.* Typhimurium DNA gyrase was evaluated as in Chapter I. Briefly, reaction mixture (30 μL) contained 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; 6.5% glycerol; 0.1 mg/mL of BSA), 1 mM ATP, relaxed pBR322 DNA (0.3 μg), 18 nM DNA gyrase subunits (with/without fluoroquinolones), 0.16 μg/mL of WQ-3810, 0.64 μg/mL of WQ-3334 and 2.56 μg/mL of WQ-4065, and various concentrations of QnrB19 (6 to 4,374 nM; 3-fold serial increases). The reactions were incubated at 35 °C for 60 min and stopped by adding 8 μL of 5X dye mix (5% SDS, 25% glycerol and 0.25 mg/mL of bromophenol blue). After incubation, the reaction mixtures were diluted 4 times with a 1× DNA sample buffer (0.1% SDS; 0.05% Bromo Phenol Blue; 10% glycerol; and 100 mM Tris-HCl, pH 8.0) and loaded onto 1% agarose gel in Tris-borate-EDTA (TBE) buffer for electrophoresis (96 min at 50 mA). The gel was stained with 0.5 μg/mL of GelRed (Wako Pure Chemical Industries Ltd.). The amount of DNA supercoiling activity was measured by tracing the intensity of the bands with software ImageJ

(http://rsbweb.nih.gov/ij). To confirm reproducibility, DNA supercoiling activity assay was carried out three times in the reaction mixture.

Impact of QnrB19 on IC_{50} of fluoroquinolones

To examine the ability of QnrB19 to provide fluoroquinolone resistance to DNA gyrase, the DNA supercoiling activity of *S.* Typhimurium DNA gyrase in the presence of QnrB19 and fluoroquinolones was investigated as previously described [54]. The reaction mixture was prepared in a final volume of 30 µL consist of DNA gyrase assay buffer, 1 mM ATP, relaxed pBR322 DNA (0.3 mg) with 18 nM DNA gyrase subunit, fluoroquinolones (3-fold serial concentration increases); 0.01 - 3.2 µg/mL of WQ-3810, 0.032 - 10 µg/mL of WQ-3334, and 0.32 - 100 µg/mL of WQ-4065, in the absence or presence of 18 nM QnrB19. Reactions, gel electrophoresis and measuring DNA supercoiling activity were performed as explained in previous sections. And the inhibitory effects of WQ-3810, WQ-3334 and WQ-4065 on DNA gyrase were assessed by determining the drug concentrations required to reduce by 50% the supercoiling activity (IC_{50}s), using the calculator at AAT Bioquest (https://www.aatbio.com/tools/ic50-calculator). To confirm reproducibility, the DNA supercoiling activity assay was carried out in triplicate.

Results

Inhibitory activities of WQ-3810, WQ-3334 and WQ-4065 against *S*. Typhimurium DNA gyrase.

IC_{50}s of WQ-3810, WQ-3334 and WQ-4065 were calculated by *in vitro* DNA gyrase assay with the increasing concentration of each quinolones to be 0.031 ± 0.003, 0.068 ± 0.016 and 0.72 ± 0.39 µg/mL, respectively (Table 2).

Impact of QnrB19 on *S*. Typhimurium DNA gyrase supercoiling activity in the presence or absence of fluoroquinolones

