INVESTIGATIONS ON THE ROLES OF EFFLUX PUMP INHIBITORS ON THE ANTIBIOTIC TOLERANCE OF NON-REPLICATING *MYCOBACTERIUM SMEGMATIS*

by

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Dedicated to my family members and you as an interested reader of my scientific findings.

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ABSTRACT

Normal healthy people are not susceptible to tuberculosis (TB) but immunocompromised and HIV positive patients are at high risk of TB. The treatment regimen (rifampin, isoniazid and amikacin) for TB patients is 6-9 months for normal patients but if *Mycobacterium tuberculosis* (Mtb) becomes multidrug resistant, it takes 20-30 months to treat. According to the World Health Organization in 2018, there were about half a million new cases among which 78% were multidrug resistant TB. This antibiotic resistance is due in part to its ability to survive in the macrophage in our body by entering a non-replicating persistent state. Mtb also contains efflux pumps that increase antibiotic tolerance by pumping out the drugs. Therefore, if the efflux pump activity can be blocked by using efflux pump inhibitors, then it might increase antibiotic susceptibility of the pathogen. In our study, we used *Mycobacterium smegmatis* (Msm) as a model organism for Mtb and subjected it to a combination of three stresses (low oxygen, low pH and low nutrients) that mimic the physiological stresses in the human body and report that these conditions produced a non-replicating state in Msm. This is the first report of the use of this combination of stresses to produce a non-replicating state in Msm. Our results show that non-replicating Msm became completely tolerant to isoniazid and displayed increased tolerance to rifampin and clarithromycin by nearly 2-fold when compared to log-phase cells. Moreover, the efflux pump inhibitor verapamil decreased the antibiotic tolerance of the nonreplicating Msm to the antibiotics by 6-10 fold and the efflux pump inhibitor piperine decreased tolerance to the antibiotics by 2-4 fold. Also, in this study we attempted to construct a gene knockout mutant lacking two potential ATP-binding cassette transporters to study their functions as drug exporters. However, we were unable to obtain homologous recombination mutants. Further studies on efflux pump inhibitors could potentially enable greater understanding of antibiotic tolerance mechanisms in non-replicating, drug tolerant Mtb and enable the development of novel therapies that shorten treatment time for tuberculosis.

CHAPTER 1. INTRODUCTION

1.1 The causative agent of tuberculosis disease and its effect on public health

Tuberculosis (TB) is the leading cause of death from a single infectious agent worldwide. Each year millions of people continue to fall sick with TB. The estimated death caused by TB was 1.45 million, among which HIV-negative persons was 1.2 million and among HIV-positive individuals was 251,000 in 2018 (WHO, 2019). Drug resistance TB case was more than half million. There are some mechanisms that are thought to be involved in drug resistance such as drug uptake inhibition, drug target modification, drug inactivation and pumping out drugs (Peterson & Kaur, 2018). Also, due to the long TB treatment regimen of 6-9 months, it is easy for patients to miss doses. Non-compliance with the regimen of drugs can play a crucial role in inducing drug resistance.

Mycobacterium tuberculosis (Mtb) is a non-motile, non-capsulated, non-spore forming bacillus surrounded by a thick glycolipid envelope outside the lipid bilayer. This acid fast, slow-growing obligate aerobe requires 18-24 hours to grow and is about 4-10 microns long and 0.5-1.0 microns wide. The membrane of Mtb contains efflux pumps which include ATP binding cassette (ABC) transporter proteins. Latent (Mtb) can remain inside the host cell for decades without causing disease. However, when the host immune system is weakened, it can be reactivated to cause active disease (Daniel, Maamar, Deb, Sirakova, & Kolattukudy, 2011).

1.2 Mtb enters a non-replicating persistent state under a combination of stresses

Mtb enters a non-replicating persistent state inside the macrophage. Mtb-infected macrophages are enclosed within granulomas under hypoxic conditions. In the macrophages, Mtb is thought to experience stresses such as low oxygen, low pH, low nutrient and high CO_2. The combined multiple stresses cause Mtb to enter into a non-replicating persistent state. It has been shown that under combined stress conditions, Mtb becomes more resistant to rifampicin and isoniazid (Deb et al., 2009).

