# Studies on fluoroquinolone resistance in *Mycobacterium tuberculosis* utilizing recombineering technology

(組換え技術を利用した結核菌のフルオロキノロン耐性に関する研究)

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**CONTENTS**

## ABBREVIATIONS

| | |
|---|---|
| TB | Tuberculosis |
| *Mtb* | *Mycobacterium tuberculosis* |
| RMP | Rifampicin |
| INH | Isoniazid |
| PZA | Pyrazinamide |
| EMB | Ethambutol |
| MDR | Multidrug-resistant |
| FQ | Fluoroquinolone |
| LVX | Levofloxacin |
| MXF | Moxifloxacin |
| DNA | Deoxyribonucleic acid |
| ATP | Adenosine triphosphate |
| GyrA | DNA gyrase subunit A |
| GyrB | DNA gyrase subunit B |
| BCG | *Mycobacterium tuberculosis* var. *bovis* Bacille Calmette–Guérin |
| AMP | Ampicillin |
| KM | Kanamycin |
| HYG | Hygromycin |
| Ni-NTA | Nickel-nitrilotriacetic acid |
| WT | Wild type |
| G88C | Amino acid substitution glycine to cysteine at position 88 |
| A90V | Amino acid substitution alanine to valine at position 90 |
| D94A | Amino acid substitution aspartic acid to alanine at position 94 |
| D94G | Amino acid substitution aspartic acid to glycine at position 94 |

| | |
|---|---|
| D94H | Amino acid substitution aspartic acid to histidine at position 94 |
| D94N | Amino acid substitution aspartic acid to asparagine at position 94 |
| D94Y | Amino acid substitution aspartic acid to tyrosine at position 94 |
| PCR | Polymerase chain reaction |
| SDS-PAGE | Sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| His | Histidine |
| CBB | Coomassie brilliant blue |
| BSA | Bovine serum albumin |
| $IC_{50}$ | Drug concentrations required to inhibit supercoiling activity by 50% |
| *hyg* | Hygromycin resistance cassette |
| MIC | Minimum inhibitory concentrations |
| $OD_{590}$ | Optical density at a wavelength of 590 nm |
| FICI | Fractional inhibitory concentration index |
| $FICI_m$ | Minimum FICI |
| NOV | Novobiocin |
| GM | Gentamicin |
| SM | Streptomycin |
| DMEM | Dulbecco's Modified Eagle's medium |
| RPMI | Roswell Park Memorial Institute |
| FBS | Fetal bovine serum |
| CFU | Colony forming unit |
| PMA | Phorbol 12-myristate 13-acetate |
| D-PBS | Dulbecco's phosphate buffered saline |
| MOI | Multiplicity of infection |

## PREFACE

TB is a global disease caused by infection with the bacillus *Mtb*. It typically affects lungs as pulmonary TB and is spread through the air when people with TB expel bacteria by coughing or sneezing. With an estimated 10 million new cases and 1.6 million deaths in 2017, TB represents one of the most serious infectious diseases throughout the world [1]. For the treatment of drug-susceptible TB, multidrug therapy using RMP, INH, PZA and EMB for 6-9 months is necessary. These drugs target essential bacterial enzymes to inhibit transcription, fatty acid synthesis and cell wall synthesis. The ample and consistent combination therapy can minimize the risks of relapse and drug resistance in TB. Nevertheless, MDR-TB, which is resistant to at least two drugs including RMP and INH, has increased significantly and become a persistent threat with 558,000 new cases in 2017 [1]. MDR-TB requires treatment with second-line drugs such as FQs and injectable agents. However, the success rate for treatment of MDR-TB is 55%, leading to an urgent need for development of new anti-TB drugs or regimens with novel modes of action and better understanding of drug-resistance mechanisms.

FQs have been recognized as effective antibacterial agents and widely used to treat bacterial infection. For the treatment of TB, LVX and MXF are frequently used with other effective drugs in treating MDR-TB patients. FQs kill bacteria via double-stranded DNA breaks by binding to DNA gyrase. DNA gyrase is a type II DNA topoisomerase that catalyzes DNA supercoiling at the expense of ATP hydrolysis. It modulates DNA topology to maintain chromosomal superstructure and has an essential role for efficient DNA replication and transcription in bacteria. This enzyme is composed of GyrA and GyrB encoded by *gyrA* and *gyrB*, respectively. According to epidemiological studies [2–4], amino acid substitutions at positions 88, 90, 94 and elsewhere in the quinolone resistance-determining region of GyrA have been found to be responsible for acquisition of FQ resistance in *Mtb* (Fig. 1). These substitutions are thought to change the formation of FQ binding pocket of the enzyme, leading to FQ resistance [5,6]. In clinical strains of *Mtb*, the

substitutions at position 90 and 94 are found predominantly, while the substitution at position 88 has been found only in limited cases. However, the substitution at position 88 has been known to confer high-level FQ resistance in *Mtb* strains. In order to understand such diverse characters of FQ-resistance substitutions, the use of clinical *Mtb* strains has some difficulties because of various genetic backgrounds and labor-intensive methodology.

To control FQ-resistant TB, I carried out researches using a recombineering technology, which are composed of two chapters. In CHAPTER 1, an inhibitory activity of WQ-3810, a novel potent FQ which showed inhibition even against FQ-resistant pathogens, was examined as an anti-TB drug. In CHAPTER 2, fitness cost of FQ-resistant *Mtb* was investigated for further understanding of FQ-resistance mechanism, which is supportive to develop new TB treatment regimens.

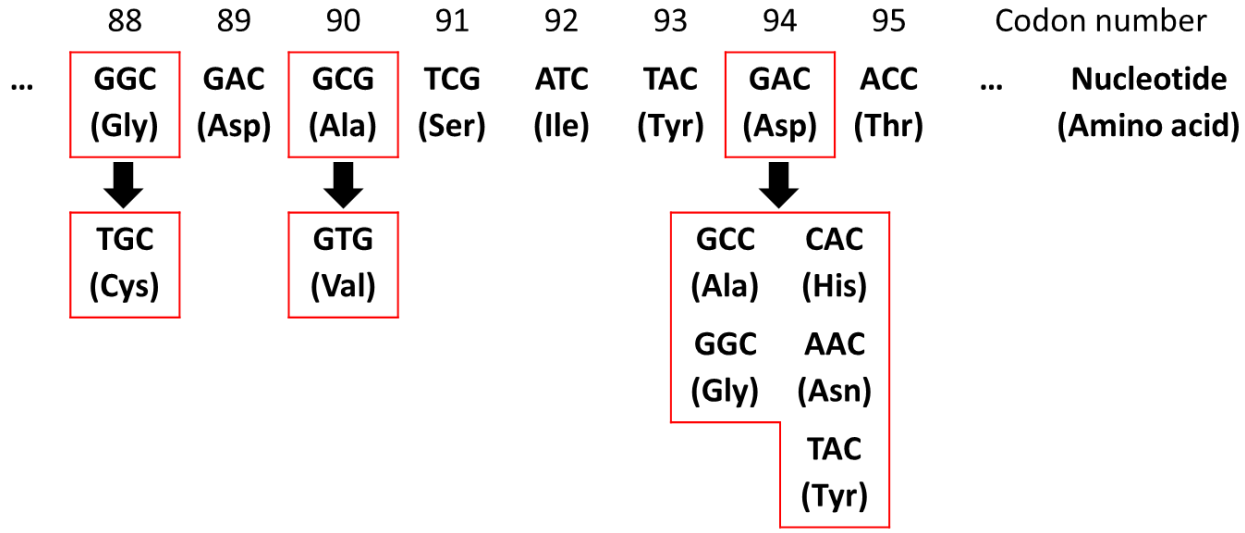



**Figure 1. Amino acid substitutions found within GyrA in FQ-resistant *Mtb*.**

The amino acids are shown below the nucleotide sequence.

# CHAPTER I:

## WQ-3810: a new fluoroquinolone with a high potential against fluoroquinolone-resistant *Mycobacterium tuberculosis*

### 1. Introduction

WQ-3810 is a FQ developed by Wakunaga Pharmaceutical Co., Ltd. with increased lipophilicity and membrane permeability [7]. This novel drug showed high DNA gyrase inhibitory activity and antibacterial activity [8] against MDR and even FQ-resistant gram-negative pathogens such as *Escherichia coli* and *Acinetobacter baumannii*. WQ-3810 is also active against FQ-resistant clinical isolates of gram-positive bacteria such as *Streptococcus pneumoniae* and methicillin-resistant *Staphylococcus aureus*. WQ-3810 has a high oral absorption rate with a low potential for side effects. However, the enzyme inhibitory activity and antimycobacterial activity of this drug has not been evaluated against *Mycobacterium* species.

To evaluate the inhibitory activity of WQ-3810 against *Mtb*, I devised two pronged approach that utilized (1) recombinant DNA gyrases derived from *Mtb* Beijing lineage, which are most prevalent in Asian countries [9]; (2) recombinant BCG strains in which the gyrase-coding genes were replaced with *Mtb* Beijing lineage variants (rBCG), and used for in vitro antibacterial activity of WQ-3810. My results suggest that WQ-3810 shows promise as a new anti-TB drug.

## 2. Materials and Methods

### 2.1. Reagents and Kits

FQs, LVX and MXF were purchased from LKT Laboratories, Inc. (St Paul, MN, USA) and WQ-3810 was a gift from Wakunaga Pharmaceutical Co., Ltd. (Tokyo, Japan) (Fig. 2). AMP, KM, HYG and INH were purchased from FUJIFILM Wako Pure Chemical Co., Ltd. (Osaka, Japan), EMB was purchased from MP Biomedicals, LLC (Solon, OH, USA). DNA Ligation Kit, Mighty Mix, and In-Fusion® HD Cloning Kit were obtained from Takara Bio Inc. (Shiga, Japan). Ni-NTA Agarose was obtained from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Restriction enzymes were purchased from New England Biolabs, Inc. (Ipswich, MA, USA). Relaxed pBR322 DNA was purchased from John Innes Enterprises Ltd (Norwich, UK). BD Difco™ Middlebrook 7H9 broth, BD Difco™ Middlebrook 7H10 agar and BD BBL™ Middlebrook OADC Enrichment were obtained from Becton, Dickinson and Co. (MD, USA), and MycoBroth was purchased from Kyokuto Pharmaceutical Industrial Co., Ltd. (Tokyo, Japan). Oligonucleotide primers used for cloning, mutagenesis and sequencing are listed in Table 1.

### 2.2. Bacterial strains and plasmids

BCG Tokyo 172 strains were provided from the Japan BCG Laboratory (Tokyo, Japan). FQ-susceptible or -resistant *Mtb* Beijing lineage strains listed in Table 2 were obtained from Osaka Institute of Public Health (Osaka, Japan). *E. coli* strain DH5α (Takara Bio Inc.) was used for DNA cloning. *E. coli* strains Rosetta-gami 2 (DE3) and BL21 (DE3) pLysS (Merck KGaA, Darmstadt, Germany) were used for protein expression. Vector plasmids pET-20b (+) and pET-19b (Merck KGaA) were used to construct plasmids for the expression of *Mtb* GyrA and GyrB, respectively. A *Mtb* GyrB expression plasmid, pTB-B, was constructed in previous study [10]. A parental plasmid for allelic exchange, pΔAHm31, was constructed, as shown in Fig. 3. Recombinase expression plasmid, pJV53, and resolvase expression plasmid, pYUB870 were gifts from Dr. Hatfull and Dr. Jacobs, respectively [11,12].

### 2.3. Construction of DNA gyrase subunit expression plasmid

A range of *Mtb* DNA gyrase expression plasmids were constructed: WT-GyrA; G88C-GyrA; A90V-GyrA; D94A-GyrA; D94G-GyrA; D94H-GyrA; D94N-GyrA and D94Y-GyrA (Table 3) [10]. Briefly, gene fragments were amplified by PCR in the reaction mixture consisted of 1x PrimeSTAR MAX DNA Polymerase (Takara Bio Inc.), 0.3 μM of each primer pair and 0.02 ng/μl of bacterial DNA from Osaka MDR-12 (Beijing lineage). Gene cassettes encoding N-terminal and C-terminal of WT-GyrA were amplified using each primer pair ON-873/ON-874 and ON-875/ON-876. Both of gene cassettes were used as templates for the amplification of complete *gyrA* cassette with a primer pair, ON-873/ON-876. Using NdeI and XhoI, the amplified products were transferred into pET20b(+) to construct 6x His-tagged WT-GyrA expression plasmid (termed pET/gyrA/WT). Mutations were introduced into the WT-*gyrA* in the plasmid by PCR with pairs of complementary primers containing the mutation of interest and ON-873 or ON-874. The *gyrA* amplicon with each mutation was digested with NdeI and HpaI and ligated into pET/gyrA/WT digested with the same restriction enzymes. A *gyrB* cassette was amplified with ON-1518/ON-886 using pTB-B as a template. The PCR product was digested by NdeI and XhoI, and transferred into pET19b to construct 6x His-tagged GyrB expression plasmid (termed pET/gyrB/WT). The nucleotide sequences of the DNA gyrase gene in the plasmids were confirmed using a BigDye Terminator (version 3.1) cycle sequencing kit and an ABI Prism 3130xl genetic analyzer (Thermo Fisher Scientific Inc.), according to the manufacturer's protocol.

### 2.4. Recombinant expression and purification of DNA gyrase subunits

Recombinant DNA gyrase subunits were expressed and purified using a slightly modified method described by Kim et al [10]. Expression of GyrA and GyrB in *E. coli* Rosetta-gami 2 (DE3) or BL21 (DE3) pLysS, respectively, was induced by addition of 1 mM isopropyl beta-D-thiogalactopyranoside and incubation at 18˚C for 40 h or at 23˚C for 5 h. After harvesting

the culture, *E. coli* was sonicated and the recombinant DNA gyrase in the supernatant was purified by affinity chromatography using Ni-NTA Agarose. The purified protein fractions were analyzed by SDS-PAGE stained with CBB. The proteins were quantified by Bradford Protein Assay Kit (Takara Bio Inc.).

### 2.5. DNA supercoiling assay and inhibitory assay

The assay monitoring ATP-dependent supercoiling activity of recombinant DNA gyrases was performed as previously described [10]. Briefly, the activity was examined in 30 µl reaction mixture consisting of 1x buffer (35 mM Tris-HCl pH 7.5, 6 mM $MgCl_2$, 1.8 mM spermidine, 24 mM KCl, 5 mM dithiothreitol, 0.36 mg/ml BSA, 6.5% w/v glycerol), 1 mM ATP, 2 nM relaxed pBR322 DNA, and 32 nM GyrA and GyrB subunits. After the mixture was incubated at 37˚C for 60 min, reactions were stopped by the addition of 7.5 µl of 5x dye mix (5% SDS, 25% glycerol, 0.25 mg/ml bromophenol blue). The relaxed DNA and the supercoiled DNA were separated by agarose gel electrophoresis. The inhibitory activities of FQs were similarly tested using FQ-mediated DNA supercoiling activity inhibition. Various amounts of LVX, MXF or WQ-3810 were used as inhibitors. The reaction mixture contained 1x buffer, 1 mM ATP, 2 nM relaxed pBR322 DNA, 4 nM GyrA and GyrB subunits and the indicated concentration of FQs in a total volume of 30 µl. As described in the methodology for the DNA supercoiling assay, the relaxed DNA and the supercoiled DNA were separated by agarose gel electrophoresis after the reactions. To assess the inhibitory activities of FQs on each DNA gyrase, the amount of supercoiled DNA in the reactions was quantified with ImageJ software (https://imagej.nih.gov/ij/) and the $IC_{50}$s of each FQ were calculated.

### 2.6. **Construction of pΔAHm31 plasmid.**

A parental plasmid for the allelic exchange, pΔAHm31, was constructed as follows. At first, a *hyg* cassette with res sites was digested from pYUB854 using XbaI and HindIII. The product was

ligated into a pUC18 vector digested by same enzymes. The constructed plasmid was termed p18HygX-Hi (Fig. 3A). To utilize single restriction enzyme sites of EcoRI and SalI in multi-cloning site, silent mutations were introduced into the *hyg* cassette. Using primers F15Hyg61-Eco and R15Hyg-744 Sal, a 684 bp fragment of *hyg* was amplified from pYUB854. Using SalI and EcoRI, a 3929 bp fragment was digested from p18HygX-Hi. Both fragments were ligated using In-Fusion® HD Cloning Kit (p18HmXb-Hi). From the p18HmXb-Hi, the mutated *hyg* cassette was digested with XbaI and NheI, and ligated into pUC18 treated with XbaI and Calf intestine Alkaline Phosphatase. The ligated plasmid was termed pAHm31. To shorten the plasmid size, an *AmpR* gene was removed. Using primers FpUC18 2668-Sca and RpUC18-1677 Sca, pAHm31 was amplified. The product was digested by ScaI and self-ligated at the ScaI site. The constructed plasmid was termed pΔAHm31 (Fig. 3B).