Figure 16 shows the interaction between QnrB19, *S.* Typhimurium DNA gyrase and fluoroquinolones. At concentrations of 6 - 18 nM, QnrB19 showed little or less impact on DNA supercoiling activity of DNA gyrase. QnrB19 exhibited a concentration-dependent inhibitory activity against DNA gyrase at concentrations higher than 54 nM, while it completely inhibited the DNA gyrase activity at the highest concentration of 4,374 nM. On the other hand, QnrB19 protected DNA gyrase from the impact of WQ-3810, WQ-3334 and WQ-4065. WQ-3810 at the concentration of 0.16 µg/mL, WQ-3334 at 0.64 µg/mL and WQ-4065 at 2.56 µg/mL reduced the DNA gyrase activity to 24.2, 11.1 and 19.3 %, respectively. QnrB19 at the concentration of 6 - 162 nM restored the DNA gyrase activity in dose dependent manner. DNA gyrase activities reduced by WQ-3810, WQ-3334 and WQ-4065 were restored by addition of 18 - 162 nM QnrB19 up to approximately 4.28 - 4.45 times (From 24.2%, and 107.7% to 103.5% of original DNA gyrase activity; Figure 16C), 5.78 - 6.80 times (From 11.1%, and 64.2% to 75.5% of original DNA gyrase activity; Figure 16C) and 4.89 - 5.18 times (From 19.3%, and 94.3% to 99.9% of original DNA gyrase activity; Figure 16C), respectively.

Effect of QnrB19 on IC_{50}s of fluoroquinolones

In a concentration-dependent manner, WQ-3810, WQ-3334 and WQ-4065 inhibited *S.* Typhimurium DNA gyrase activity. Addition of 18 nM QnrB19, the same concentration to DNA gyrase, DNA supercoiling activity was reduced by the inhibition of WQ-3810, WQ-3334 and WQ-4065 (WQ-3810; Figure 17A and D, WQ-3334; Figure 17B and E, and WQ-4065; Figure 17C and F). Table 2 shows the effect of QnrB19 on IC_{50}s of fluoroquinolones against *S.* Typhimurium DNA gyrase. The IC_{50} of WQ-3810, WQ-3334 and WQ-4065 had been increased to 14.2-, 13.5- and 12.7 -folds by QnrB19, respectively.

Discussion

Fluoroquinolones are drugs of choice for treating NTS infections, however, acquisition of resistance by *Salmonella* pathogens has limited the use of these drugs. WQ-3810 with a unique substituent at the R1 groups (6-amino-3,5-difluoropyridine-2-yl) and R7 groups (3-isopropylaminoazetizine-1-yl) has shown high activity against fluoroquinolone resistant pathogens. Two novel fluoroquinolones, WQ-3334 and WQ-4065 with similar structure to WQ-3810 has also been developed. However, there are limited reports on the correlation between structural feature and inhibitory activities of these compounds against *S.* Typhimurium DNA gyrase. In addition, there is no report on the impact of Qnr families against these compounds.

In our previous *in-silico* study, interaction of the R1-group in WQ-3810 with aspartic acid at position 464 in GyrB was revealed to enhance the inhibitory effect of this compound against DNA gyrase of *M. leprae* [44]. WQ-3810 and WQ-3334 have identical R1 group; 6-amino-3,5-difluoropyridine-2-yl. This might add high inhibitory activity (IC_{50}; 0.068 $\pm$ 0.106 μg/mL as in Table 2) to WQ-3334. Only one distinct substituent can be found between WQ-3810 and WQ-3334 (CH_3; WQ-3810, Br; WQ-3334) which might be the cause of 2.2-fold higher IC_{50} in WQ-3334. In contrast, WQ-4065 with 6-ethylamino-3,5-difluoropyridine-2-yl at the R1 position showed 23.2-fold higher IC_{50} than WQ-3810. Only the addition of ethyl group at position 6 in R1 group affected the DNA gyrase inhibitory activity much.