1.3 Lipids are the energy source for latent Mtb

Latent Mtb stores fatty acids as triacylglycerol (TAG) which can act as an energy source during the latent state (Daniel et al., 2004). Mtb uses host fatty acids to accumulate lipid droplets. After lipid accumulation, the Mtb cell acquires a dormancy-like phenotype (Deb et al., 2009). It was shown that radiolabeled fatty acids from human macrophages were directly incorporated into Mtb. The study also showed that the composition of Mtb TAG and host TAG was nearly identical. The incorporated fatty acids are used to synthesize TAG that potentially could serve as an energy source during latent period (Daniel et al., 2011). The fatty acid uptake process in Mtb is not clearly understood. However, it is established that TAG acts as an energy source for Mtb in the non-replicating state.

1.4 Drug-resistance mechanisms in Mtb:

Antibiotic resistance is a major source of morbidity and mortality worldwide. Most microorganisms have the capability of developing antibiotic resistance by modifying a drug target, limiting uptake of a drug, inactivating a drug and active efflux of a drug(Peterson & Kaur, 2018; Reygaert, 2018). One of the mechanisms of drug resistance is pumping out the drug from bacteria by efflux pumps. Efflux pumps are membrane proteins that are involved in the transport of antimicrobial drugs (Rodrigues, Parish, Balganesh, & Ainsa, 2017). Efflux pumps help transporting various molecules across the cell-wall membranes. There are five different classes of bacterial efflux pumps with different structural morphologies, energy sources and substrate specificities (Fig. 1). The five superfamilies include the resistance-nodulation-cell division (RND) superfamily, the multidrug and toxic compound extrusion (MATE) superfamily, adenosine triphosphate binding cassette (ABC) superfamily, the major facilitator superfamily (MFS) and the small multidrug resistance (SMR) superfamily (Pule et al., 2016).There are a total of 267 predicted transporters to exist in Mtb, of which 129 belong to the ABC family; 30, to the major facilitator superfamily (MFS); and 14, to the resistance-nodulation-cell division (RND) family (Antonova, Gryadunov, & Zimenkov, 2018).

1.5 Efflux pump inhibitors and their effects on antibiotic susceptibility of Mtb:

Verapamil is a chemically synthesized calcium (Ca^{2+}) channel blocker which inhibits efflux pump activity by reduction of the transmembrane potential (Lee & Tsien, 1983). Verapamil is used clinically to treat migraines, cardiac disorders and headaches (Wit & Cranefield, 1974). Verapamil interferes with the generation of the proton motif force and inhibits ATP-dependent multidrug transporters and efflux pumps in prokaryotes. A recent study showed that verapamil improved the activity of bedaquiline against *Mycobacterium abscessus in vitro* and in macrophages (Viljoen et al., 2019). Verapamil shows significant inhibitory activity against mycobacterial efflux pumps (Pule et al., 2016). The minimal inhibitory concentration (MIC) of isoniazid was reported to be reduced by the efflux pump inhibitor verapamil in Mtb isolates that were resistant to isoniazid (Jaiswal et al., 2017).

There are some efflux pump inhibitors that are found in nature such as reserpine which is isolated from the roots of *Rauwolfia vomitoria* Afz. Reserpine inhibits vesicular monoamine transporters thereby blocking uptake and storage of dopamine into synaptic vesicles (Plummer, Earl, Schneider, Trapold, & Barrett, 1954). Clinically it is used in treating hypertension and psychiatric disorders (Iwu & Court, 1977). It is one of the potential efflux pump inhibitors that target the RND superfamily (Fig. 1). Depending on the antibiotics, it increases susceptibility 4-15 fold in mycobacteria. (Pule et al., 2016).