### 2.7. Construction of plasmids containing allelic exchange substrates

Plasmids containing allelic exchange substrates to allow homologous recombination between plasmid-borne *Mtb* DNA gyrase genes and BCG chromosomal orthologues were constructed (Table 3) following the previously described recombineering protocols [13]. The genomic DNAs were extracted from BCG Tokyo 172 strain and *Mtb* Beijing lineage strains with or without FQ-resistance mutations (Osaka MDR-12 (WT), Osaka MDR-23 (G88C), Osaka MDR-14 (A90V), Osaka MDR-5 (D94A), Osaka MDR-13 (D94G) and Osaka MDR-79 (D94H)). An approximately 4.8 kb fragment containing *gyrA* and *gyrB* was amplified using ON-1547/ON-1548. Similarly, a 0.7 kb fragment with Rv0007, a gene located downstream of *gyrAB*, was amplified using ON-1558/ON-1559. The former *gyrAB* PCR product was digested with HindIII and XbaI, and cloned upstream of *hyg* in a parental pΔAHm31 plasmid. The latter Rv0007 product was directly cloned downstream of *hyg* in the KpnI-digested plasmid using In-Fusion® HD Cloning Kit. To construct the substrates containing novel FQ mutations, D94N or D94Y, the pΔAHm/gyrAB/WT plasmid containing WT allelic exchange substrate was used as a PCR template. A 2.5 kb fragment

and a 2.3 kb fragment were amplified using ON-1566/ON-1568 and ON-1567/ON-1569 (D94N) or ON-1566/ON-1570 and ON-1567/ON-1571 (D94Y). The amplified products were ligated to the pΔAHm/gyrAB/WT plasmid digested with HindIII and XbaI, using In-Fusion® HD Cloning Kit. The nucleotide sequence of the plasmid was confirmed by standard sequencing.

### 2.8. Preparation of recombinant BCG

The rBCG strains in which the DNA gyrase genes were replaced with *Mtb* DNA gyrase orthologues (rBCG/*Mtb*) were established by a modified recombineering protocol [13]. All strains were cultivated in Middlebrook 7H9 broth containing 10% OADC enrichment, 0.2% glycerol and 0.05% tyloxapol or on Middlebrook 7H10 agar supplemented with 10% OADC enrichment, 0.2% glycerol and 0.05% tyloxapol unless otherwise indicated. The pJV53, encoding a recombinase, was transformed into electrocompetent BCG cells, the recombinase expression was induced by the addition of 0.2% acetamide, and induced-BCG cells were again prepared as electrocompetent cells. The plasmids containing allelic exchange substrates were linearized by HindIII and transformed into the recombinase-expressing cells. After plating the transformed cells on Middlebrook 7H10 agar with 20 μg/ml HYG and incubating to select transformants, colonies were screened by colony PCR using ON-1564/ON-1565, which were designed to amplify the whole exchanged sequence from outside the region. Then, homologous recombination was confirmed by sequencing using primers listed in Table 1. The colonies were incubated in Middlebrook 7H9 broth for 2 weeks and plated on Middlebrook 7H10 agar without drugs. After the loss of pJV53 from the BCG was confirmed by PCR using ON-1562/ON-1563, the BCG cells were again prepared as electrocompetent cells. pYUB870, encoding a res-site-specific resolvase (γδ-tnpR), was transformed into the recombinants to remove the res sites flanking the *hyg* cassette. After selection using Middlebrook 7H10 agar with 20 μg/ml KM, the absence of the *hyg* cassette was confirmed by PCR using ON-1564/ON-1565. The recombinants were cultured in Middlebrook 7H9 broth without antibiotics, and the loss of pYUB870 after continuous incubation and confirmed by PCR using

ON-1562/ON-1563.

### 2.9. Drug susceptibility test

Parental BCG and rBCG strains were analyzed to determine the MICs of selected drugs. The broth microdilution method was carried out in duplicate according to the manufacturer's instructions. A bacterial culture ($OD_{590}$ = 0.15) was diluted 40-fold with MycoBroth and 100 μl of the dilution was added to each well of a sterile round bottom microtitre plate containing the 2-fold serially diluted drugs (100 μl/well). After the test plate was incubated for 14 days at 37˚C, the MIC was defined as the lowest concentrations of antibiotics that inhibited visible bacterial growth. The panel of drugs was LVX, MXF, WQ-3810, EMB and INH.

### 2.10. Checkerboard assay

Recombinant BCG strains, which have WT or G88C DNA gyrases, were used to assess the interaction between FQs and cell wall synthesis inhibitors. Using a checkerboard assay [14], FICI of each agent was determined in the presence of sub-inhibitory concentrations of another. A bacterial culture ($OD_{590}$ = 0.15) was diluted 40-fold with MycoBroth and 100 μl of the dilution was added to each well of a sterile round bottom microtitre plate containing the serially diluted concentrations of FQs (50 μl/well) and INH or EMB (50 μl/well). After the test plate was incubated for 14 days at 37˚C, the FICI of each combination between agents was calculated using the following equation: FICI = (MIC of drug A in the presence of drug B / MIC of drug A alone) + (MIC of drug B in the presence of drug A / MIC of drug B alone). $FICI_m$s in the tested combinations are shown and $FICI_m \leq 0.5$ is regarded as synergistic.

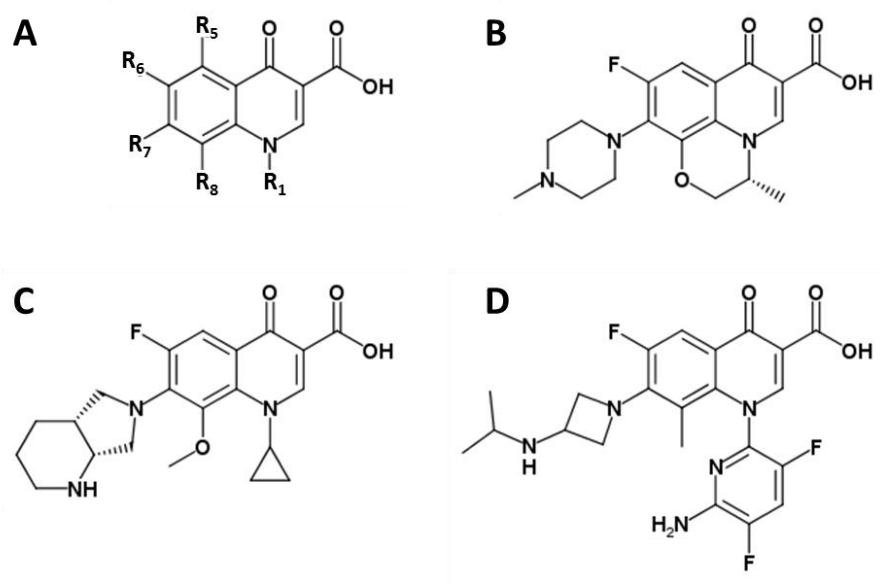



**Figure 2. Structure of FQs used in this study.**

(A) The basic structure of quinolones. (B) LVX. (C) MXF. (D) WQ-3810.

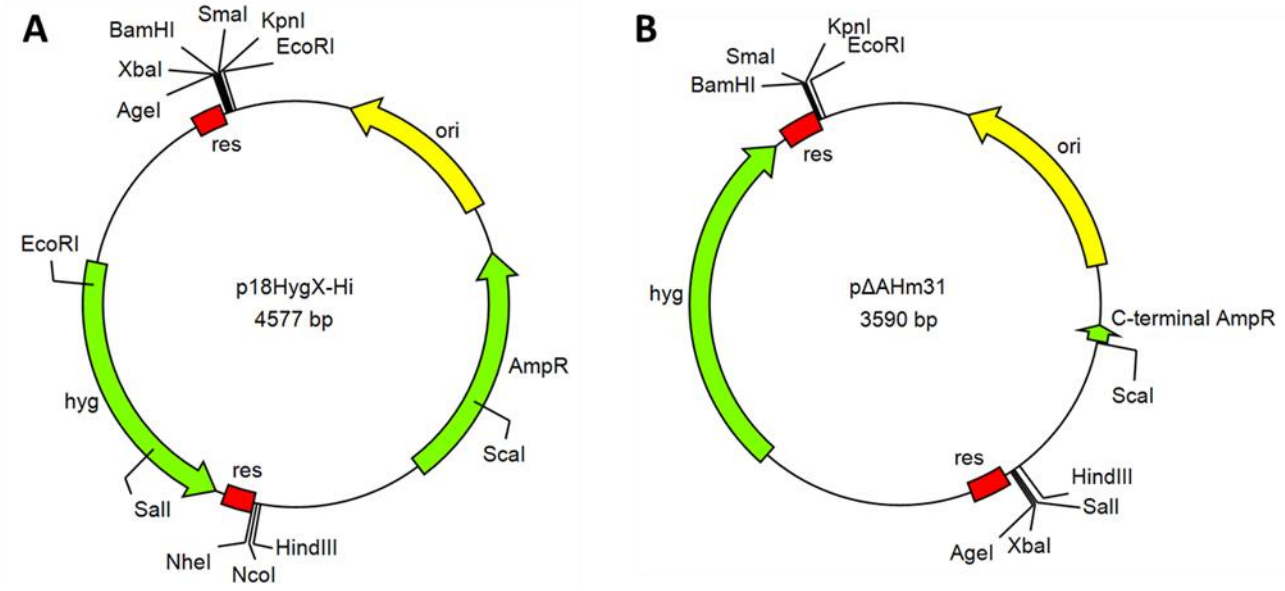



**Figure 3. Construction of pΔAHm31 plasmid.**

(A) p18HygX-Hi. (B) pΔAHm31.

**Table 1. Oligonucleotide sequences of primers used in this study.**

| Primer names | Sequence (Nucleotide position) | Comment |
|---|---|---|
| *gyrA* and *gyrB* cloning primers for expression plasmids | | |
| ON-873 | CCCATATGACAGACACGACGTTGCCGCC (1-23 in *gyrA*), NdeI site | WT-GyrA F |
| ON-874 | GTTAACCGGGCTTCGGTGTACCTCATCG (377-404 in *gyrA*), HpaI site | WT-GyrA R |
| ON-875 | GGTTAACCCCGTTGGCGATGGAGATGC (398-424 in *gyrA*), HpaI site | WT-GyrA F |
| ON-876 | GGCTCGAGTTAATGATGATGATGATGATGATTGCCCGTCTGGTCTGCGCCG (2493-2517 in *gyrA*), XhoI site and 6x His tag | WT-GyrA R |
| ON-1518 | CCCATATGGCTGCCCAGAAAAAGAAGGCCC (118-142 in *gyrB*), NdeI site | GyrB F |
| ON-886 | GGCTCGAGTTAGACATCCAGGAACCGAACATCC (2121-2145 in *gyrB*), XhoI site | GyrB R |
| Complementary primers for site-directed mutagenesis of expression plasmids | | |
| k-4 | TCGACGCGTC**GCA**GTGCGGGTGG (252-274 in *gyrA*) | G88C-GyrA R |
| k-3 | CCACCCGCAC**TGC**GACGCGTCGA (252-274 in *gyrA*) | G88C-GyrA F |
| k-10 | CGTAGATCGA**CAC**GTCGCCGTGC (258-280 in *gyrA*) | A90V-GyrA R |
| k-9 | GCACGGCGAC**GTG**TCGATCTACG (258-280 in *gyrA*) | A90V-GyrA F |
| ON-1527 | CGCACCAGGGT**GGC**GTAGATCGACG (269-293 in *gyrA*) | D94A-GyrA R |
| ON-1528 | CGCGTCGATCTAC**GCC**ACCCTGGTGCG (267-293 in *gyrA*) | D94A-GyrA F |
| k-85 | TGCGCACCAGGGT**GCC**GTAGATC (273-295 in *gyrA*) | D94G-GyrA R |
| k-84 | GATCTAC**GGC**ACCCTGGTGCGCA (273-295 in *gyrA*) | D94G-GyrA F |
| ON-1529 | TGCGCACCAGGGT**GTG**GTAGATCGACGC (268-295 in *gyrA*) | D94H-GyrA R |
| ON-1530 | ACGCGTCGATCTAC**CAC**ACCCTGGTGCG (266-293 in *gyrA*) | D94H-GyrA F |
| ON-1531 | TGCGCACCAGGGT**GTT**GTAGATCGACGC (268-295 in *gyrA*) | D94N-GyrA R |
| ON-1532 | ACGCGTCGATCTAC**AAC**ACCCTGGTGCG (266-293 in *gyrA*) | D94N-GyrA F |
| ON-1533 | TGCGCACCAGGGT**GTA**GTAGATCGACGC (268-295 in *gyrA*) | D94Y-GyrA R |
| ON-1534 | ACGCGTCGATCTAC**TAC**ACCCTGGTGCG (266-293 in *gyrA*) | D94Y-GyrA F |

**Table 1. Oligonucleotide sequences of primers used in this study (continued).**

| Primer names | Sequence (Nucleotide position) | Comment |
|---|---|---|
| Construction of pΔAHm31 | | |
| F15Hyg61-Eco | CTGCGGAACGACCAGGA**G**TTCTGGGAGCCGCTG, silent mutation in EcoRI site | |
| R15Hyg-744 Sal | ATAGACGTCGGTGAAGTC**C**ACGATCCCGGTGAC, silent mutation in SalI site | |
| FpUC18 2668-Sca | TTAAAGTACTATCACGAGGCCCTTTCGTC, ScaI site | |
| RpUC18-1677 Sca | GCATAGTACTAGACAGATCGCTGAGATAGG, ScaI site | |
| Cloning primers for plasmids containing allelic exchange substrate | | |
| ON-1547 | TCTAAGCTTCGGGACGCACCAGGAAGAAAGATG, HindIII site | upstream F |
| ON-1548 | TATTCTAGATAGCTGCCCGATTCCTCCTCAGATCG, XbaI site | upstream R |
| ON-1558 | GGTTGCTAGAGGATCCCCGGAGGAGGAATCGGGCAGCTAGGC identical region to end of pΔAHm31 digested with KpnI | downstream, in fusion F |
| ON-1559 | CATGATTACGAATTCGAGCTCGCGACCGTGATCATCCAGACGAAG identical region to end of pΔAHm31 digested with KpnI | downstream, in fusion R |

**Table 1. Oligonucleotide sequences of primers used in this study (continued).**

| Primer names | Sequence (Nucleotide position) | Comment |
|---|---|---|
| Mutagenesis primers for plasmids containing allelic exchange substrate | | |
| ON-1566 | CGGCCAGTGCCAAGCTTCGG<br>identical region to end of pΔAHm31 digested with HindIII | upstream, in fusion F |
| ON-1567 | CGATATTTGATTTAGGATACACCGGTTCTAGATAGCTGCC<br>identical region to end of pΔAHm31 digested with XbaI | upstream, in fusion R |
| ON-1568 | CAGGGT**GTT**GTAGATCGACGCGTCGCC<br>identical region to end of another mutated fragment | D94N, in fusion R |
| ON-1569 | GCGTCGATCTAC**AAC**ACCCTGGTGCGCATG<br>identical region to end of another mutated fragment | D94N, in fusion F |
| ON-1570 | CAGGGT**GTA**GTAGATCGACGCGTCGCC<br>identical region to end of another mutated fragment | D94Y, in fusion R |
| ON-1571 | GCGTCGATCTAC**TAC**ACCCTGGTGCGCATG<br>identical region to end of another mutated fragment | D94Y, in fusion F |

**Table 1. Oligonucleotide sequences of primers used in this study (continued).**

| Primer names | Sequence (Nucleotide position) | Comment |
|---|---|---|
| *gyrA* and *gyrB* sequencing primers | | |
| ON-841 | TCGGACGCGTATGCGATATC (448-467 in *gyrB*) | |
| ON-897 | CCTGAGACCACTCGTACCCGTCGCG (538-562 in *gyrB*) | |
| ON-888 | ACATCAACCGCACCAAGAACGC (905-926 in *gyrB*) | |
| ON-1100 | CGGCACGTAAGGCACGAGAG (1373-1392 in *gyrB*) | |
| ON-512 | GGCTTCGGTGTACCTCATCG (377-396 in *gyrA*) | |
| ON-877 | TGCCGTATCAGGTCAACCACGAC (821-843 in *gyrA*) | |
| ON-1166 | CGATGACGATGCGTAAACCGACC (927-949 in *gyrA*) | |
| ON-878 | GATCCGGGCGTCGGAGACCGTC (1221-1242 in *gyrA*) | |
| ON-879 | GGCAAGGGCGTGCAGGGTGCGG (1621-1642 in *gyrA*) | |
| ON-880 | TCTCGGCCAACGGGCAGTCCATC (2021-2043 in *gyrA*) | |
| Screening primers | | |
| ON-1562 | TGTCGGGCAATCAGGTGCGACAATC (96-120 in *KanR2*) | PCR to check the presence of pJV53 or pYUB870 |
| ON-1563 | TGGCAAGATCCTGGTATCGGTCTGCG (666-691 in *KanR2*) | PCR to check the presence of pJV53 or pYUB870 |
| ON-1564 | CACGTCGATCGGCCCAGAACAAGG ((-125)-(-102) in *gyrB*) | PCR to check sequence and $HYG^r$ cassette deletion |
| ON-1565 | GCGTTGTTCAACAGGTCACCGACG (717-740 in Rv0007) | PCR to check sequence and $HYG^r$ cassette deletion |

Mutated codons are shown in bold letters.