QnrB19 had little effect on DNA supercoiling activity of DNA gyrase (18 nM GyrA and 18 nM GyrB) at the concentration of 6 - 18 nM; however, increasing amount of QnrB19 exerted the ability to reduce the activity of DNA gyrase in concentration dependent manner and at the highest concentration of 4,374 nM QnrB19 in the absence of fluoroquinolones completely inhibited DNA supercoiling activity as in Chapter I. In this study, QnrB19 showed

the ability to protect DNA gyrase from the effect of WQ-3810 (0.16 µg/mL), WQ-3334 (0.64 µg/mL) and WQ-4065 (2.56 µg/mL) in a concentration dependent manner (Figure 16). At the concentration of 18 - 162 nM, QnrB19 showed restoring ability on WQ-3810, WQ-3334 and WQ-4065 inhibited DNA supercoiling activity of DNA gyrase by approximately 4.28 to 4.45-folds, 5.78 to 6.80-folds and 4.89 to 5.18-folds, respectively (Figure 16C). This was in good agreement with the results in Chapter I, where at the concentration of 54 nM, QnrB19 showed the maximum restoring ability on the nalidixic acid (50 µg/mL), norfloxacin (1 µg/mL) and ciprofloxacin (0.5 µg/mL) inhibited DNA supercoiling activity of DNA gyrase by approximately 1.8, 3.6 and 7.1-folds, respectively. Comparing the results in this study with those in Chapter I, QnrB19 showed restoring ability on the novel fluoroquinolones (WQ-3810, WQ-3334 and WQ-4065) inhibited DNA gyrase activities similar to norfloxacin and ciprofloxacin, but higher than old quinolone, nalidixic acid. Thus, it is worth noting that QnrB19 was shown to protect DNA gyrase activity from varieties of quinolones including old and new quinolone with distinct properties.

The IC_{50}s of WQ-3810, 3334 and 4065 in the presence of QnrB19 had been increased to 14.2-, 13.5- and 12.7-folds, respectively. In Chapter I, the presence of same molar concentration of QnrB19 as DNA gyrase caused 3.0- and 2.7-folds increase of IC_{50}s of ciprofloxacin with cyclopropyl substituents and norfloxacin with an ethyl group at R1 position, respectively, while the IC_{50} of nalidixic acid was not affected. The structural differences, especially at R1 group, between WQ-compounds and other quinolones examined in our previous study might be the cause of the extent of IC_{50}s increase. This differs from the findings of the current study where WQ-3810, WQ-3334 and WQ-4065 has shown high IC_{50}s (Table 2) suggesting that QnrB19 might compete with fluoroquinolones through the R1 and R8 group.

In our previous studies, IC_{50}s obtained by DNA supercoiling assay targeting wild type *S.* Typhimurium DNA gyrase and MICs obtained by conventional culture-based drug

susceptibility tests have been compared. Ciprofloxacin showed IC_{50} of 0.25 ± 0.05 μg/mL and MIC of 0.0078 μg/mL (32-fold lower than IC_{50}). Nonetheless, nalidixic acid exhibited an IC_{50} of 65.1 ± 19.08 μg/mL and MIC of 4 μg/mL (16-fold lower than IC_{50}) [47]. In contrast, MIC of WQ-3810 was 2.4-fold lower than IC_{50} as in our previous work [42]. These facts exhibited that the correlation between IC_{50}s and MICs varies between quinolones. MICs of WQ-3810, WQ-3334 and WQ-4065 were assumed to be around 0.013, 0.03 and 0.3 μg/mL against wild type *Salmonella* in accordance with the data obtained in our previous study on the fold decrease of MIC from IC_{50} in WQ-3810 [42]. The Clinical and Laboratory Standards Institute (CLSI) 2019 defined MIC breakpoint for *Salmonella* spp. resistance to ciprofloxacin, norfloxacin and nalidixic acid to be ≥ 1, ≥ 16 and ≥ 32 μg/mL, respectively. Therefore, three WQ-compounds would be good candidate drugs for the treatment of salmonellosis. Nevertheless, the estimated MIC of WQ-3810, WQ-3334 and WQ-4065 were 0.18, 0.38 and 3.8 μg/mL from the data on IC_{50}s in the presence of QnrB19, respectively, WQ-3810 and WQ-3334 could be predicted as potent drugs against *Salmonella* carrying QnrB19.