Another efflux pump inhibitor piperine used in ancient traditional medicine is present in black pepper and can be isolated from *Piper nigrum* (Bhardwaj et al., 2002). Mtb gene sequencing revealed that it contains twenty cytochrome p450 enzymes that can be potential drug target (Ouellet, Johnston, & Ortiz de Montellano, 2010). Piperine inhibits cytochrome p450-mediated pathways. A previous study showed that it also inhibits glucuronidation activity in animal models (Pule et al., 2016). Piperine potentially inhibits multidrug efflux pumps of Mtb (Sharma et al., 2010). Studies show that piperine could decrease the MIC of ethidium bromide and ciprofloxacin by 2-fold (Jin et al., 2011; Pule et al., 2016).

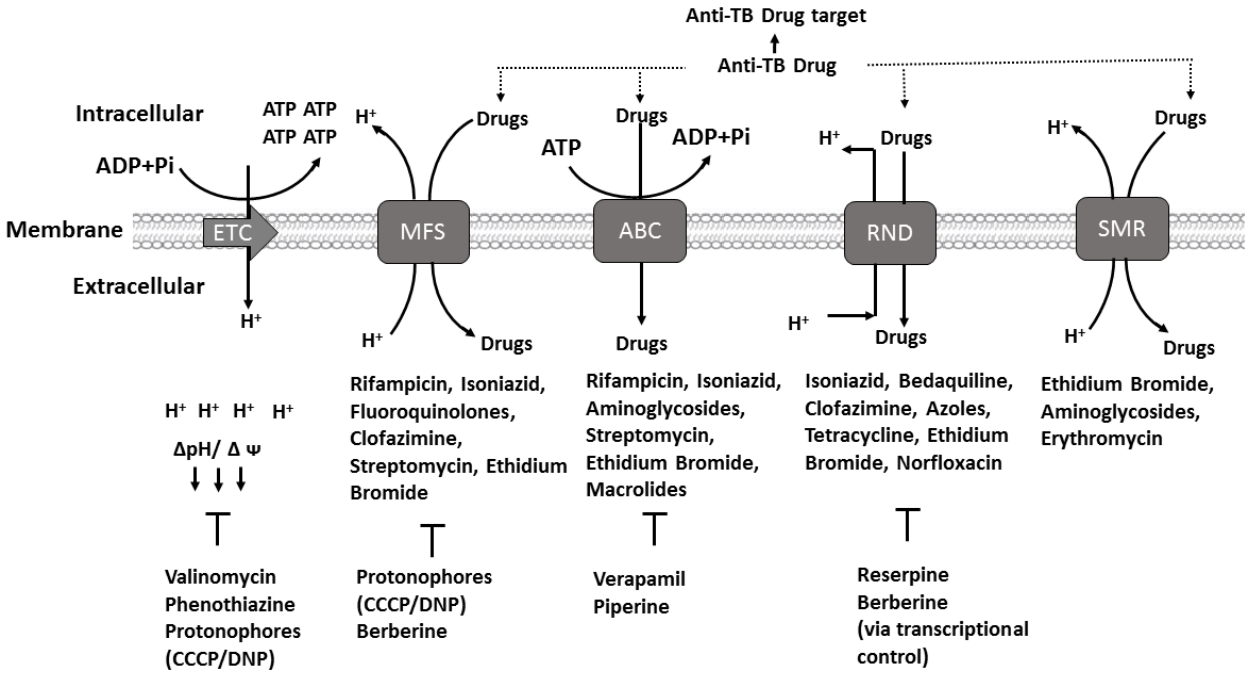

Figure 1. **Mechanism of action of efflux pump inhibitors on transporter protein families involved in antibiotic efflux.** Five classes of efflux pumps in bacterial membranes and inhibitors that act on them are shown (Pule et al., 2016). Verapamil inhibits ABC transporters and reserpine inhibits RND family transporters.