**Table 2. *Mtb* and rBCG strains used in this study.**

| Strain | Mutations based on genome sequence of *Mtb* str. Beijing/NITR203 registered at NCBI (RefSeq: NC_021054.1) | | Characteristics | Source |
|---|---|---|---|---|
| | *gyrB* | *gyrA* | | |
| *Mtb* Beijing lineage strains | | | | |
| Osaka MDR-12 | - | - | $RMP^r$, $INH^r$ | Osaka Institute of Public Health |
| Osaka MDR-23 | - | G262T(G88C) | $RMP^r$, $FQ^r$, $INH^r$ | Osaka Institute of Public Health |
| Osaka MDR-14 | - | C269T(A90V) | $RMP^r$, $FQ^r$, $INH^r$ | Osaka Institute of Public Health |
| Osaka MDR-5 | - | A281C(D94A) | $RMP^r$, $FQ^r$, $INH^r$ | Osaka Institute of Public Health |
| Osaka MDR-13 | - | A281G(D94G) | $RMP^r$, $FQ^r$, $INH^r$ | Osaka Institute of Public Health |
| Osaka MDR-79 | - | G280C(D94H) | $RMP^r$, $FQ^r$, $INH^r$ | Osaka Institute of Public Health |
| Parental BCG and rBCG strains | | | | |
| BCG Tokyo 172* | G513A, C1167T, G1207T(A403S) | C984T, G1323T, C1440T, T1842C | | Japan BCG Laboratory |
| rBCG/BCG* | G513A, C1167T, G1207T(A403S) | C984T, G1323T, C1440T, T1842C | res | This study |
| rBCG/Mtb/WT | - | - | res | This study |
| rBCG/Mtb/G88C | - | G262T(G88C) | res, $FQ^r$ | This study |
| rBCG/Mtb/A90V | - | C269T(A90V) | res, $FQ^r$ | This study |
| rBCG/Mtb/D94A | - | A281C(D94A) | res, $FQ^r$ | This study |
| rBCG/Mtb/D94G | - | A281G(D94G) | res, $FQ^r$ | This study |
| rBCG/Mtb/D94H | - | G280C(D94H) | res, $FQ^r$ | This study |
| rBCG/Mtb/D94N | - | G280A(D94N) | res, $FQ^r$ | This study |
| rBCG/Mtb/D94Y | - | G280T(D94Y) | res, $FQ^r$ | This study |

*BCG specific mutations in gyrase coding genes

res: remaining res sequence downstream of gyrase coding genes

**Table 3. Plasmids used in this study.**

| Names | Characteristics | Source/reference |
|---|---|---|
| *Mtb* DNA gyrase subunit expression plasmids | | |
| pET20b(+) | Vector for protein expression, $AMP^r$ | Merck KGaA |
| pET/gyrA/WT | 6x His-tagged WT-GyrA coding sequence in pET20b(+) | This study |
| pET/gyrA/G88C | 6x His-tagged G88C-GyrA coding sequence in pET20b(+) | This study |
| pET/gyrA/A90V | 6x His-tagged A90V-GyrA coding sequence in pET20b(+) | This study |
| pET/gyrA/D94A | 6x His-tagged D94A-GyrA coding sequence in pET20b(+) | This study |
| pET/gyrA/D94G | 6x His-tagged D94G-GyrA coding sequence in pET20b(+) | This study |
| pET/gyrA/D94H | 6x His-tagged D94H-GyrA coding sequence in pET20b(+) | This study |
| pET/gyrA/D94N | 6x His-tagged D94N-GyrA coding sequence in pET20b(+) | This study |
| pET/gyrA/D94Y | 6x His-tagged D94Y-GyrA coding sequence in pET20b(+) | This study |
| pET19b | Vector for protein expression, $AMP^r$ | Merck KGaA |
| pTB-B | 6x His-tagged GyrB coding sequence in pET19b | [10] |
| pET/gyrB/WT | 6x His-tagged GyrB coding sequence (5'- 117 bp was deleted) in pET19b | This study |

**Table 3. Plasmids used in this study (continued).**

| Names | Characteristics | Source/reference |
|---|---|---|
| Plasmids containing allelic exchange substrates | | |
| pUC18 | Vector for cloning allelic exchange substrate, $AMP^r$ | TaKaRa |
| p18HygX-Hi | *hyg* cassette with res sites in pUC18; $AMP^r$, $HYG^r$ | This study |
| p18HmXb-Hi | *hyg* cassette with res sites in pUC18; excludes EcoRI and SalI from *hyg*, $AMP^r$, $HYG^r$ | This study |
| pAHm31 | *hyg* cassette with res sites in pUC18; excludes EcoRI and SalI from *hyg*, $AMP^r$, $HYG^r$ | This study |
| pΔAHm31 | *hyg* cassette with res sites in pUC18; excludes EcoRI and SalI from *hyg*, excludes AmpR sequence, $HYG^r$ | This study |
| pΔAHm/gyrAB/BCG | BCG-*gyrAB* and Rv0007 fragments flanking *hyg* cassette in pΔAHm31 | This study |
| pΔAHm/gyrAB/WT | WT-*Mtb-gyrAB* and Rv0007 fragments flanking *hyg* cassette in pΔAHm31 | This study |
| pΔAHm/gyrAB/G88C | G88C-*Mtb-gyrAB* and Rv0007 fragments flanking *hyg* cassette in pΔAHm31 | This study |
| pΔAHm/gyrAB/A90V | A90V-*Mtb-gyrAB* and Rv0007 fragments flanking *hyg* cassette in pΔAHm31 | This study |
| pΔAHm/gyrAB/D94A | D94A-*Mtb-gyrAB* and Rv0007 fragments flanking *hyg* cassette in pΔAHm31 | This study |
| pΔAHm/gyrAB/D94G | D94G-*Mtb-gyrAB* and Rv0007 fragments flanking *hyg* cassette in pΔAHm31 | This study |
| pΔAHm/gyrAB/D94H | D94H-*Mtb-gyrAB* and Rv0007 fragments flanking *hyg* cassette in pΔAHm31 | This study |
| pΔAHm/gyrAB/D94N | D94N-*Mtb-gyrAB* and Rv0007 fragments flanking *hyg* cassette in pΔAHm31 | This study |
| pΔAHm/gyrAB/D94Y | D94Y-*Mtb-gyrAB* and Rv0007 fragments flanking *hyg* cassette in pΔAHm31 | This study |
| Plasmids used for allelic exchange | | |
| pJV53 | Shuttle plasmid expressing recombinase under the control of acetamide, $KM^r$ | [11] |
| pYUB854 | *hyg* cassette with res sites was used to construct pΔAHm31, $HYG^r$ | [12] |
| pYUB870 | Shuttle plasmid expressing resolvase, $KM^r$ | [12] |

## 3. Results

### 3.1. Expression and purification of recombinant DNA gyrase subunits

Using a Bradford Protein Assay to calculate protein concentration, 1.0 to 3.5 mg of purified DNA gyrase subunits were harvested from 500 ml cultures of the *E. coli*. The purity of the recombinant proteins was confirmed by SDS-PAGE (Fig. 4). All the recombinant proteins were obtained at high purity (>95%) with molecular weights of 93 kDa and 74 kDa for GyrA and GyrB, respectively.

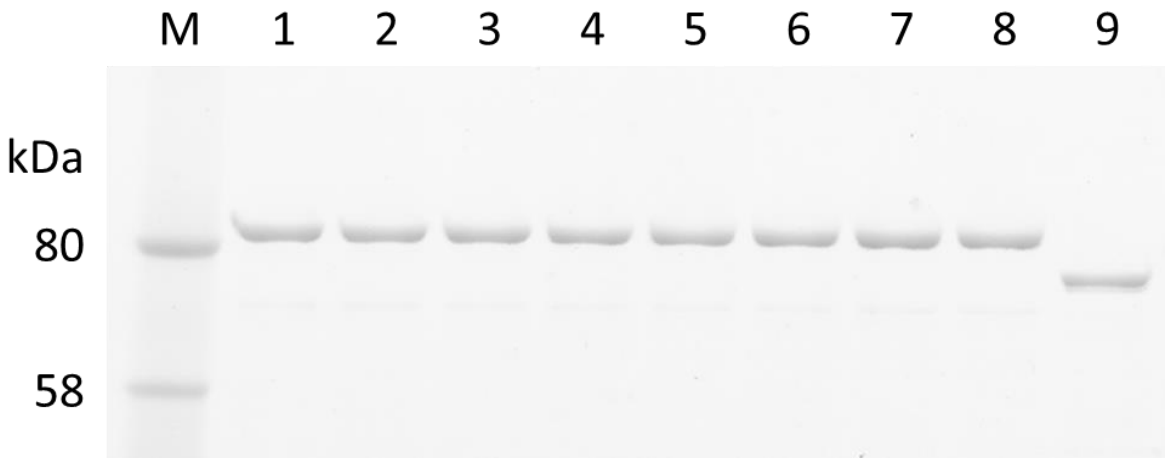



**Figure 4. Purification of recombinant *Mtb* GyrA and GyrB.**

The His-tagged proteins were expressed by *E. coli* and purified by Ni-NTA Agarose. Approximately 300 ng of each protein was loaded on SDS-PAGE and stained with CBB. Lanes: M, protein size marker; 1, WT-GyrA; 2, G88C-GyrA; 3, A90V-GyrA; 4, D94A-GyrA; 5, D94G-GyrA; 6, D94H-GyrA; 7 D94N-GyrA; 8, D94Y-GyrA; 9, GyrB.

### 3.2. DNA supercoiling activity of *Mtb* DNA gyrases

DNA supercoiling activities using combinations of WT or each mutant GyrA and GyrB were examined using relaxed pBR322 DNA as a substrate in the presence or absence of ATP (Fig. 5). DNA gyrase activities were only observed when ATP and recombinant DNA gyrase subunits were all present. In this assay, I confirmed the enzyme activities of purified recombinant gyrases using sufficient concentrations (32 nM). In an experiment using various concentrations (2 to 64 nM) of enzymes, mutants had slightly weak activities compared with WT enzyme (data shown in CHAPTER II). Because excessive amount of enzyme might interfere the exact results of inhibitory assay, I used lower concentrations (4 nM) than sufficient concentrations, for subsequent assays.

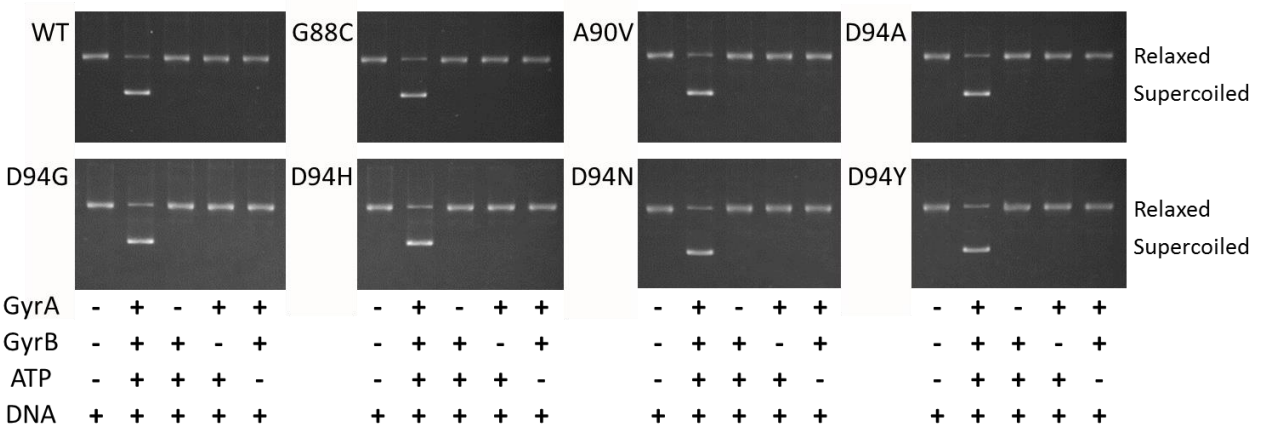



**Figure 5. ATP-dependent supercoiling activity of DNA gyrases.**

The reaction mixture (30 μl) containing 32 nM GyrA (WT or each mutant), 32 nM GyrB and 2 nM relaxed DNA and 1 mM ATP was incubated at 37˚C. After 60 min, the reactions were terminated by SDS and samples were electrophoresed through a 1% agarose gel.

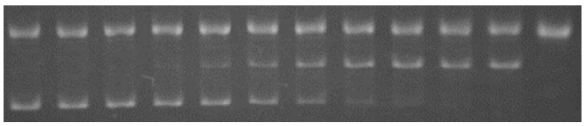
### 3.3. Inhibition of DNA gyrases by FQs

Inhibition of DNA supercoiling activities by FQs were observed in agarose gel electrophoresis (Fig. 6) and $IC_{50}$s of each FQ were determined to estimate the inhibitory activity of FQs (Fig. 7). This revealed that the inhibitory activity of WQ-3810 against WT DNA gyrase ($IC_{50}$s: 3.04 μg/ml) had no significant difference with that of MXF ($IC_{50}$s: 2.01 μg/ml) but was higher than that of LVX ($IC_{50}$s: 5.84 μg/ml). Similarly, WQ-3810 as well as MXF showed lower $IC_{50}$s against mutant DNA gyrases than LVX. Hence, against mutants with A90V and D94A substitutions, lower concentrations of WQ-3810 were required to inhibit the enzyme activity ($IC_{50}$s: 15.8 and 11.2 μg/ml, respectively), while $IC_{50}$s of LVX were 96.7 and 91.9 μg/ml. $IC_{50}$s of WQ-3810 were comparable to those of MXF ($IC_{50}$s of 7.50 and 10.0 μg/ml, respectively). Similarly, WQ-3810 and MXF showed lower $IC_{50}$s against other mutations at codon 94, in the range from 39.5 to 118 μg/ml and from 33.5 to 74.5 μg/ml, respectively, compared to those of LVX which ranged from 315 to 589 μg/ml. Interestingly, even against the G88C mutant, which has been known to be one of the highest resistance-conferring mutations in both clinical isolates [3,4] and experimentally-derived strains [15,16], WQ-3810 showed a considerably lower $IC_{50}$s of 50.0 μg/ml when compared with LVX and MXF ($IC_{50}$s: 518 and 296 μg/ml, respectively).