Novel fluoroquinolones, WQ-3810, WQ-3334 and WQ-4065 with unique difluoropyridine derivatives at R1 groups were assessed in this study to observe the inhibitory activity against wild type *S.* Typhimurium DNA gyrase and interaction with plasmid-encoded quinolone resistance protein QnrB19. Results show that in the absence and the presence of QnrB19, WQ-3810 and WQ-3334 which has 6-amino-3,5-difluoropyridine-2-yl at the R1 groups showed stronger inhibitory activity against *S.* Typhimurium DNA gyrases with a lower IC_{50}s than WQ-4065 which have a 6-ethylamino-3,5-difluoropyridine-2-yl at the R1 position. The results suggested that WQ-3810 and WQ-3334 could be the good candidate drug for wild type *Salmonella* and *Salmonella* carrying QnrB19.

Summary

Recently, WQ-3810 was showed to have strong inhibitory activity against several pathogens including *S.* Typhimurium and was highlighted to be as a new candidate antimicrobial for treating patients infected by various fluoroquinolone-resistant pathogens. The unique potentiality of this compound is attributed to R1 groups (6-amino-3,5-difluoropyridine-2-yl). Nonetheless, two recently synthesized novel fluoroquinolones, WQ-3334 and WQ-4065 have a similar R1 group.

The aims of this study are to elucidate the abilities of three novel quinolones against *S.* Typhimurium and that with a plasmid encoded quinolone resistant protein, QnrB19, by *in vitro* DNA supercoiling assay utilizing recombinant DNA gyrase. The IC_{50}s of WQ-3810, WQ-3334 and WQ-4065 against *S.* Typhimurium DNA gyrase were 0.031 ± 0.003, 0.068 ± 0.016 and 0.72 ± 0.39 μg/mL, respectively, and MICs were assumed to be around 0.013, 0.03 and 0.3 μg/mL. QnrB19 increased IC_{50}s to be 0.44 ± 0.05, 0.92 ± 0.34 and 9.16 ± 2.21 μg/mL (the predicted MICs; 0.18, 0.38 and 3.8 μg/mL), respectively.

WQ-3810 and WQ-3334 showed stronger inhibitory activity against *S.* Typhimurium DNA gyrases even in the presence of QnrB19 than WQ-4065. The results suggested these two compounds to be the good candidate drug for wild type *Salmonella* and *Salmonella* carrying QnrB19.