Efflux pump inhibitors could serve as adjunct anti-mycobacterial therapies. However, there are no studies on the effect of these efflux pump inhibitors on the antibiotic resistance of non-replicating *Mycobacterium smegmatis* (Msm) which is a non-pathogenic model organism for Mtb (Reyrat & Kahn, 2001)

1.6 Mechanisms of action of commonly used anti-tuberculosis drugs:

Antibiotics can be classified as bacteriostatic or bactericidal depending on the organism and drug concentration. Macrolides are one of the most commonly used antibiotic classes that consist of a large macrocyclic lactone ring to which one or more deoxy sugars may be attached. Erythromycin, azithromycin and clarithromycin are commonly used macrolides. Clarithromycin is derived from erythromycin and it inhibits bacterial protein synthesis by binding to the bacterial 50S ribosomal subunit. The macrolide can bind to the large subunit of the bacterial ribosome. It binds to the nascent peptide exit channel and inhibits protein synthesis (Gaynor & Mankin, 2003). It also inhibits peptidyl transferase activity and interferes with amino acid translocation during the

translation and protein assembly process. Bacteria use two mechanisms to survive in the presence of macrolides. They can pump out the macrolides or they can modify the drug target site. The second mechanism comes in the form of mutations in 23S rRNA or site specific post-translational modification of ribosomal proteins (Gaynor & Mankin, 2003).

Ofloxacin is a quinolone antimicrobial agent which inhibits bacterial topoisomerase IV and DNA gyrase enzymes activity(Drlica & Zhao, 1997; Fisher, Mizuuchi, O'Dea, Ohmori, & Gellert, 1981; Takiff et al., 1994). Those enzymes are required for transcription, DNA replication, repair and recombination. Quinolones produce double-stranded breaks in the bacterial chromosome and exhibit bactericidal activity by increasing the concentration of enzyme-DNA cleavage complexes. Quinolone physically blocks subsequent ligation reactions of permanent chromosomal breaks caused by enzyme-DNA-quinolone. Other DNA repair mechanisms are affected by the accumulation of a large number of broken DNA which ultimately leads to bacterial cell death (Bhagwat et al., 2019).

Isoniazid is a prodrug which need to be activated by bacterial enzyme KatG. This enzyme acts as a catalase-peroxidase that has other activities including peroxynitritase and NADH oxidase. *KatG* and *inhA* gene mutation shows 2 to16-fold reduction in isoniazid MIC (Jaiswal et al., 2017). Studies have shown that KatG-activated isoniazid generates reactive species that form adducts with NAD+ and NADP+. Produced reactive species in combination with NAD+ and NADP+ are thought to inhibit nucleic acid and lipid biosynthesis (Hazbon et al., 2006; Ramaswamy et al., 2003; Rawat, Whitty, & Tonge, 2003; Timmins & Deretic, 2006; Zhang, Heym, Allen, Young, & Cole, 1992).

Rifampicin stops the growth of actively growing bacteria by inhibiting bacterial DNA-dependent RNA polymerase by forming a stable drug-enzyme complex. RNA polymerase is involved in DNA transcription. Mycobacterial resistance to rifampicin is caused by mutations in β subunit of RNA polymerase leading to a change in the structure of the β subunit of RNA polymerase which subsequently inhibits binding of rifampin on the ribosomal subunit (Palomino & Martin, 2014; Wehrli, 1983).

Amikacin is a commonly used aminoglycoside and a derivative of kanamycin. During translation, amikacin blocks the function of the bacterial 30S ribosomal subunit, making it unable to produce proteins. The common resistance mechanism of aminoglycoside is enzymatic modification of aminoglycosides by aminoglycoside-modifying enzymes such as

acetyltransferases, phosphotransferase and nucleotidyltransferases (Krüüner, Jureen, Levina, Ghebremichael, & Hoffner, 2003; Ramirez & Tolmasky, 2017; Zaunbrecher, Sikes, Metchock, Shinnick, & Posey, 2009).