LVX (μg/ml)

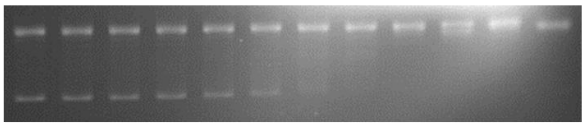
WT

0 0.25 0.5 1 2 4 8 16 32 64 128 R



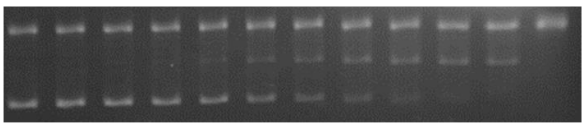
G88C

0 32 64 128 256 512 1024 2048 4096 8192 16384 R



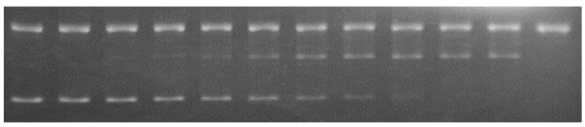
A90V

0 4 8 16 32 64 128 256 512 1024 2048 R



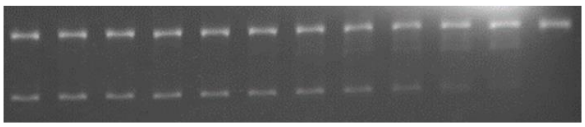
D94A

0 4 8 16 32 64 128 256 512 1024 2048 R



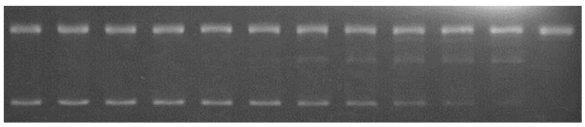
D94G

0 4 8 16 32 64 128 256 512 1024 2048 R



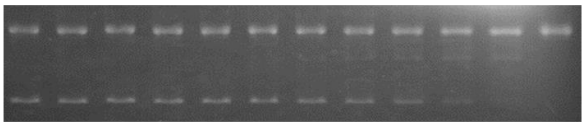
D94H

0 4 8 16 32 64 128 256 512 1024 2048 R



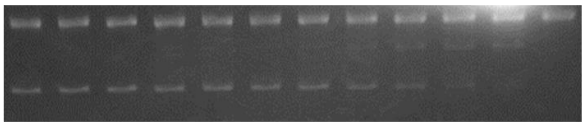
D94N

0 4 8 16 32 64 128 256 512 1024 2048 R



D94Y

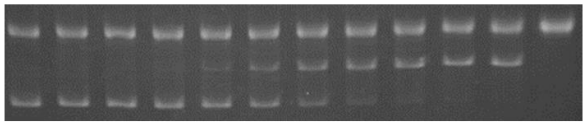
0 4 8 16 32 64 128 256 512 1024 2048 R



MXF (μg/ml)

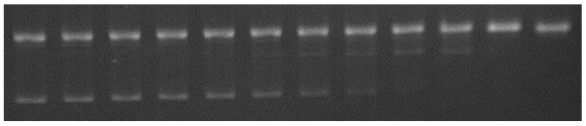
WT

0 0.063 0.125 0.25 0.5 1 2 4 8 16 32 R



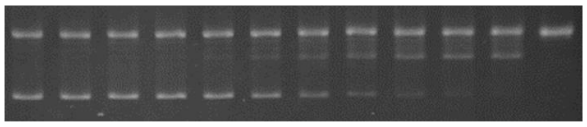
G88C

0 8 16 32 64 128 256 512 1024 2048 4096 R



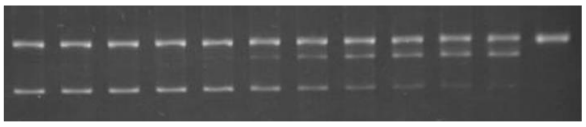
A90V

0 0.25 0.5 1 2 4 8 16 32 64 128 R



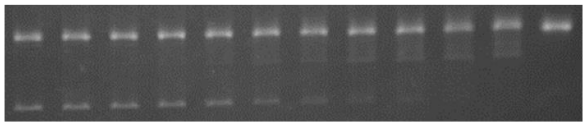
D94A

0 0.25 0.5 1 2 4 8 16 32 64 128 R



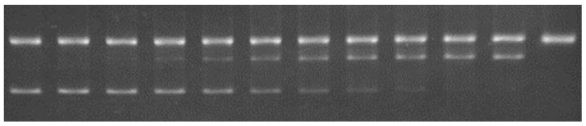
D94G

0 2 4 8 16 32 64 128 256 512 1024 R



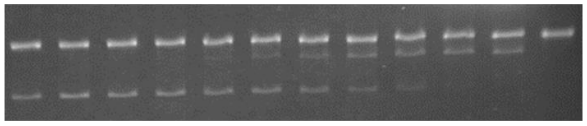
D94H

0 2 4 8 16 32 64 128 256 512 1024 R



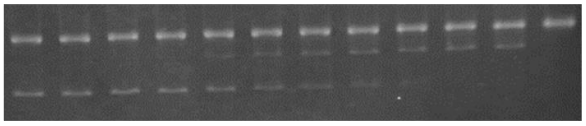
D94N

0 2 4 8 16 32 64 128 256 512 1024 R



D94Y

0 2 4 8 16 32 64 128 256 512 1024 R



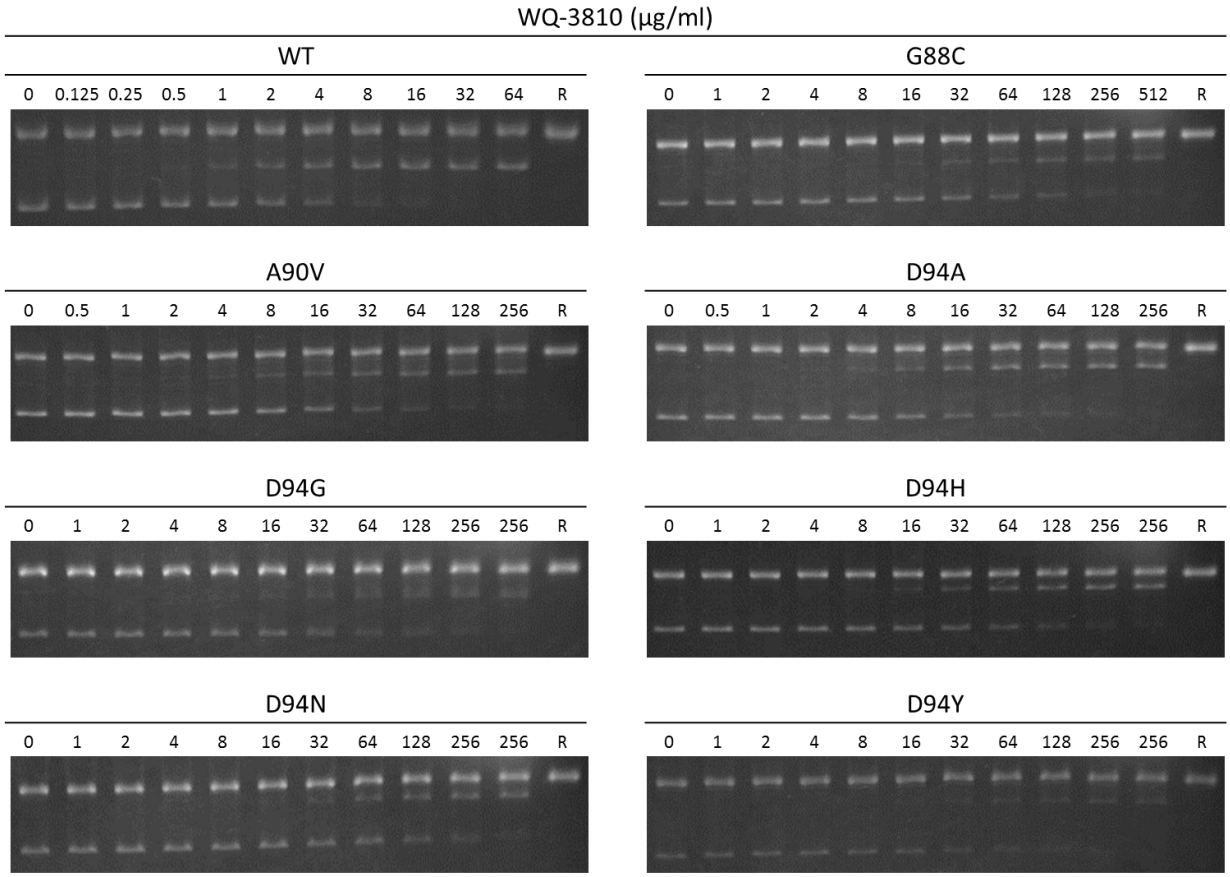



**Figure 6. FQ activities against recombinant *Mtb* DNA gyrase.**

The reaction (30 μl) containing 4 nM GyrA (WT or each mutant), 4 nM GyrB and 2 nM relaxed DNA, 1 mM ATP and indicated concentrations of FQ was incubated at 37˚C. After 60 min, the reaction was terminated by SDS and samples were electrophoresed through a 1% agarose gel. R: relaxed DNA.

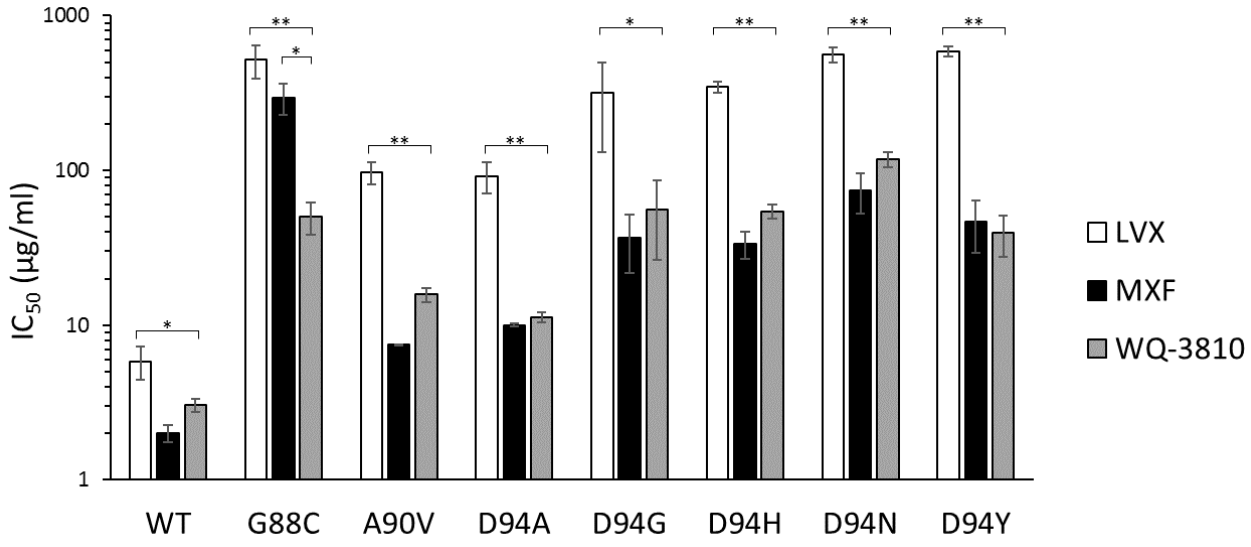



**Figure 7. FQ activities against recombinant *Mtb* DNA gyrase.**

Amount of DNA supercoiled by DNA gyrase was quantified from agarose gel electrophoresis in triplicate. From the proportion of supercoiled DNA, the $IC_{50}$s of three FQs were calculated. After assumptions of normality and homogeneity of variances were tested using Shapiro-Wilk test and Bartlett's test respectively, statistically significant differences in $IC_{50}$ from WQ-3810 were described using Dunnett's test (*, $p<0.05$; **, $p<0.01$).

### 3.4. Antibacterial activity of FQs against rBCG strains

Since genetic backgrounds and phenotypic characteristics among clinical strains are diverse and often cryptic, the rBCG strains whose DNA gyrase genes were replaced with *Mtb* orthologues were used to estimate the direct relationships between FQs and FQ resistance-associated mutations in GyrA. As shown in Table 4, MICs of each FQ against WT and mutant rBCG strains were determined to evaluate the antibacterial activity. LVX and WQ-3810 exhibited relatively high MICs against rBCG strains, ranging from 0.25 to 8 μg/ml and from 0.5 to 8 μg/ml, compared with MICs of MXF in a range from 0.0625 to 2 μg/ml. However, the ratios of the $MIC_{mutant}$ / $MIC_{WT}$ for WQ-3810 were lower than for the other tested FQs, ranging from 4- to 16-fold. In particular, the presence of the G88C mutation did not increase the MIC for WQ-3810 (16-fold) as much it increased the MICs for LVX and MXF (64- and 128-fold, respectively).

**Table 4. Drug susceptibility of rBCG strains.**

| | MIC (μg/ml) | | | | | | | | | | MIC (μg/ml) with 1 μg/ml EMB | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Strain | LVX | | MXF | | WQ-3810 | | EMB | | INH | | MXF | | WQ-3810 | |
| BCG Tokyo 172 | 0.25 | | 0.0625 | | 0.5 | | 2 | | 0.0625 | | 0.0625 | | 0.125-0.0625 | |
| rBCG/BCG | 0.25 | | 0.0625 | | 0.5 | | ND | | ND | | 0.0625 | | 0.0625 | |
| rBCG/Mtb/WT | 0.25 | (1) | 0.0625 | (1) | 0.5 | (1) | 2 | (1) | 0.03125 | (1) | 0.0625 | (1) | 0.0625 | (1) |
| rBCG/Mtb/G88C | 16 | (64) | 8 | (128) | 8 | (16) | 2 | (1) | 0.03125 | (1) | 4 | (64) | 0.5 | (8) |
| rBCG/Mtb/A90V | 2 | (8) | 0.5 | (8) | 2 | (4) | ND | | ND | | 0.25 | (4) | 0.25 | (4) |
| rBCG/Mtb/D94A | 2 | (8) | 0.5 | (8) | 2 | (4) | ND | | ND | | 0.25 | (4) | 0.25 | (4) |
| rBCG/Mtb/D94G | 8 | (32) | 2 | (32) | 4-8 | (8-16) | ND | | ND | | 1 | (16) | 0.5 | (8) |
| rBCG/Mtb/D94H | 8 | (32) | 1-2 | (16-32) | 4 | (8) | ND | | ND | | 1 | (16) | 0.5 | (8) |
| rBCG/Mtb/D94N | 8 | (32) | 2 | (32) | 8 | (16) | ND | | ND | | 2 | (32) | 1 | (16) |
| rBCG/Mtb/D94Y | 8 | (32) | 1-2 | (16-32) | 2-4 | (4-8) | ND | | ND | | 1 | (16) | 0.5 | (8) |

Concentration ranges were as follows: LVX (0.03125-32 μg/ml), MXF (0.01563-32 μg/ml), WQ-3810 (0.00781-32 μg/ml), EMB (0.25-8 μg/ml), INH (0.00781-4 μg/ml) . Numbers in brackets: ratio of the $MIC_{mutant}$ / $MIC_{WT}$. ND: not determined.

### 3.5. Effect of cell wall synthesis inhibitors on the MIC of WQ-3810 against rBCG strains

Interaction between FQs and cell wall synthesis inhibitors such as EMB and INH was analyzed using a checkerboard assay. As shown in Table 5, only the combination of WQ-3810 and EMB against both WT and G88C rBCG strains exhibited a synergistic relation, with an $FICI_m$ of 0.5, while WQ-3810 also showed a lower $FICI_m$ than MXF in combination with INH. Additionally, the MICs of FQs against rBCG strains were examined with a sub-lethal concentration of EMB (1 μg/ml) to see any synergistic relations (Table 4). The MICs of WQ-3810 against rBGG strains were determined to be between 0.0625 and 1 μg/ml, while those of MXF ranged from 0.0625 to 4 μg/ml. As shown in Fig. 8, the addition of a sub-lethal concentration of EMB to the drug susceptibility test increased the correlation between $IC_{50}$s and MICs of WQ-3810 (correlation coefficient value, R = 0.982), and caused an approximately 8-fold decrease in the MIC of WQ-3810.