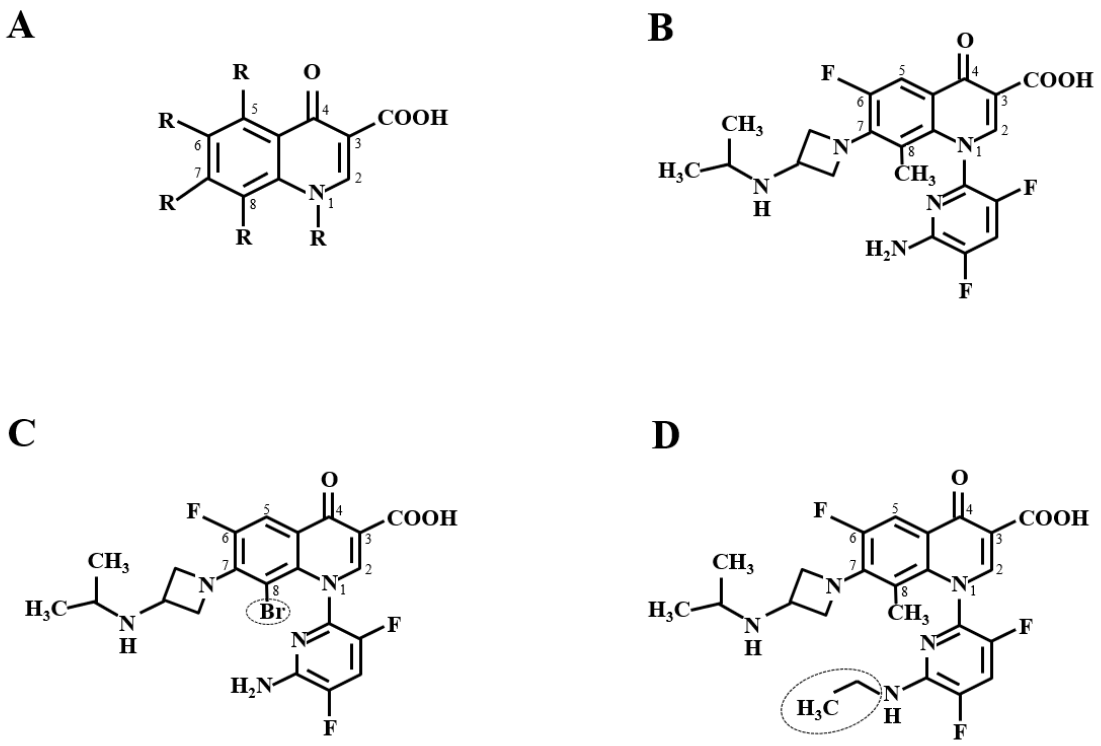

Figure 15. Chemical structures of fluoroquinolones used in the present study.