1.7 Energy sources of ABC transporter proteins and efflux pumps and their effects on antibiotic susceptibility of non-replicating drug resistant Mtb:

ATP is used as an energy source for ABC transporter proteins. Since ABC transporters can act as exporters, they can help bacteria survive in the presence of antibiotic by exporting drug molecules. It has been discovered that the ABC transporter Rv3723/LucA and the Mce1 transporter complex are involved in fatty acid import into Mtb. The authors showed that a network of shared and dedicated proteins are involved in fatty acid import in Mtb (Nazarova et al., 2019). It has been shown previously by our lab that the Mtb ABC transporter Rv1272c is responsible for enhancing the import of long chain fatty acid in *E.coli* (Martin & Daniel, 2018).In the latent state, Mtb accumulates TAG-containing lipid droplets from the host which act as an energy source. Dormant Mtb metabolically incorporates oleic acid into TAG. Deletion of proteins that are related to accumulation of lipids decrease phenotypic tolerance of Mtb to antibiotic such as rifampicin (Daniel, Kapoor, Sirakova, Sinha, & Kolattukudy, 2016).

CHAPTER 2. HYPOTHESIS AND OBJECTIVES

We postulate that *Mycobacterium smegmatis* (Msm) subjected to the combination of stresses (low O_2, low pH and low nutrient levels) will display a higher level of phenotypic antibiotic tolerance (resistance) compared to Msm in log-phase growth. We further postulate that efflux pump inhibitors will decrease the observed antibiotic resistance. We also postulate that metabolic energy sources such as ATP, oleic acid, glucose and glycerol could enhance the antibiotic resistance observed by providing energy for bacterial efflux pump transporter proteins that pump out antibiotics.

We selected Msm because it is a fast-growing and non-pathogenic model organism for Mtb. We investigated whether Msm cells entered a non-replicating state under a combination of stresses that are thought to be experienced inside the human body (Deb et al., 2009). There are no studies on the roles of efflux pump inhibitors on the antibiotic tolerance of non-replicating Msm under multiple-stressed conditions. Therefore, we examined the effects of efflux pump inhibitors and carbon sources such as glycerol and oleic acid on the antibiotic tolerance of Msm in log-phase growth and under the combination of stresses that induce it to enter a non-replicating state.

Based on amino acid sequence identities, we identified MSMEG_5008 and MSMEG_5009, two proteins in Msm, homologous with the Mtb ABC transporters previously studied in our lab (Martin & Daniel, 2018). We hypothesize that these two gene products could be involved in importing long-chain fatty acid from the host cell into mycobacterial cell. We attempted to construct targeted gene knockout mutants lacking these two genes in Msm.

CHAPTER 3. MATERIALS AND METHODS

3.1 Mycobacterial growth and culture under combined multiple-stress conditions

Mycobacterium smegmatis (Msm) *mc²155* (ATCC, Manassas, VA) cells were cultured in Dubos medium (BD Life Sciences, Sparks, MD) with 5 % (v/v) glycerol. A saturated starter culture was grown in Dubos medium from glycerol stocks stored in ultracold freezer and used as inoculum for a 100 ml Dubos broth culture which was grown with shaking at 37 °C to an optical density at 600 nm (OD_{600}) of 0.2. The cells were then centrifuged and resuspended in diluted (10 %) Dubos medium containing 0.5 % (v/v) glycerol adjusted to pH 5.0 and aliquoted into 96-well plates (240 µl/well of 10^8 colony-forming-units (CFU)/ml cells). The 96-well plates with Msm cultures in the wells at a head-space ratio of 0.5 were sealed with airtight plastic film to allow the cultures to gradually deplete the oxygen and enter a hypoxic state. Complete sealing produced vapor inside the well when plate was incubated at 37 °C for five days. Methylene blue was added to selected wells as an indicator of hypoxia. The plates were incubated at 37 °C. Thus, the cells were subjected simultaneously to hypoxia, acidic pH (adjusted pH to 5 in medium) and nutrient starvation (10 % Dubos medium). Our *in vitro* model is a modification of an earlier combined multiple stress model that was shown to cause Mtb to enter a non-replicating, drug-tolerant state (Deb et al., 2009). Single colony was grown in agar plate and count colony on different OD_{600}. Based on viable cell counts at different OD_{600}, the correlation of OD_{600} to CFU was established.