**Table 5. Interaction between FQs and cell wall synthesis inhibitors.**

| Strain / drug combinations | MIC of FQ (μg/ml) | MIC of cell wall synthesis inhibitor (μg/ml) | FIC of FQ | FIC of cell wall synthesis inhibitor | FICI | $FICI_m$ |
|---|---|---|---|---|---|---|
| rBCG/Mtb/WT | | | | | | |
| MXF + EMB | 0.0625 | 0 | 1 | 0 | 1 | 1 |
| | 0 | 2 | 0 | 1 | 1 | |
| WQ-3810 + EMB | 0.5 | 0 | 1 | 0 | 1 | 0.5 |
| | 0.25 | 0.125 | 0.5 | 0.0625 | 0.5625 | |
| | 0.125 | 0.5 | 0.25 | 0.25 | 0.5 | |
| | 0.0625 | 1 | 0.125 | 0.5 | 0.625 | |
| | 0 | 2 | 0 | 1 | 1 | |
| MXF + INH | 0.0625 | 0 | 1 | 0 | 1 | 1 |
| | 0 | 0.0625 | 0 | 1 | 1 | |
| WQ-3810 + INH | 0.5 | 0 | 1 | 0 | 1 | 0.75 |
| | 0.25 | 0.015625 | 0.5 | 0.25 | 0.75 | |
| | 0.125 | 0.03125 | 0.25 | 0.5 | 0.75 | |
| | 0 | 0.0625 | 0 | 1 | 1 | |

Concentration ranges were as follows: MXF (0.0625-32 μg/ml), WQ-3810 (0.0625-32 μg/ml), EMB (0.0625-4 μg/ml), INH (0.00195-0.125 μg/ml).

Synergistic activity was evaluated by calculating FICI. $FICI_m \leqq 0.5$ is regarded as synergistic.

**Table 5. Interaction between FQs and cell wall synthesis inhibitors (continued).**

| Strain / drug combinations | MIC of FQ (μg/ml) | MIC of cell wall synthesis inhibitor (μg/ml) | FIC of FQ | FIC of cell wall synthesis inhibitor | FICI | $FICI_m$ |
|---|---|---|---|---|---|---|
| rBCG/Mtb/G88C | | | | | | |
| MXF + EMB | 8 | 0 | 1 | 0 | 1 | 0.75 |
| | 4 | 0.5 | 0.5 | 0.25 | 0.75 | |
| | 0 | 2 | 0 | 1 | 1 | |
| WQ-3810 + EMB | 8 | 0 | 1 | 0 | 1 | 0.5 |
| | 2 | 0.5 | 0.25 | 0.25 | 0.5 | |
| | 0.5 | 1 | 0.0625 | 0.5 | 0.5625 | |
| | 0 | 2 | 0 | 1 | 1 | |
| MXF + INH | 8 | 0 | 1 | 0 | 1 | 0.75 |
| | 2 | 0.03125 | 0.25 | 0.5 | 0.75 | |
| | 0 | 0.0625 | 0 | 1 | 1 | |
| WQ-3810 + INH | 8 | 0 | 1 | 0 | 1 | 0.625 |
| | 4 | 0.015625 | 0.5 | 0.25 | 0.75 | |
| | 1 | 0.03125 | 0.125 | 0.5 | 0.625 | |
| | 0 | 0.0625 | 0 | 1 | 1 | |

Concentration ranges were as follows: MXF (0.0625-32 μg/ml), WQ-3810 (0.0625-32 μg/ml), EMB (0.0625-4 μg/ml), INH (0.00195-0.125 μg/ml).

Synergistic activity was evaluated by calculating FICI. $FICI_m \leqq 0.5$ is regarded as synergistic.

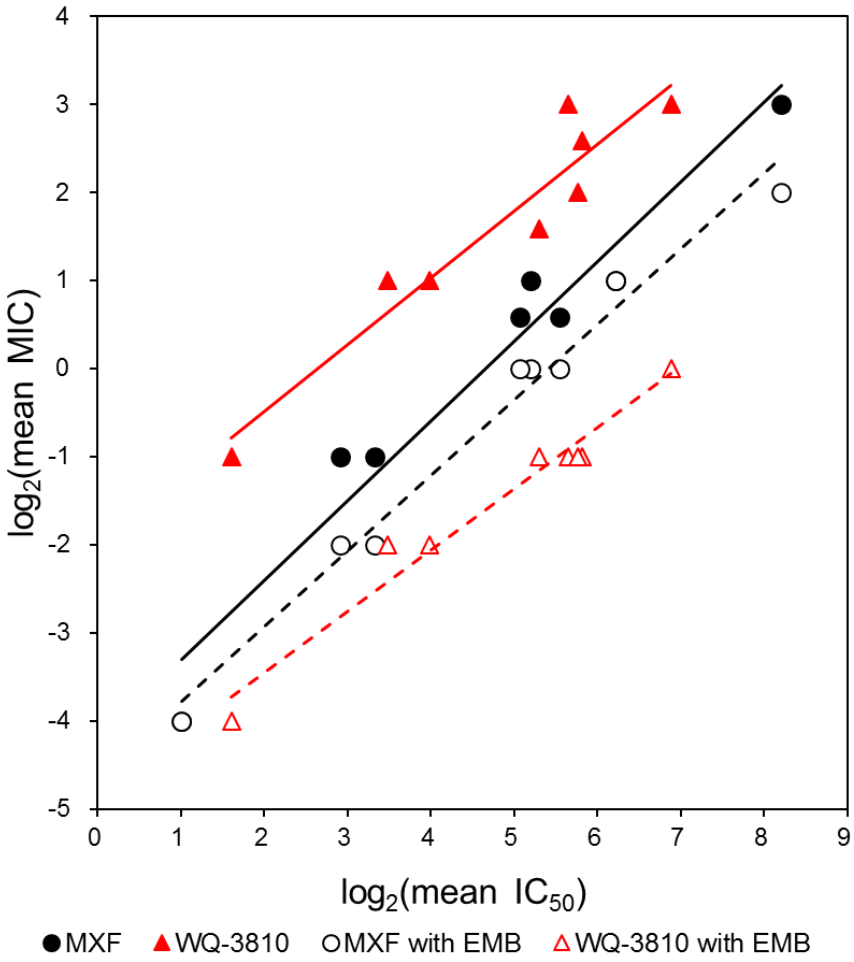



**Figure 8. Correlation between $IC_{50}$s and MICs of FQs.**

$Log_2(IC_{50})$s of MXF or WQ-3810 against each recombinant DNA gyrase were plotted on x axis. $Log_2$(MIC)s of corresponding FQs alone or with EMB (1 μg/ml) against each rBCG strain were plotted on y axis. R indicates correlation coefficient value.

## 4. Discussion

In this study, I evaluated the enzyme inhibitory activity of WQ-3810 using recombinant *Mtb* DNA gyrases. For in vitro evaluation of the FQs, recombinant DNA gyrases derived from the *Mtb* H37Rv strain, a type strain, have been used generally. On the other hand, in this study, the recombinant DNA gyrases were derived from the Beijing lineage of *Mtb* that harbors polymorphisms at positions Q21, T95 and D668 in the GyrA subunit of DNA gyrase. These are different amino acids from these in the *Mtb* H37Rv subunit which instead possesses E21, S95 and G668, respectively. Even though these positions have not been thought to confer FQ resistance [2,17], position 95 is located in the quinolone resistance-determining region. Moreover, the DNA gyrase polymorphisms in the *Mtb* Beijing lineage are highly conserved among the globally dominant *Mtb* strains [17–19]. Therefore, in my study the *Mtb* Beijing lineage DNA gyrase was used to evaluate the inhibitory activity of FQs. Comparing the results of $IC_{50}$s of conventional FQs against Beijing lineage and H37Rv DNA gyrases from this study with the published data [5,16,20,21], most of the mutations conferred equivalent FQ resistance to both Beijing and H37Rv DNA gyrases. However, the G88C mutation conferred much higher resistance, with 89-fold and 147-fold increases in $IC_{50}$s of LXV and MXF in this study, while other reports showed 13- or 20-fold and 8.8-fold increases in $IC_{50}$s of LVX and MXF, respectively, against *Mtb* H37Rv DNA gyrases [5,16]. This agrees with reports that clinical *Mtb* isolates harboring the G88C mutation showed considerably higher MIC for FQs [3,4]. In addition, the G88C mutation has been observed so far only in clinical strains with amino acids Q21, T95 and D668 in the GyrA subunit of DNA gyrase as per data in the NCBI database (www.ncbi.nlm.nih.gov). Considering that the polymorphisms in the *Mtb* H37Rv DNA gyrase are found infrequently in epidemiological studies [18,19] and that FQ resistance has been more frequently reported in Beijing lineage isolates [22,23], greater studies of the *Mtb* Beijing lineage DNA gyrase are strongly warranted so as to evaluate the exact functioning of this enzyme.

Some researchers have previously published on the application of recombinant

mycobacteria to FQ evaluation. For example, Malik et al. produced A90V and D94G *Mtb* strains using representative laboratory strains such as H37Rv, Erdman and CDC1551, but the number of analyzed *gyrA* mutations was limited [24]. Moreover, they used temperature sensitive bacteriophage and cosmids for homologous recombination and didn't remove the HYG-resistance gene from the recombinants, which might have had subtle influences on susceptibility tests. In a similar vein, Yoshida et al. produced recombinant *M. smegmatis* and BCG strains with an expression plasmid carrying *Mtb* H37Rv DNA gyrase genes [15]. Since the *Mtb* DNA gyrase was expressed on the plasmid ectopically, it might have affected the expression level of the enzyme and interfere with the results of drug susceptibility tests. In this study, I produced rBCG strains in which their own DNA gyrase-coding genes were replaced with *Mtb* Beijing lineage variants by electroporation of a recombinase-expressing plasmid and allelic exchange substrates. Since the HYG selection marker was removed from the chromosomal DNA, it would not have any off target effects on gene transcription. The *Mtb* DNA gyrase genes were inserted via homologous recombination into the identical chromosomal locus in the BCG strain ensuring the genes were in the correct genomic contact and under the same transcriptional controls as in *Mtb*. Of course BCG also belongs to the same complex as *Mtb* with an identical 16S rRNA sequence and 99.9% similarity at the nucleotide level [17,25]. Hence, the applications of rBCG strains produced in my study were useful to precisely clarify the intracellular relationship between FQs and mutations in DNA gyrase.

According to the results of previous studies [26,27], the combination of a 5-amino-2,4-difluorophenyl group at N-1 position, azetidinyl group at C-7 position, and Cl or Br or Me group at C-8 position of the quinolone ring gives potent antibacterial activity against diverse bacterial species. In addition, the presence of a 2,4-difluorophenyl group at the N-1 position further increased the in vitro potency as well as in vivo efficacy in mice model compared to the cyclopropyl group at the same position in MXF and ciprofloxacin [26]. Additionally, the *m*-amino group on the N1 phenyl ring enhanced the bactericidal activity, especially against FQ-resistant strains [27]. The *m*-amino group might be effective as an electron acceptor in the FQ binding pocket.

Therefore, WQ-3810, developed from these analogues, also had good antibacterial activity against FQ-resistant pathogens. Kazamori et al reported the potent antibacterial activity of WQ-3810 against FQ-resistant clinical isolates of *E. coli, A. baumannii*, *S. pneumoniae* and *S. aureus* [8]. They also analyzed the inhibitory activity of WQ-3810 against *E. coli* DNA gyrase using recombinant proteins [8]. While WQ-3810 and LVX exhibited comparable inhibitory activity against WT DNA gyrase, the inhibitory activity of WQ-3810 was nine-fold more potent against DNA gyrase with substitutions of S83L and D87N (corresponding to the substitutions at the positions 90 and 94 in *Mtb*) in GyrA than that of LVX. The inhibitory activity of WQ-3810 was also analyzed against the *Mtb* Beijing lineage DNA gyrase in my study. WQ-3810 showed potency even against the LVX-resistant enzymes and inhibited DNA gyrase carrying the G88C substitution more strongly than MXF. These findings might be because of the unique conformational features at the N1, C7 and C8 substituents of the quinolone ring in WQ-3810.

Both enzyme assay and bacterial assay showed that WQ-3810 has potent antimycobacterial activities, in particular against variants carrying the G88C mutation which is responsible for high-level FQ resistance in clinical *Mtb* isolates [3,4]. According to the crystal structure of DNA gyrase, the G88C amino acid substitution is located at the beginning of α-helix 4 of the breakage-reunion domain of GyrA [28], which is assumed to play an important role in making the FQ binding pocket, while amino acids 90 and 94 make a water-metal ion interaction with the C3/C4 keto acid of FQ [6,29]. Therefore, the G88C substitution can change the conformation of the loop connecting α-helix 3 and 4 and affect the depth of the FQ binding pocket, leading to FQ resistance [30]. In addition, some groups have reported another FQ binding mode in DNA gyrase where position 88 of GyrA directly binds the C7 substituent of FQs through GyrA-based cross-linking [31,32]. Hence, substitutions at position 88 can disrupt the interaction between DNA gyrase and FQs. Considering the potent activity of WQ-3810 against G88C mutants shown in my current study, C7-azetidinyl-substituted-FQs might bind the FQ-binding pocket of DNA gyrase without a strong interaction at position 88. In epidemiological studies of *Mtb* clinical isolates the G88C substitution

has been identified at a low frequency, but it is easily produced under the pressure of novel potent C8-methoxy FQs such as PD161144, PD161148 and DC-159a [16,31]. This indicates that the newly developed FQs might increase the occurrence of the G88C mutation in *Mtb* clinical isolates. Conversely, WQ-3810 is still efficacious against variants carrying the G88C mutation, as well and other mutations, compared with conventional FQs.

Contrary to the potency of WQ-3810 against DNA gyrase, the FQ susceptibility test with rBCG strains showed that WQ-3810 had a relatively weak antimycobacterial activity compared with MXF. The lower efficacy of WQ-3810 against mycobacteria indicates that permeability of WQ-3810 may be less for mycobacteria than other bacterial species because mycobacteria have a thick lipid-rich cell wall composed of mycolic acids, which form a primary hydrophobic barrier [33]. Many researchers have tried to develop lipophilic FQs to enhance their penetration into the bacterial cytoplasm and oral absorption properties [34]. Similarly, Itoh et al designed and synthesized WQ-3810 by the introduction of an alkyl group into 7-(3-aminoazetidin-1-yl) FQ so as to increase its lipophilicity [7]. Some reports show that such FQs have high potency in general against bacterial species, but other reports indicate lipophilicity of FQs has little importance for activity against mycobacteria [35]. To clarify the efficacy of WQ-3810 in mycobacteria, a checkerboard assay with cell wall synthesis inhibitors was performed so as to obstruct the formation of lipid-rich cell wall and hence potentially increase permeability. WQ-3810 showed lower $FICI_m$s than MXF in combination with INH or EMB. In particular, the combination of WQ-3810 and EMB showed synergistic activity against FQ-susceptible and -resistant rBCG strains. In addition, sub-lethal concentrations of EMB enhanced bactericidal activity of WQ-3810 but not MXF. Those results showed that WQ-3810 has antimycobacterial potency equal to or even greater than MXF in combination with cell wall synthesis inhibitors. While INH inhibits cell wall biosynthesis by blocking the biosynthesis of mycolic acids, EMB inhibits the arabinosyl-transferases, leading disruption of arabinogalactan in the cell wall and liberation of mycolic acid residues as well [36]. Therefore, EMB might make the mycobacterial membrane more permeable to compound,

especially lipophilic antibiotics [37]. In current situation, utilities of other cell wall synthesis inhibitors such as delamanid and prothionamide could also be recommended as second-line anti-TB drugs for synergizing WQ-3810 potency.

In conclusion, my study evaluated the efficacy of WQ-3810 against *Mtb* using recombinant *Mtb* DNA gyrases and rBCG strains. The DNA gyrase inhibitory assay revealed that WQ-3810 showed inhibition against LVX-resistant DNA gyrases. In addition, WQ-3810 was efficacious even against a DNA gyrase variant harboring a G88C mutation which conferred the highest resistance against MXF. Furthermore, the combination of WQ-3810 and EMB exerted antimicrobial potency equal to or even greater than MXF. In high TB burden countries, FQs can be easily obtained at pharmacies and readily prescribed by medical doctors. In such countries, the ease of FQ availability not only drives a high rate of FQ-resistance acquisition in MDR-TB [38] but also non-MDR-TB shows FQ mono-resistance [39,40]. FQ-resistant TB is difficult to treat, but multi-drug therapy that includes WQ-3810 and EMB might have potential as a new, potent anti-TB treatment regimen that can be effective even against FQ-resistant *Mtb* in clinical settings.