A; Basic structure, B; WQ-3810, C; WQ-3334, D; WQ-4065

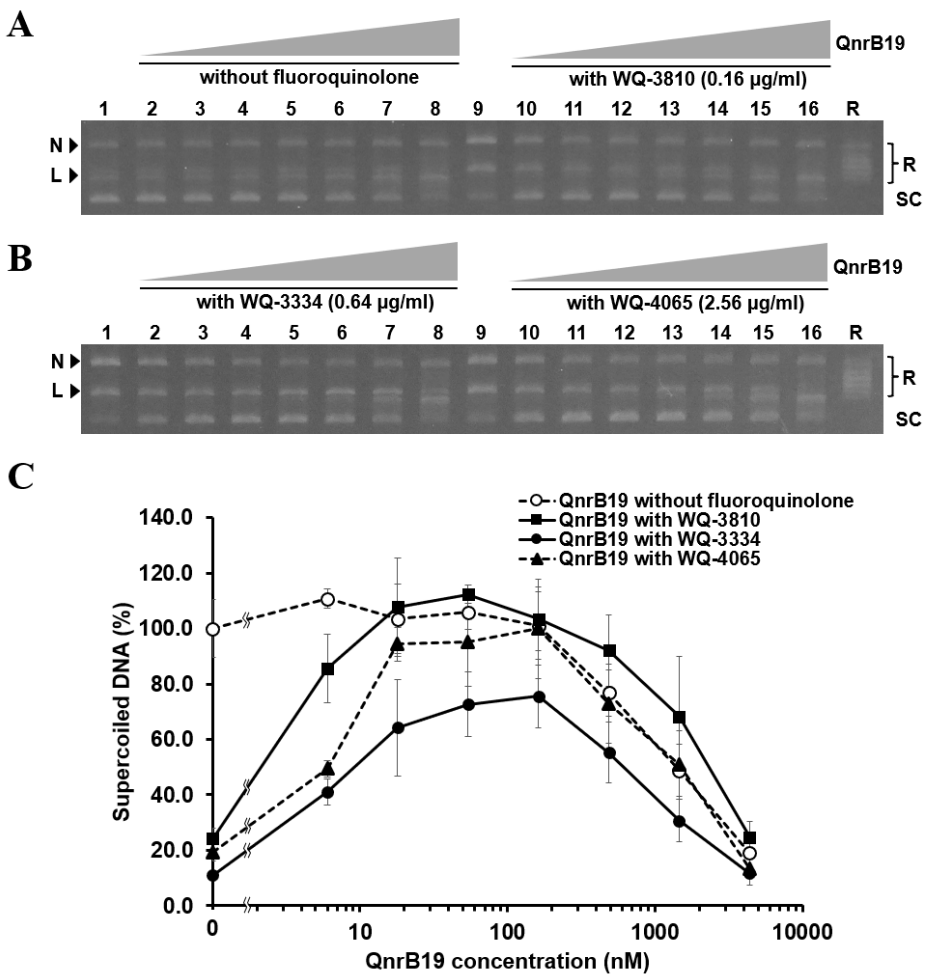

Figure 16. Inhibition of wild type *S.* Typhimurium DNA gyrase by fluroquinolones and protection by QnrB19

Reaction mixtures were diluted 4 times with 1× DNA sample buffer and 10 μL of them were analyzed by electrophoresis in 1% agarose gels. Lanes 2 - 16: contained 0.3 μg relaxed plasmid pBR322, reaction buffer and 18 nM DNA gyrase subunit. (A) Lane 1 - 8: with the absence of fluoroquinolone; lane 9 - 16: with the presence of 0.16 μg/mL WQ-3810. (B) Lane 1 - 8: with the presence 0.64 μg/mL WQ-3334; lane 9 - 16: with the presence of 2.56 μg/mL WQ-4065. Lanes 2 and 10: with 6 nM QnrB19; lanes 3 and 11: with 18 nM QnrB19; lanes 4 and 12: with 54 nM QnrB19; lanes 5 and 13: with 162 nM QnrB19; lanes 6 and 14: with 486 nM QnrB19; lanes 7 and 15: with 1458 nM QnrB19; lanes 8 and 16: with 4374 nM QnrB19; lane 17: relaxed plasmid pBR322. R, SC, L and N denote relaxed, supercoiled, liner and nicked form of pBR322 DNA, respectively. In panel C, dotted lines with thick circles denote the effect of QnrB19 on the DNA supercoiling activity of DNA gyrase (expressed as % activity comparing to that without fluoroquinolones). Dotted lines with filled triangles denote those with the presence of 2.56 μg/mL WQ-4065. Solid lines with filled squares and filled circles denote those with the presence of 0.16 μg/mL WQ-3810 and 0.64 μg/mL WQ-3334, respectively.