3.2 Determination of antibiotic resistance and effect of efflux inhibitors using a multiwell plate assay

We used the following antibiotics in our assays: clarithromycin (Alfa Aesar, Ward Hill, MA), ofloxacin (Alfa Aesar, Ward Hill, MA), rifampin (Acros Organics, NJ), Isoniazid (Alfa Aesar, Ward Hill, MA), Amikacin (MP Biomedicals, Solon, OH) and Erythromycin (MP Biomedicals, Solon, OH). The following efflux pump inhibitors (EPIs) were used in our assays: Verapamil (TCI America, Portland, OR), Piperine (Alfa Aesar, Ward Hill, MA), CCCP (Alfa Aesar, Ward Hill, MA), Reserpine (Alfa Aesar, Ward Hill, MA) and Berberine (TCI America, Portland, OR). Antibiotics and inhibitors were dissolved in dimethyl sulfoxide or pH-adjusted buffer as appropriate. Msm cells were grown under three combined stresses as described above. At days 3,

5 and 7, cells were collected using centrifugation (at 8000g, 20 minutes) and spent medium was sterilized using 0.22 µm filter (Thermo Fisher Scientific, Waltham, MA). Cells were diluted to $5\times10_4$ cells/well in the sterilized spent medium (Singh, Akhtar, Mishra, & Sarkar, 2011). The spent medium obtained from cells incubated under hypoxia for five days was used since oxygen and nutrients were expected to be depleted in that medium. CFUs were determined by agar plating. The effects of ATP, fatty acid, glucose and glycerol on modulating antibiotic resistance and efflux pump inhibitor action were determined. ATP (final concentration of 0.25 µM), glucose and glycerol (final concentrations of 10 mM each) were added to cells 15 min prior to addition of EPIs. Cells were then incubated with the EPIs for 15 min prior to addition of antibiotics. Cells were incubated for 16 h with oleic acid at a final concentration of 100 µM before addition of efflux inhibitors and antibiotics as above. The antibiotics were used at their previously reported minimal inhibitory concentrations (MICs) and the EPIs were used at concentrations reported in previous studies (Hurst-Hess, Rudra, & Ghosh, 2017; Menichini, Lari, & Rindi, 2020; Viljoen et al., 2019). (Hurst-Hess et al., 2017). The EPIs (Verapamil, Reserpine, Piperine, CCCP and Berberine) were added at the following concentrations respectively (50 µg/ml, 40 µg/ml, 30 µg/ml, 25 µg/ml, 30 µg/ml) to separate wells containing the Msm cultures and the cells were incubated with the efflux pump inhibitors at 37 °C for 15 min. Then antibiotics were then added to the indicated final concentrations - Clarithromycin (0.50 µg/ml), Ofloxacin (1 µg/ml), Rifampin (1 µg/ml), Isoniazid (20 µg/ml), Amikacin (0.625 µg/ml) and Erythromycin (2.5 µg/ml) in the cultures pre-incubated with the efflux inhibitors. After adding antibiotics, plates were then re-sealed and incubated at 37°C for two more days. Cell viability was determined using a modified protocol of the XTT-menadione assay reported previously (Singh et al., 2011). Briefly, XTT (Biotium, Fremont, CA) was added to a final concentration of 125 µM (24 µl/well of 0.84 mg/ml, dissolved in PBS) and the plates were incubated at 37 °C for 20 min. 2.6 µl of menadione (Alfa Aesar, Ward Hill, MA; 1.032 mg/ml, final concentration 60 µM) was added to each well and incubated at 37 °C for 40 min. Absorbance at 470 nm was measured using an accuSkan™ GO UV/Vis Microplate Spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