**Summary**

FQ resistance in *Mtb,* caused by amino acid substitutions in DNA gyrase, has been increasingly reported worldwide. WQ-3810 is a newly developed FQ that is highly active against FQ-resistant pathogens; however, its activity against *Mtb* has not been evaluated. Herein I examined the efficacy of WQ-3810 against *Mtb* through the use of recombinant *Mtb* DNA gyrases. In addition, in vitro antimycobacterial activity of WQ-3810 was evaluated against recombinant BCG strains in which gyrase-coding genes were replaced with *Mtb* variants containing resistance-conferring mutations. WQ-3810 showed a higher inhibitory activity than LVX against most recombinant DNA gyrases with FQ-resistance mutations. Furthermore, WQ-3810 showed inhibition even against a DNA gyrase variant harboring a G88C mutation which is thought to confer the highest resistance against FQs in clinical *Mtb* isolates. In contrast, the FQ susceptibility test showed that WQ-3810 had relatively weak mycobactericidal activity compared with MXF. However, the combination of WQ-3810 and EMB showed the greatest degree of synergistic activity against recombinant strains. Since FQs and EMB have been used in multi-drug therapy for TB, WQ-3810 might represent a new, potent anti-TB drug that can be effective even against FQ-resistant *Mtb* strains.

## CHAPTER II:

## Fitness cost of fluoroquinolone-resistant *Mycobacterium tuberculosis*

### 1. Introduction

*Mtb* is intrinsically resistant to various antibiotics due to its thick lipid-rich cell wall [33], and only limited drugs are effective for TB treatment. In clinically relevant *Mtb* strains, resistance mechanisms against those drugs are strongly associated with target alterations caused through the acquisition of specific chromosomal mutations [41]. However, its alterations often cause a fitness cost such as reduced bacterial growth in the absence of the drugs, because the drug target is generally essential for bacteria [42]. In *Mtb*, the fitness costs of resistance acquisition to some of anti-TB drugs such as INH, RMP or KM have been studied very well [43–45]. So far, some of researchers have studied fitness cost in FQ-resistant mycobacteria using experimentally-derived *M. smegmatis* strains [46,47] or BCG strains with an expression plasmid carrying *Mtb* H37Rv DNA gyrase genes [15]. However, its detailed mechanism was still unclear and the influence of chromosomal mutations in DNA gyrase-coding genes needed to be analyzed. To analyze the fitness cost in FQ-resistant *Mtb*, I utilized recombinant *Mtb* DNA gyrases and rBCGs constructed in CHAPTER I and evaluated the influence of the FQ-resistance mutations on enzymatic and bacterial phenotype.

## 2. Materials and Methods

### 2.1. Reagents

MXF were purchased from LKT Laboratories, Inc. (St Paul, MN, USA). NOV, INH, RMP, KM, PZA and GM were purchased from FUJIFILM Wako Pure Chemical Co., Ltd. (Osaka, Japan). EMB and SM was purchased from MP Biomedicals, LLC (Solon, OH, USA) and Sigma-Aldrich (St. Louis, MO, USA), respectively. Relaxed pBR322 DNA was purchased from John Innes Enterprises Ltd (Norwich, UK). BD Difco™ Middlebrook 7H9 broth, BD Difco™ Middlebrook 7H10 agar and BD BBL™ Middlebrook OADC Enrichment were obtained from Becton, Dickinson and Co. (MD, USA), and 2% Ogawa medium was purchased from Kyokuto Pharmaceutical Industrial Co., Ltd. (Tokyo, Japan).

### 2.2. Enzyme dose-dependent DNA supercoiling assay

The enzyme dose-dependent supercoiling activity of recombinant DNA gyrases was tested as described in CHAPTER I. Recombinant DNA gyrase subunits purified in CHAPTER I were used in this assay. The reaction mixture contained 1x buffer, 1 mM ATP, 2 nM relaxed pBR322 DNA and indicated concentrations of GyrA and GyrB subunits in a total volume of 30 µl. After the reaction, the relaxed DNA and the supercoiled DNA were separated by agarose gel electrophoresis. To assess the DNA supercoiling activities of each DNA gyrase, the amount of relaxed DNA and supercoiled DNA in the reactions was quantified with ImageJ software (https://imagej.nih.gov/ij/). One unit was defined as the amount of DNA gyrase that is necessary to supercoil 95% DNA.

### 2.3. Growth assay

In this assay, growth rates of parental BCG and rBCG strains constructed in CHAPTER I were analyzed. Glycerol stocks of the strains were spread on Ogawa medium and incubated for 14 days at 37˚C. Appeared colony was suspended into 4 ml Middlebrook 7H9 broth containing 10% OADC enrichment, 0.2% glycerol and 0.05% tyloxapol, and cultured until $OD_{590}$ became 0.15±

0.01. The bacterial culture was diluted 100-fold with 4 ml Middlebrook 7H9 broth and incubated again. After $OD_{590}$ became 0.15±0.01, the $OD_{590}$ was measured every day for 4 days. During the incubation, the culture was vortexed for 20 seconds every day.

### 2.4. Pellicle formation assay

Pellicle formation of parental BCG and rBCG strains (WT and G88C) were examined. The appeared colony on Ogawa medium was suspended into 4 ml Middlebrook 7H9 broth and cultured until $OD_{590}$ became 0.15±0.01. The bacterial culture was diluted 100-fold with 4 ml Sauton's medium (4g/l L-Asparagine Monohydrate, 50 mg/l Ammonium Iron (III) Citrate, 2 g/l Trisodium Citrate Dihydrate, 5.92 g/l $K_2HPO_4$, 3.27 g/l $NaH_2PO_4$, 0.5 g/l $MgSO_47H_2O$, 1 mg/l $ZnSO_47H_2O$, 6% Glycerol, pH 7.1) and incubated without shaking for 5 weeks at 37˚C.

### 2.5. Growth assay with anti-TB drugs

Growth of WT and G88C rBCG strains were observed in this assay. The strains were transferred from Ogawa medium to 4 ml Middlebrook 7H9 broth and cultured until $OD_{590}$ became 0.15±0.01. The bacterial culture was diluted 100-fold in a new 4 ml Middlebrook 7H9 broth containing indicated concentrations of each anti-TB drug such as MXF, NOV, INH, RMP, EMB, SM, KM or PZA, and incubated for 18 days at 37˚C. PZA was added in original broth (pH 6.8) and acidified broth (pH 5.9) with $KH_2PO_4$. During the incubation, the culture was vortexed for 20 seconds every day and the $OD_{590}$ was measured every two days.

### 2.6. Cell culture

Murine leukemia macrophage cell line RAW 264 (RCB0535) and human acute monocytic leukemia cell line THP-1 (RCB1189) were purchased from RIKEN BioResource Research Center (Ibaraki, Japan). RAW 264 was maintained in DMEM (Sigma-Aldrich) and THP-1 was maintained in RPMI-1640 medium (FUJIFILM Wako Pure Chemical Co., Ltd.) supplemented with 10%

heat-inactivated FBS (Biosera, Nuaille, France). Cells were cultured in a humidified 5% $CO_2$ atmosphere at 37˚C.

### 2.7. BCG infection in cell lines

WT and G88C rBCG strains were grown on Ogawa medium. The bacteria were transferred to into 4 ml Middlebrook 7H9 broth and cultured until $OD_{590}$ became 0.15±0.01. The pre-cultured bacteria were diluted 100-fold with Middlebrook 7H9 broth and the bacteria were grown to the log phase with shaking. The bacteria were harvested as inoculum and frozen at -80˚C with 15% glycerol. The CFU of glycerol stocks was determined by plating serial 10-fold dilutions on Middlebrook 7H10 agar (supplemented with 10% OADC enrichment, 0.2% glycerol and 0.05% tyloxapol) and counting colonies after 3-week incubation.

RAW 264 cells ($1 \times 10^5$) were cultured in 400 μl DMEM containing 10% FBS for 1 day in 24-well cell culture plates at 37˚C at 5% $CO_2$ before BCG infection. THP-1 cells ($2 \times 10^5$) were cultured in 400 μl RPMI medium containing 10% FBS in the presence of PMA (10 ng/ml) for 2 days in 24-well cell culture plates at 37˚C at 5% $CO_2$. After washing twice with D-PBS, cells were cultured in RPMI medium again for 1 day. BCG infection was carried out as previously described [48]. Briefly, after the cells were washed twice with D-PBS, the cells were cultured in 400 μl each medium with rBCG strains at MOI 2.5, MOI 0.25 or MOI 0.025 for 2 h at 37˚C. Following removal of the medium, the cells were washed twice with D-PBS and cultured in 400 μl complete medium containing GM (50 μg/ml) for 4 h at 37˚C. After washing twice with D-PBS, cells were cultured in complete medium with GM (10 μg/ml). The infected cells were lysed by 0.025% SDS at days 1, 3 and 5 post infection and the numbers of intracellular bacteria were determined by plating and counting as explained above.

## 3. Results

### 3.1. DNA supercoiling activity of *Mtb* DNA gyrases

DNA supercoiling activities in combinations of WT or each mutant GyrA and GyrB were examined using relaxed pBR322 DNA as a substrate in the presence of ATP. WT and all mutant DNA gyrases showed enzyme dose-dependent increase in the amount of supercoiled DNA in agarose gel electrophoresis (Fig.9). According to the calculation, DNA supercoiling activity of DNA gyrase with A90V substitution ($2.9 \times 10^5$ U/mg) had no significant difference with WT enzyme ($2.6 \times 10^5$ U/mg) (Fig. 10). On the other hand, other mutant showed almost 2-fold weaker activities than WT, ranging from $1.2 \times 10^5$ to $1.4 \times 10^5$ U/mg (Fig. 10).

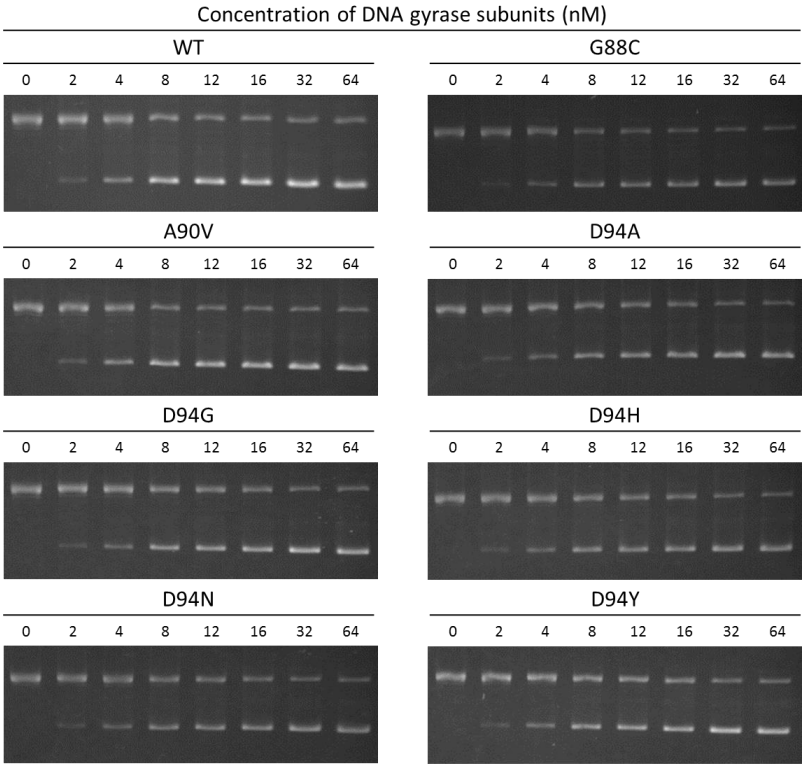



**Figure 9. Enzyme dose-dependent supercoiling activity of DNA gyrases.**

The reaction mixture (30 μl) containing indicated concentrations of GyrA (WT or each mutant) and GyrB, 2 nM relaxed DNA and 1 mM ATP was incubated at 37˚C. After 60 min, the reactions were terminated by SDS and samples were electrophoresed through a 1% agarose gel.

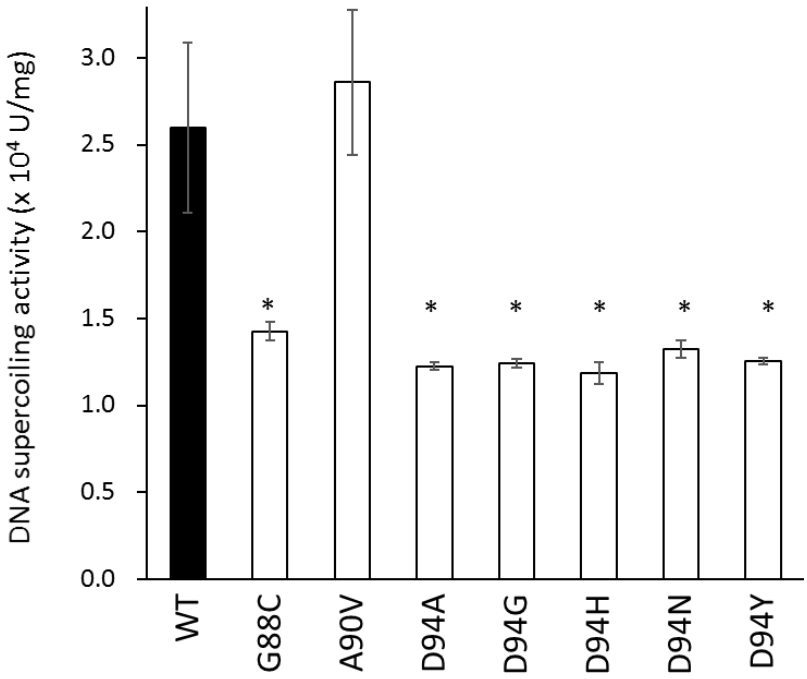



**Figure 10. DNA supercoiling activity of DNA gyrases.**

Proportion of supercoiled pBR322 in applied DNA was calculated from agarose gel electrophoresis in triplicate. One unit of enzyme activity was defined as the amount of DNA gyrase that converted 95% of relaxed DNA to the supercoiled form. Statistically significant differences in the enzyme activity from WT were described using Dunnett's test (*, $p<0.01$).

### 3.2. Growth and pellicle formation of rBCG strains

Growth of parental BCG and rBCG strains was observed in Middlebrook 7H9 broth (Fig. 11). There were no big differences in growth of all mutant recombinants comparing with WT and parental strains. Because mycobacteria have a high lipid content on cell surface, they form a pellicle in standing culture without detergent [49]. Thus, pellicle formation of parental BCG and rBCG strains (WT and G88C) were examined in Sauton's medium. Parental BCG and WT rBCG strains form pellicles at the air-water interface (Fig. 12). In contrast, the ability of pellicle formation is lower in G88C rBCG strain.

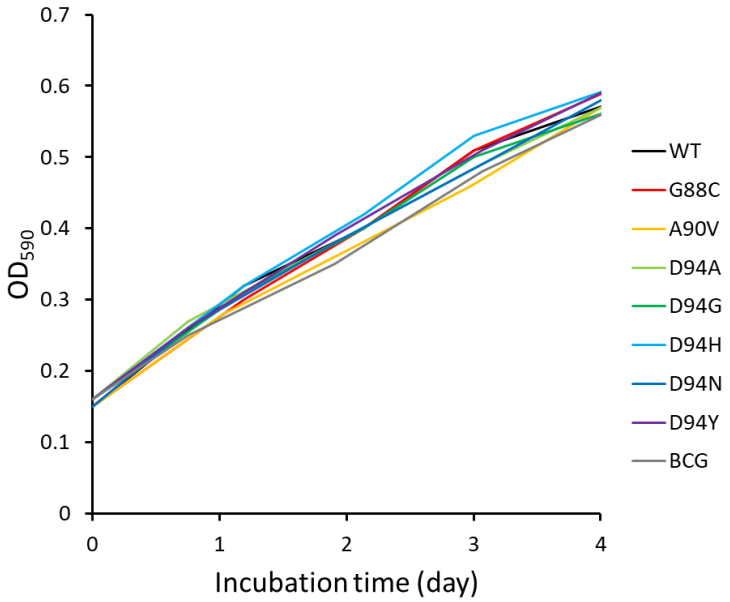



**Figure 11. Growth of rBCG strains.**

Growth of parental BCG and rBCG strains was observed. After the $OD_{590}$ reached $0.15 \pm 0.01$, it was measured for 4 days at 37˚C. During the incubation, the culture was vortexed for 20 seconds every day.