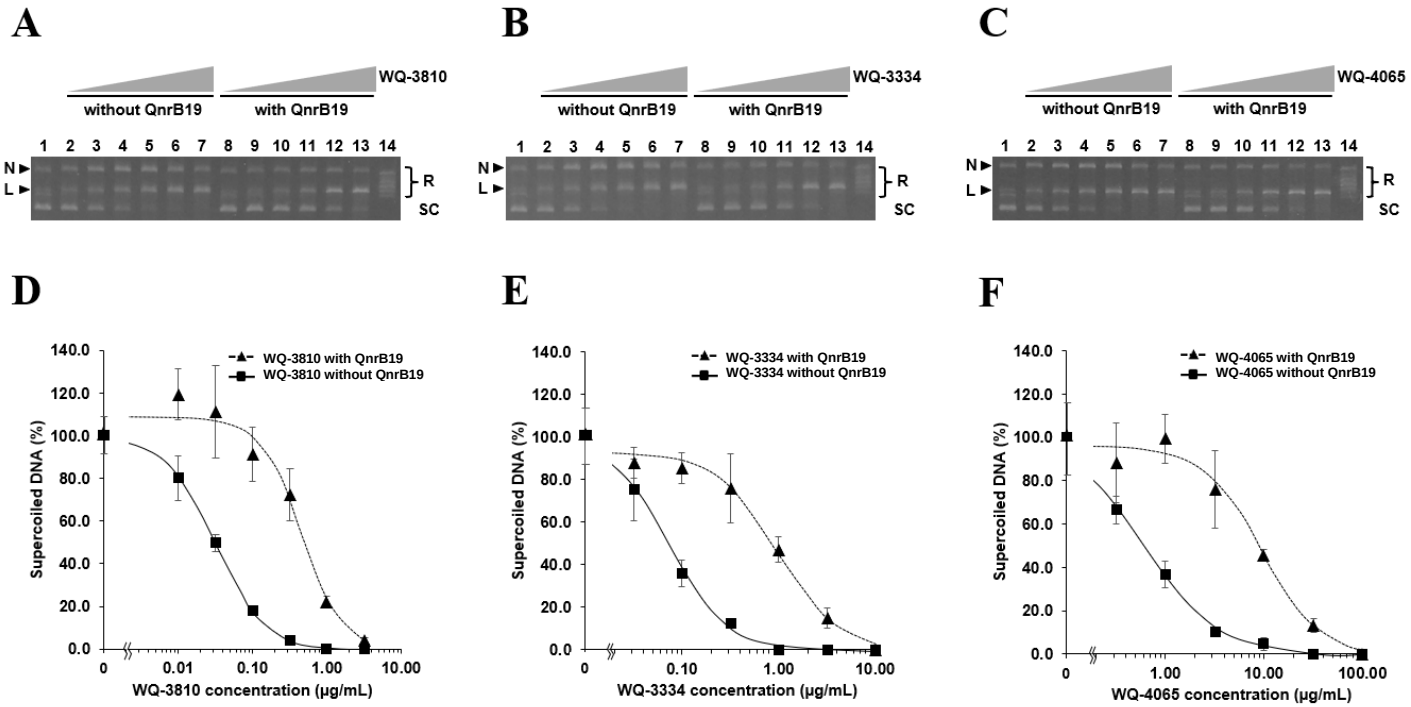

Figure 17. Effect of QnrB19 on IC_{50}s of fluoroquinolones

Reaction mixtures were diluted 4 times with 1× DNA sample buffer and 10 μL of them were analyzed by electrophoresis in 1% agarose gels. Lane 1 - 13: Reaction mixture contained 0.3 μg relaxed plasmid pBR322, reaction buffer and 18 nM DNA gyrase subunit; Lanes 2 - 7: with 0.01 - 3.2 μg/mL WQ-3810 (A), with 0.032 - 10 μg/mL WQ-3334 (B), with 0.32 - 100 μg/mL WQ-4065 (C) in the absence of QnrB19; lanes 8 - 13: with 0.01 - 3.2 μg/mL WQ-3810 (A), with 0.032 - 10 μg/mL WQ -3334 (B), with 0.32 - 100 μg/mL WQ-4065 (C) in the presence of 18 nM QnrB19; lane 14: relaxed plasmid pBR322. Solid lines with filled squares in panels D, E and F denote the impact of WQ-3810, WQ-3334 and WQ-4065 on the DNA supercoiling activity of DNA gyrase (expressed as % activity comparing to that without QnrB19), respectively. Dotted lines with filled triangles denote those in the presence of 18 nM QnrB19.

Table 2. Impact of IC_{50}s of fluoroquinolones on WT *S.* Typhimurium DNA gyrase with/without QnrB19

Fluoroquinolones	R-1[a]	R-6[a]	R-7[a]	R-8[a]	IC_{50}[b] (μg/mL)		Ratio (Without: With QnrB19)
					Without QnrB19	With QnrB19	
WQ-3810	6-amino-3,5-difluoropyridine-2-yl	F	3-isopropylaminoazetizine-1-yl	CH_3	0.031 ± 0.003	0.44 ± 0.05	1 : 14.2
WQ-3334	6-amino-3,5-difluoropyridine-2-yl	F	3-isopropylaminoazetizine-1-yl	Br	0.068 ± 0.016	0.92 ± 0.34	1 : 13.5
WQ-4065	6-ethylamino-3,5-difluoropyridine-2-yl	F	3-isopropylaminoazetizine-1-yl	CH_3	0.72 ± 0.39	9.16 ± 2.21	1 : 12.7

[a] Position of substituents in the structures of fluoroquinolones.