3.3 Construction of M. smegmatis gene-knockout mutant

3.3.1 Genomic DNA isolation from *M. smegmatis*

The isolation of genomic DNA was done by following a modified procedure published previously (Medjahed & Singh, 2010). Briefly, Msm wild-type cells were grown in 3.5 ml Middlebrook 7H9 medium with Albumin-Dextrose-Catalase (ADC) enrichment and Tween 80 (BD Life Sciences, Sparks, MD) at 37 °C until cells reached an OD_{600} of 0.8 - 1.0. The cells were resuspended in 250 µl lysis buffer I (10 mM Tris.Cl, pH 8.0; 1 mM EDTA, pH 8.0; 0.5 % [v/v] Tween-80 and 500 µg/ ml lysozyme), mixed gently by pipetting and incubated for two hours at 37 °C Then 250 µl of lysis buffer II (10 mM Tris.Cl, pH 8.0; 1 mM EDTA, pH 8.0; 400 µg/ ml proteinase K and 1 % [w/v] SDS) was added and mixed gently by pipetting and incubated for another two hours at 37 °C. Then the tubes were incubated at 55 °C for two hours. After incubation, 500 µl of 25:24:1 phenol/ chloroform/ isoamyl alcohol was added and shaken vigorously by hand to mix. After microcentrifugation for 30 min at 16,000 X g at room temperature, the aqueous layer was transferred to a fresh, sterile 1.5 ml microcentrifuge tube. The extraction was repeated with 25:24:1 phenol/ chloroform/ isoamyl alcohol and the aqueous layer was collected. Then, 100 µl of 5 M NaCl was added and mixed gently and after microcentrifugation for 5 min at 16,000 X g at room temperature, 500 µl of aqueous layer was transferred to fresh sterile microcentrifuge tube. To the 500 µl aqueous solution, 350 µl of chilled absolute ethanol was added and mixed gently by inverting until genomic DNA had precipitated out of solution. It was incubated for 5 min at room temperature and microcentrifuged for 10 min at 16,000 X g at room temperature. After aspiring the supernatant, 500 µl of 70 % ethanol was added to wash the DNA pellet. Then, after mixing gently by inversion, the tube was microcentrifuged for 10 min at 16,000 X g at room temperature. The supernatant was aspired carefully, and the pellet was air dried for 10-15 min. Finally, the DNA pellet was dissolved in 25 µl of ultrapure water at room temperature or 4 °C.

3.3.2 Preparation of *MSMEG*_5008-5009 allelic exchange substrate

We used a recombineering approach towards disruption of the *MSMEG_5008-5009* open reading frames following modifications of previously reported procedures (Medjahed & Singh, 2010; J. van Kessel & F Hatfull, 2008; J. C. van Kessel & Hatfull, 2007). The allelic exchange substrate (AES) was prepared by polymerase chain reaction (PCR) amplification of the upstream

and downstream flanks of the ORF from the genomic DNA of *M. smegmatis*. The Q5 Hot Start High-Fidelity DNA polymerase (New England BioLabs, Ipswich, MA) was used for the PCR amplification. The bleomycin resistance cassette was amplified from the plasmid pMSG360zeo (Addgene, Watertown, MA). Specific primers were designed to amplify the DNA fragments with appropriate overlaps using the NEBuilder Assembly Tool (http://nebuilder.neb.com; accessed July 2019) for Gibson assembly. The primers used are listed in Table 1.

Table 1.Primers used for generation of MSMEG_5008-5009 AES construct.

Primer Name	Sequence (5'→ 3')	Q5 Annealing Temperature
5009 UP_fwd 5009 UP_rev	GGCAGCCCGACGAGTTGG ACTTGGCCATACCGTCGGCAGGTACAGC	68 °C
5008Bleo_fwd 5009Bleo_rev	TGCCGACGGTATGGCCAAGTTGACCAGTG CCGCGTCACGTCAGTCCTGCTCCTCGGC	64 °C
5009DN_fwd 5009DN_rev	GCAGGACTGACGTGACGCGGATCTGATC AACCCTTCCCGACGTCGAAG	64 °C

The PCR products were purified from agarose gel using the GeneJET Gel Extraction Kit (Thermo Scientific) and assembled using the NEBuilder HiFi DNA Assembly Master Mix (New England BioLabs, Ipswich, MA) following the manufacturer's protocol. The HiFi reaction product was used as template for PCR amplification of the 1396 bp linear AES DNA using the high-fidelity Q5 DNA polymerase with the primers 5009 UP_fwd and 5009DN_rev at an annealing temperature of 71 °C. The AES product was purified from the agarose gel as described above.