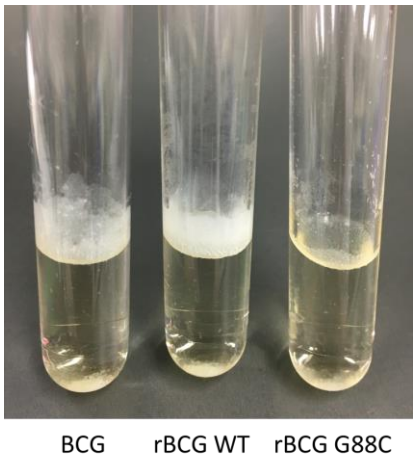



**Figure 12. Pellicle formation of BCG and rBCG strains.**

Pellicle formation of parental BCG and rBCG strains (WT and G88C) were examined. The bacterial strains were cultured in standing Sauton's medium for 5 weeks at 37˚C.

### 3.3. Growth of rBCG strains under pressure of anti-TB drugs.

The pellicle formation can influence on tolerance to antibiotics in mycobacteria [49,50]. Therefore, consequences of the G88C mutation in DNA gyrase on the bacterial susceptibility to drugs were investigated (Fig. 13). The G88C rBCG strain grew at same speed as WT strain in the absence of any drugs (Fig. 13A) but showed increased resistance to MXF (Fig. 13B). While 4 μg/ml MXF was able to induce growth arrest in the mutant, the WT growth was inhibited at 0.0625 μg/ml. These concentrations were corresponding to the results of MICs in CHAPTER I. On the other hand, the mutant caused increased susceptibilities to some of antibiotics. Treatment with NOV, an aminocoumarin antibiotic that inhibits DNA gyrase by targeting the GyrB subunit [51], caused delayed growth of the mutant strain even at 2 μg/ml and completely inhibited at 32 μg/ml, while the growth of WT strain was completely inhibited at 64 μg/ml (Fig. 13C). Furthermore, the G88C rBCG strain showed slower growth than WT strain at 0.0625 μg/ml of INH and 0.0391-0.0781 μg/ml of RMP (Fig. 13D and E). The mutant strain can grow similarly with WT under the pressure of other anti-TB drugs such as EMB, SM, KM and PZA (Fig. 14).

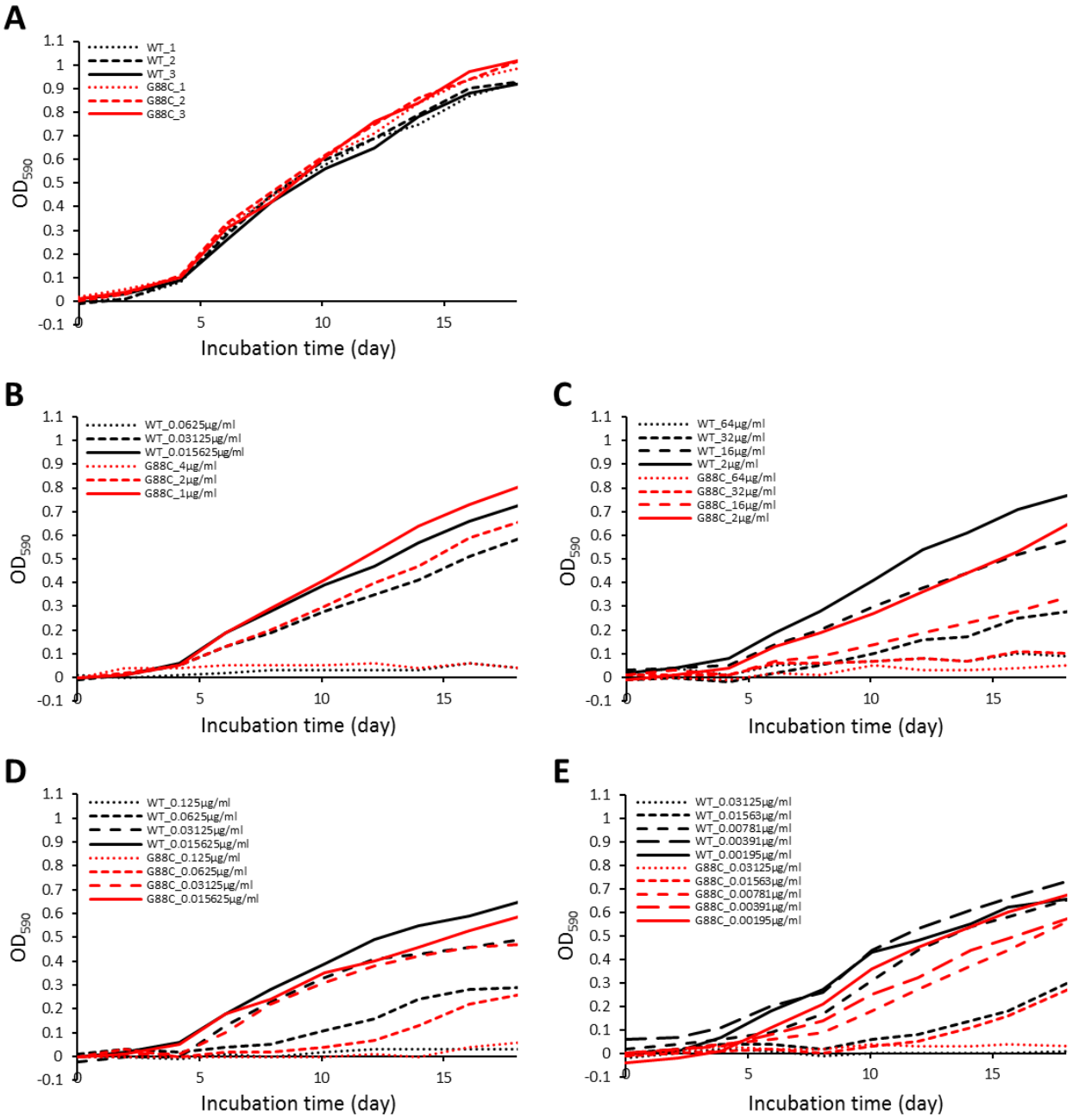



**Figure 13. Growth curve of rBCG strains under pressure of anti-TB drugs (1).**

WT and G88C rBCG strains were cultured without antibiotics (A) or with sublethal concentrations of each anti-TB drugs (B-E). The $OD_{590}$ was measured for 18 days at 37˚C. During the incubation, the culture was vortexed for 20 seconds every day. Concentration ranges were as follows: (B) MXF (0.015625-0.0625 μg/ml for WT, 1-4 μg/ml for G88C), (C) NOV (2-64 μg/ml), (D) INH (0.015625-0.125 μg/ml) and (E) RMP (0.00195-0.03125 μg/ml). The bacterial strains were cultured in triplicate only in the culture without any antibiotics.

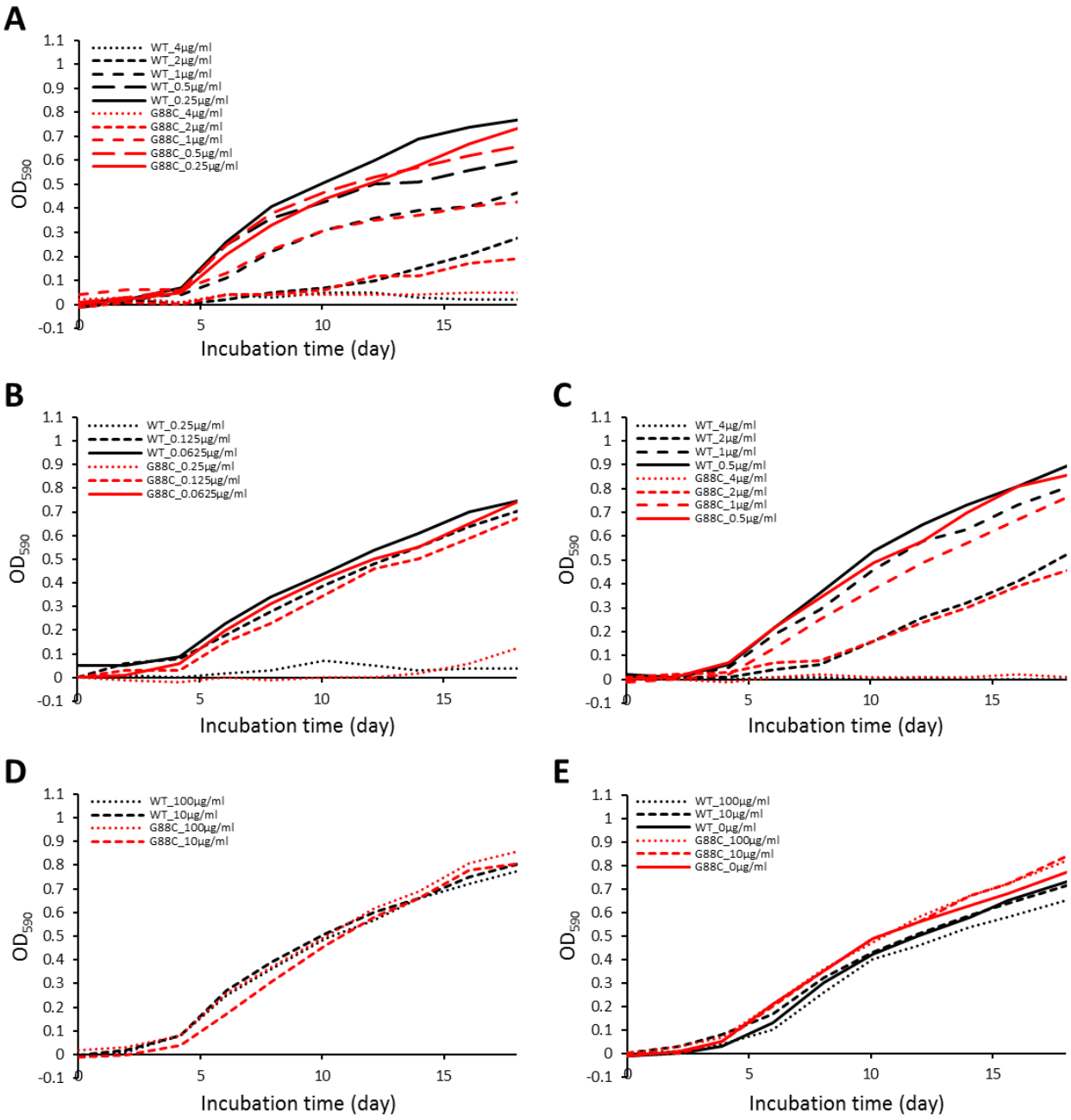



**Figure 14. Growth curve of rBCG strains under pressure of anti-TB drugs (2).**

WT and G88C rBCG strains were cultured with sublethal concentrations of each anti-TB drugs. The $OD_{590}$ was measured for 18 days at 37˚C. During the incubation, the culture was vortexed for 20 seconds every day. Concentration ranges were as follows: (A) EMB (0.25-4 μg/ml), (B) SM (0.0625-0.25 μg/ml), (C) KM (0.5-4 μg/ml), (D) PZA (10-100 μg/ml) and (E) PZA (0-100 μg/ml) using Middlebrook 7H9 broth at pH 5.9.

### 3.4. Intracellular viability of rBCG strains

To evaluate the influence of G88C mutation on intracellular viability, RAW 264 and PMA-differentiated THP-1 cell lines were infected with WT or G88C rBCG strains at various MOIs (Fig. 15). In RAW 264 cells, the intracellular bacterial numbers of G88C strain were lower than WT strain even at day 1 of post-infection (Fig. 15A). After 5-day incubation of G88C strain at MOI 0.025, the number of viable intracellular bacteria decreased by 10-fold. In PMA-differentiated THP-1 cells, the intracellular bacterial numbers of the mutant strain were comparable to those of WT at MOI 0.025 (Fig. 15B). In contrast, the intracellular bacterial numbers of G88C strain were less than those of WT under the conditions at the high MOIs (Fig. 15B).

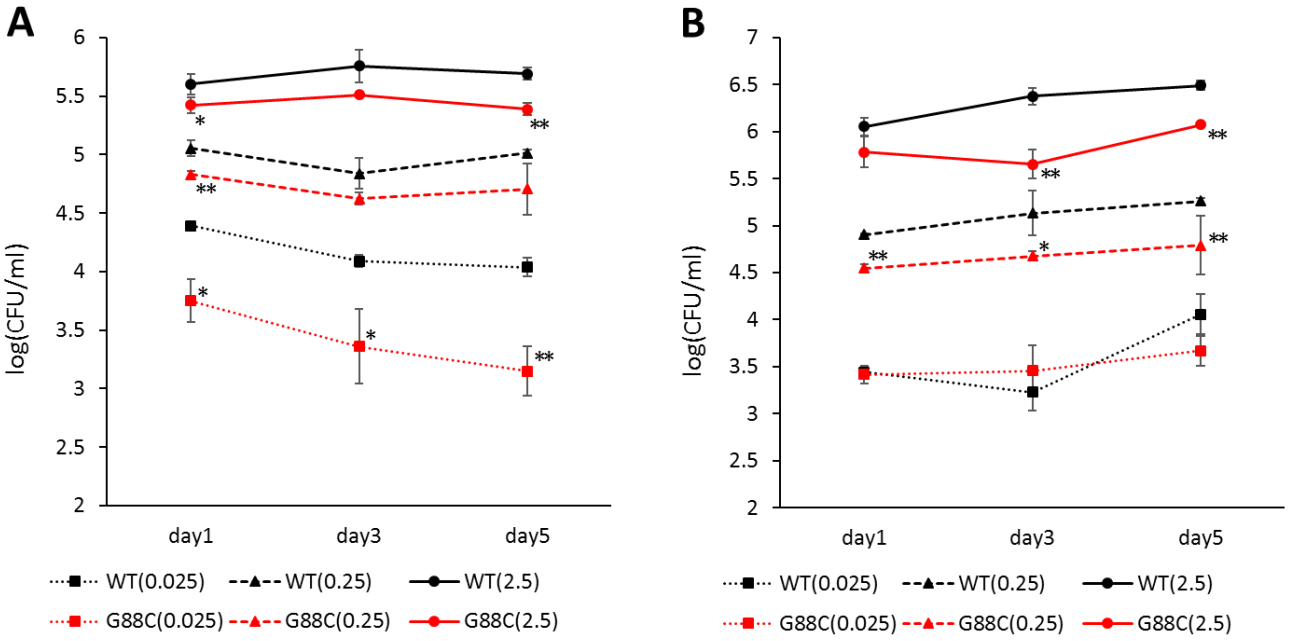



**Figure 15. Intracellular viability of rBCG strains.**

(A) RAW 264 and (B) PMA-differentiated THP-1 cell lines were infected with WT or G88C rBCG strains at MOI 0.025, 0.25 or 2.5. CFUs were measured at 1, 3 and 5 days after infection. Mean ± SD for three independent experiments is shown. Using Student's t-test, statistically significant differences with WT were indicated (*, $p<0.05$; **, $p<0.01$).

## 4. Discussion

In this study, I evaluated the influences of FQ-resistance mutations on DNA supercoiling activity of the DNA gyrase. Only one mutation, A90V, didn't show any influence on DNA gyrase supercoiling activity (Fig. 9 and 10). It indicates that clinical *Mtb* isolates with A90V mutation can be maintained in patients without fitness cost. Indeed, this mutant type has been isolated more frequently than others despite of weak FQ resistance [52,53]. On the other hands, the other amino acid substitutions reduced not only FQ susceptibility (shown in CHAPTER I) but also supercoiling activity of DNA gyrase (Fig. 9 and 10). The reduced activities of D94G, D94H and D94N *Mtb* DNA gyrases were also observed in other studies [20,21] and similar results were obtained from another study using G89C and D95G *M. leprae* DNA gyrases (corresponding to the substitutions at the positions 88 and 94 in *Mtb*) [54].