[b] IC_{50}: Concentrations of fluoroquinolones to inhibit the supercoiling activity of DNA gyrase by 50%.

CONCLUSION

Fluoroquinolones have been used for the drugs of choice to treat non-typhoidal *Salmonella* (NTS) infection in humans and animals. However, plasmid-mediated quinolone resistance (PMQR) mechanism has emerged in Enterobacteriaceae. Plasmid-encoded quinolone resistance protein Qnr is an important factor among PMQR in bacterial resistance to quinolones. This protein interacts with DNA gyrase and reduces susceptibility to quinolones.

However, as there are limited information of the predominant Qnr types in NTS isolates, I investigated the prevalence of Qnr in 692 reports published from 2008 to 2017 and found that 4,459 Enterobacteriaceae isolates had Qnrs (1,917 QnrB, 1,545 QnrS, 498 QnrA, 459 QnrD, 27 QnrVC, 12 QnrC and 1 QnrE). And QnrB19 was found in the highest number of isolates among Qnr reported in NTS isolates.

In Chapter I, *in vitro* assays for wild type *S.* Typhimurium DNA gyrases, QnrB19 and quinolones (nalidixic acid, ciprofloxacin and norfloxacin) were performed. The IC_{50}s of norfloxacin and ciprofloxacin against DNA gyrases were increased around 3-fold by the addition of QnrB19 and the results exhibited that the contribution of QnrB19 did not associate with the difference between R1 group of these two quinolones. The IC_{50} of nalidixic acid was 59- and 110-folds higher than norfloxacin and of ciprofloxacin in the absence of QnrB19, respectively, and this was similar to the that in the presence of 18 nM QnrB19. These results showed that the fluorine at R6 and/or piperazine at R7 and/or hydrogen at R8 group might associate with the ability of QnrB19 to increase IC_{50}s. QnrB19 was shown for the first time *in vitro* to have ability to grant non-classical quinolone resistance to *S.* Typhimurium DNA gyrase in connection with the structure at R6 and/or R7 and/or R8.

In Chapter II, I identified the activity of novel fluoroquinolones with the potentiality attributed by unique R1 group, 6-amino-3,5-difluoropyridine-2-yl, by *in vitro* assay. I

compared IC_{50} of WQ-3810, WQ-3334 and WQ-4065 with specific features of quinolones at positions R1, R6, R7 or R8. WQ-3810 and WQ-3334 (6-amino-3,5-difluoropyridine-2-yl at the R1 group) showed stronger inhibitory activity against *S.* Typhimurium DNA gyrases with a lower IC_{50}s than WQ-4065 (6-ethylamino-3,5-difluoropyridine-2-yl at the R1). Moreover, WQ-3810 and WQ-3334 showed greater inhibitory activity against *S.* Typhimurium DNA gyrases even in the presence of QnrB19. The results suggested that novel fluoroquinolones, WQ-3810 and WQ-3334 could be the good candidate drug for wild type *Salmonella* and *Salmonella* carrying QnrB19.

Comparison of conventional quinolones (nalidixic acid, norfloxacin and ciprofloxacin) in Chapter I and novel fluoroquinolones (WQ-3810, WQ-3334 and WQ-4065) in Chapter II showed that 6-amino-3,5-difluoropyridine-2-yl group at the R1 in WQ-3810 and WQ-3334 added a strong inhibitory activity against *S.* Typhimurium DNA gyrases to quinolones with very low IC_{50}. IC_{50}s of WQ-3810 and WQ-3334 were greatly increased in the presence of QnrB19, however, similar to those of ciprofloxacin and norfloxacin. Structural at R1 may cause the different impact by QnrB19 on the inhibitory activities of quinolones against DNA gyrase. The knowledge obtained in my study can be applied to design new compounds against bacteria carrying QnrB19 or other pentapeptide repeat proteins.

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