Because the DNA gyrase activity is essential for efficient DNA replication and transcription, I expected that these mutations should have deleterious effects on bacterial growth. The influences of FQ-resistance mutations on mycobacterial growth have been assessed in previous studies. According to the study by Borrell et al., experimentally selected *M. smegmatis* strains carrying D94G or D94Y had no fitness cost, while G88C and D94N mutants showed significantly lower fitness [46]. Similarly, Luo et al. reported no or only a low effect on the growth rate of the experimentally selected *M. smegmatis* strains with A90V, D94G, D94H or D94N substitutions [47]. Yoshida et al. utilized recombinant *M. smegmatis* and BCG strains with an expression plasmid carrying *Mtb* H37Rv DNA gyrase genes [15] and reported that the growth rates were decreased due to G88C, D94A and D94H substitutions in *M. smegmatis* and G88C and D94G substitutions in BCG. However, the influences of FQ-resistance mutations on *Mtb* still remain unclear because *M. smegmatis* is genetically not close to *Mtb* such as BCG and the ectopic expression of DNA gyrase might affect the expression level of the enzyme. Therefore, I used recombinant BCG strains whose DNA gyrase-encoding genes on chromosome were replaced with *Mtb* genes carrying each mutation in order to examine the influences of the FQ-resistance mutations on bacterial phenotype. Contrary

to the reduced enzymatic activities of mutant DNA gyrases, the mutant rBCG strains didn't cause disadvantage on growth rate (Fig. 11). Though I expected the decreasing DNA gyrase activity can cause slower growth in mutant rBCG strains following topological change of DNA, it has been known that the topological homeostasis is maintained by autoregulation of DNA topoisomerases such as topoisomerase I and DNA gyrase in mycobacteria [55,56]. Sublethal concentration of NOV, a competitive inhibitor of ATP binding by DNA gyrase, caused relaxation of the DNA followed by relaxation stimulated transcription of DNA gyrase [55]. Simultaneously, expression of topoisomerase I was downregulated by supercoiling sensitive transcription [56]. Hence, I speculate that the sublethal effect by the reduced enzymatic activity due to FQ-resistance mutations might be compensated by regulation of DNA topoisomerases. To confirm the speculation of compensatory mechanism, I should perform further analysis to assess the expression level of each genes associated with the maintenance of DNA topology.

The activities of DNA topoisomerases are also related to bacterial morphology. Conditional knock-down *M. smegmatis* strains of topoisomerase I or DNA gyrase showed loss of pellicle formation in standing cultures [57,58]. I predicted that the perturbation of DNA topology by FQ-resistance mutations can also alter the surface phenotype of rBCG strains. In pellicle formation assay, the G88C substitution, which showed the highest fitness cost in the previous studies [15,46], caused the reduced ability of pellicle formation in the rBCG strain (Fig.12). It might be caused by concomitant decreases in glycopeptidolipid expression or cell division along with disturbance of DNA topology [57,58].

The altered surface property can result in a distinct effect on permeability of the bacterial cell wall [49,50]. Sublethal concentrations of INH and RMP, first-line anti-TB drugs, reduced the growth rate of G88C rBCG strain slightly more than that of WT (Fig. 13). The similar results were observed in the previous study using the conditional knock-down *M. smegmatis* strain of DNA gyrase [58]. Therefore, the reduction in the enzymatic activity of G88C DNA gyrase can increase the cell permeability to some drugs compared with WT. In addition, the rBCG strain carrying G88C

substitution revealed much higher susceptibility to NOV than WT strain. It indicated that the enzymatic activity of DNA gyrase, when reduced by the FQ-resistance mutation, had further suppression by NOV, targeting the ATP-binding site of GyrB subunit different from FQ-binding site of GyrA in the enzyme. As with NOV, other DNA topoisomerase inhibitors such as SPR720, an aminobenzimidazole inhibitor of GyrB subunit in clinical trial [59] might be effective to disturb the topological maintenance of mycobacterial DNA, for treatment of FQ-resistant TB.

Mycobacteria causes pulmonary infection generally through the phagocytosis by macrophages. The altered surface property can also influence on intracellular viability of mycobacteria [60,61]. As a result of the infection of rBCG strains in RAW 264 cells, the G88C mutation in DNA gyrase decreased intracellular viability at different MOIs (Fig.15). On the other hands, the infection of rBCG strains in PMA-differentiated THP-1 cell lines showed that the G88C mutation in DNA gyrase decreased intracellular bacterial numbers only at MOI 0.25 and MOI 2.5 (Fig.15). It is probably because the BCG strains are much susceptible to human macrophages at the lower MOI, leading the lower numbers of intracellular bacteria even at day 1 of the post-infection. Contrast to the stable growth in Middlebrook 7H9 broth without any antibiotics, the rBCG strain with G88C substitution was highly sensitive to some anti-TB drugs and macrophage phagocytosis. These results exhibit the FQ-resistant mycobacteria has less ability to survive under the environmental stress. Therefore, the G88C substitution has been identified at a low frequency in *Mtb* clinical isolates by epidemiological studies [3,4]. This agree with less colonization property and low frequency of *Salmonella enterica* serovar Typhimurium strains with G81C (corresponding to the G88C in *Mtb*) in GyrA [62].

In conclusion, I showed that the amino acid substitutions in DNA gyrase can confer not only FQ resistance but also decline in enzymatic activity. Furthermore, it was demonstrated that G88C substitution can alter the surface property of bacteria followed by the increasing susceptibilities to other anti-TB drugs and macrophage phagocytosis. Because the DNA gyrase activity is essential for maintaining DNA topology, the substitutions in the enzyme can disrupt

efficient transcription, leading changes in mycobacterial phenotypes as fitness cost. My results in this study can provide a deeper understanding to develop new drugs and regimens targeting the fitness cost in FQ-resistant *Mtb*.

## 5. Summary

Since most of drug-resistance mechanisms in *Mtb* are due to modification of drug targets caused by chromosomal mutations, the mutations tend to change not only drug binding site but also molecular active site of the target. Hence, some of drug-resistance mutations in *Mtb* frequently cause fitness cost, reduced bacterial ability to replicate or survive in return for drug-resistance acquisition. In this study, I analyzed the influences of FQ-resistance mutations on the enzymatic and bacterial phenotype using recombinant *Mtb* DNA gyrase, an FQ target enzyme, and rBCG strains in which DNA gyrase-coding genes were replaced with *Mtb* genes. According to DNA supercoiling assay and growth assay, while most of the FQ-resistance mutations reduced the enzymatic activity of DNA gyrase, they don't affect growth rates in rBCG strains in absence of any drugs, probably because of autoregulation of DNA topoisomerases. However, constructed rBCG strain with G88C in DNA gyrase showed loss of pellicle formation and was highly sensitive to some anti-TB drugs and macrophage phagocytosis. These results indicate that the mutation in DNA gyrase might influence on tolerance to environmental stress due to the altered surface property following the perturbation of DNA topology. This study can provide a deeper understanding of the fitness cost to develop new drugs and regimens targeting the cost in FQ-resistant *Mtb*.

## CONCLUSION

FQs are antibiotics that inhibit the DNA gyrase activity and have been used for treatment of MDR-TB. However, FQ resistance in *Mtb* have caused a serious public health concern in the world. To control FQ resistance in TB and other mycobacterial infection, development and evaluation of novel DNA gyrase inhibitors and basic studies on the acquisition of FQ resistance in mycobacteria are required. In the present studies, I utilized (1) recombinant *Mtb* DNA gyrases; (2) recombinant BCG strains in which the DNA gyrase-coding genes were replaced with *Mtb* variants, to examine the FQ-resistant *Mtb*.

In the CHAPTER I, I revealed that WQ-3810, a newly developed FQ, can have a higher inhibitory activity than LVX against recombinant DNA gyrases with FQ-resistance mutations. In addition, I elucidated the inhibitory activity of WQ-3810 even against a DNA gyrase harboring a G88C mutation which is thought to confer the highest FQ resistance in clinical *Mtb* isolates. While WQ-3810 have relatively weaker bactericidal activity than MXF in single use, the combination of WQ-3810 and EMB show the great synergistic activity against recombinant strains. WQ-3810 represent a new, potent anti-TB drug that is effective even against FQ-resistant *Mtb* strains, especially in a combination with cell wall synthesis inhibitors.

In the CHAPTER II, I demonstrated the influences of FQ-resistance mutations on enzymatic and bacterial phenotypes as fitness cost in the acquisition of the drug resistance. While most of the FQ-resistance mutations reduced the DNA supercoiling activity of DNA gyrase, they don’t affect growth rates in rBCG strains in the absence of any drugs. However, rBCG strain harboring a G88C mutation in DNA gyrase showed loss of pellicle formation and was highly sensitive to some anti-TB drugs and macrophage phagocytosis. Particularly, the G88C substitution was associated with surface property and sensitivity to environmental stresses in mycobacteria.

In my thesis, I solved the difficulties in prediction of the drug permeability and assessing the influence of DNA gyrase activity on fitness cost in mycobacteria, and showed the importance of

utilizing both the target enzyme and bacterial strain for deeper understanding of FQ resistance in *Mtb*. The approach and findings from the current studies could be helpful for future studies on development and evaluation of novel drugs and regimens for TB treatment.

## ACKNOWLEDGEMENTS

I would like to express the deepest gratitude and appreciation to my supervisor, Prof. Yasuhiko Suzuki from Division of Bioresources, Hokkaido University Research Center for Zoonosis Control. He gives me warm encouragement and generous support throughout my research works and all activities in my PhD course, including writing this dissertation. I greatly appreciate the insightful advice and enormous support offered by Assoc. Prof. Chie Nakajima from Division of Bioresources. I am also particularly grateful to Prof. Hideaki Higashi from Division of Infection and Immunity, and Assoc. Prof. Norikazu Isoda from Unit of Risk Analysis and Management for the meticulous and valuable comments to improve my research activities and this dissertation. I would like to thank Dr. Tetsu Mukai from Department of Mycobacteriology, Leprosy Research Center, National Institute of Infectious Diseases, Assoc. Prof. Takashi Matsuba from Division of Bacteriology, Department of Microbiology and Immunology, Faculty of Medicine, Tottori University, Dr. Aki Tamaru from Department of Microbiology, Osaka Institute of Public Health, and Prof. Stephen V. Gordon from UCD School of Veterinary Medicine, University College Dublin for offering great techniques, knowledges and numerus contribution to my research works. I am deeply grateful to Prof. Anne J. Lenaerts, Prof. Dean C. Crick and Assist. Prof. Gregory T. Robertson from Mycobacteria Research Laboratories, Department of Microbiology, Immunology and Pathology, Colorado State University, Assist. Prof. Apichai Tuanyok from Emerging Pathogens Institute, University of Florida, and Prof. Nyam-Osor Purevdorj from School of Veterinary Medicine, Mongolian University of Life Sciences for the invaluable support.

I truly thank my seniors, Dr. Hyun Kim, Dr. Kazumasa Yokoyama, Dr. Kanjana Changkaew, Dr. Siriporn Kongsoi, Dr. Ruchirada Changkwanyeun, Mr. Eddie Solo, Dr. Hassan Diab, Dr. Fuangfa Utrarachkij, Dr. Marvin A. Villanueva, Dr. Tomoyuki Yamaguchi, Dr. Jeewan Thapa, Dr. Yogendra Shah, Dr. Nan Aye Thida Oo, Dr. Lai Lai San and Dr. Charitha Mendis for teaching me the experimental techniques, knowledges and attitude toward research activity; my lab

mates, Mr. Kentaro Koide, Mr. Jong-Hoon Park, Ms. Ruttana Pachanon, Ms. Dipti Shrestha, Mr. Thoko Flav Kapalamula, Ms. Mwangala Lonah Akapelwa, Ms. Risa Tsunoda, Ms. Wimonrat Tanomsridachchai, Mr. Lawrence P. Belotindos, Ms. Precious Bwalya, Mr. Joseph Yamweka Chizimu and Ms. Nami Ajima for making a very warm and cozy atmosphere in our division; and staff members, Ms. Yukari Fukushima, Ms. Haruka Suzuki, Ms. Miki Nakagawa, Ms. Yayoi Kameda, Ms. Yuko Hidaka and Ms. Midori Yoshida for the technical support and benignity.

I would like to thank Wakunaga Pharmaceutical Co., Ltd. for providing antibiotics. I am also grateful to Dr. Jacobs and Dr. Hatfull for supplying plasmids. I truly appreciate Japan BCG Laboratory for providing bacterial strain. Research works were supported in part by a grant from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan, for the Joint Research Program of the Research Center for Zoonosis Control, Hokkaido University, and in part by Japan Agency for Medical Research and Development (AMED).

I would like to offer my special thanks to my parents for their emotional and financial support, and Marina Aizu for her ongoing and unwavering support to continue my research life.

# 和文要旨

結核は結核菌に由来する感染症であり、世界中で年間約 1000 万人の新規罹患者と約 160 万人の死者を出している。多剤併用療法により治療可能ではあるが、複数の薬剤に耐性を獲得した多剤耐性結核の発生が公衆衛生上非常に深刻な問題となっている。フルオロキノロンは、二次選択薬として多剤耐性結核に処方され、中でもレボフロキサシンおよびモキシフロキサシンの利用が勧められている。フルオロキノロンは、DNA トポロジーを調節する DNA ジャイレースの機能を抑制することで、結核菌の増殖を阻害する。しかしながら、DNA ジャイレース A サブユニット上の 88 位、90 位、94 位のアミノ酸置換によって、フルオロキノロンの結合を回避することで、結核菌はフルオロキノロン耐性を獲得することが知られている。本研究では、フルオロキノロン耐性結核の発生及び蔓延を制御するために、遺伝子組換え技術を駆使し、新規フルオロキノロン WQ-3810 のフルオロキノロン耐性結核菌に対する阻害活性と、フルオロキノロン耐性化が結核菌にもたらす影響を評価した。

## 1. WQ-3810：新規フルオロキノロンのフルオロキノロン耐性結核菌に対する阻害作用

WQ-3810 は、新しく開発されたフルオロキノロンであり、従来のフルオロキノロンに耐性を獲得した大腸菌やアシネトバクター属菌にも殺菌作用を示すことが分かっている。本研究では、フルオロキノロン耐性変異を持つ組換え結核菌 DNA ジャイレースに加えて、結核菌 DNA ジャイレースを発現する組換え BCG 菌を用いて、WQ-3810 の酵素活性阻害作用および殺菌作用を調べた。DNA ジャイレースを用いた酵素活性阻害試験の結果、

WQ-3810 は、全ての変異型に対してレボフロキサシンよりも強い阻害活性を示すのみでなく、臨床結核菌株の中で最も高いフルオロキノロン耐性を与えるとされる G88C 変異型 DNA ジャイレースに対しても優れた阻害活性を発揮した。加えて、組換え BCG 菌を用いたフルオロキノロン感受性試験の結果、WQ-3810 は、単独ではモキシフロキサシンよりも弱い殺菌作用しか示さなかったが、細胞壁合成阻害活性を有する一次選択抗結核薬であるエタンブトールとの併用により、G88C 変異型を含む変異型組換え BCG 菌株に対してモキシフロキサシンより強い殺菌作用を有していることが分かった。以上の結果から、WQ-3810 と細胞壁合成阻害薬との併用療法は、フルオロキノロン耐性結核菌をも制御できる新しい抗結核治療法として、今後の臨床応用が期待された。

**2. フルオロキノロン耐性結核菌における fitness cost**

結核菌では主に薬剤標的酵素の薬剤結合部の構造を変化させる遺伝子変異により薬剤耐性化が生じている。通常、薬剤の標的酵素は菌の生育に必須であるため、変異を起こすと活性が低下し、菌の生育が妨げられる。耐性化の代償で求められるこのような負荷を fitness cost と言うが、結核菌におけるフルオロキノロン耐性変異が生じさせる fitness cost についての詳細は不明であった。本研究では、前述した組換え DNA ジャイレースおよび組換え BCG 菌を用いて、その酵素活性と菌増殖能などの表現型を調べることで、結核菌における fitness cost を評価した。組換え DNA ジャイレースの活性を DNA 超螺旋化試験により評価した結果、A90V 変異型を除いた全ての変異型において、野生型よりも低い活性が確認されたものの、組換え BCG 菌を用いた増殖能評価の結果、全ての変異型組換え菌は野生型と

同等の増殖速度を示すことが分かった。しかしながら、G88C 変異型組換え BCG 菌において、菌膜形成能の消失と、その他の抗結核薬やマクロファージの食作用に対する抵抗性の低下が観察された。これらの結果は、DNA ジャイレース上のアミノ酸置換が、DNA トポロジーの不安定化により結核菌の表面的性質を変化させ、環境ストレスへの抵抗性に影響を与えていることを示唆している。

本研究の成果から、新規フルオロキノロンが特に細胞壁合成阻害薬との併用により、既存薬と同等以上の抗結核作用を有すること、フルオロキノロン耐性をもたらす変異が抗酸菌において生存に不利益な影響を与えることが示唆された。また、本研究で作出された組換え DNA ジャイレースや組換え BCG 菌の利用が、結核菌のフルオロキノロン耐性に関する研究に有効であることが実証されたとともに、結核の新しい治療薬や治療法の開発・評価に助力できるアプローチとなると期待している。

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