3.3.3 Preparation of electrocompetent *M. smegmatis*

Electrocompetent cells were prepared by following a previously published protocol (J. van Kessel & F Hatfull, 2008). Wild type *M. smegmatis* mc^2 155 was inoculated in 3 ml 7H9 + ADC broth and grown with shaking (100 rpm) at 37 °C for two days. This starter culture was used to inoculate 50 ml of 7H9 + ADC medium in a 250-ml sterile flask which was incubated with shaking until OD_{600} reach 0.8-1.0. Msm cells were transferred from flask to centrifuge tubes and kept on ice for 2 hours. The cells were pelleted by centrifugation at 3600 X g for 10 min at 4 °C. After

carefully discarding the supernatant, the cells were washed with 25 ml of 10 % sterile glycerol by gently pipetting until clumps of cell were dissolved and centrifuged to pellet the cells. This process of washing the cells by centrifugation was repeated with 12.5 ml, 6.25 ml and 5 ml of 10 % glycerol. After this, the cells were finally resuspended in 2 ml of 10 % glycerol. The electrocompetent cells were aliquoted (100 µl) into 1.5 ml centrifuge tubes and were used fresh or stored at -80 °C.

3.3.4 Transformation of electrocompetent Msm cells with pJV53

Wild type *M. smegmatis* mc^{2}155 cells were transformed with the pJV53 plasmid expressing the mycobacterial recombination proteins Che9c 60-61 (Addgene, Ipswich, MA) by electroporation to generate the recombineering strain. Briefly, electrocompetent Msm cells were transformed following a previously published protocol (J. van Kessel & F Hatfull, 2008) using a BioRad MicroPulser electroporation unit (Bio-Rad Laboratories, Hercules, CA).The pJV53 plasmid (100ng) was pipetted into cells which were mixed gently and incubated for 10 min on ice and transferred into a chilled electroporation cuvette. The electroporation condition applied was 2.5 kV, 1000Ω, 25µF, 5-6 ms. Then, 1 ml of 7H9 (containing ADC and Tween 80) was added and the cells were transferred to a 15 ml sterile culture tube and incubated for 2 hours at 37 °C at 220 rpm with shaking for recovery. The cells were plated on Middlebrook 7H10-Kan (50 ug/ml) agar plates which were incubated at 37 °C for growth of colonies. The Msm transformants were screened for the presence of the pJV53 kanamycin resistance gene using the primers listed in Table 2. The expected PCR product size is 800 bp.

Table 2. Primers used for screening pJV53-containing Msm

Primer Name	Sequence (5'→ 3')	Q5 Annealing Temperature
KanFW2 KanRV2	ATGAGCCATATTCAACGGGAAACGTC GATTAGAAAAACTCATCGAGCATCAAATGAAACT	68 °C

3.3.5 Generation of *M. smegmatis* recombineering strain

Msm cells containing pJV53 were grown in 5 ml of 7H9 + ADC broth containing Kanamycin and Tween 80 at 37 °C for two days to get saturated culture. The culture was used to inoculate 100 ml of 7H9 induction medium (No ADC+ Kanamycin 50 µg/ml + 0.2 % Succinate)

which was incubated at 24 °C overnight When the cells reached OD_{600} of 0.4-0.5, expression of the mycobacterial recombination proteins on pJV53 was induced with 0.2 % acetamide. The culture was incubated with shaking for another 3 h at 24 °C. These recombineering Msm were made electrocompetent following the procedure described above for wild type Msm.

3.3.6 Transformation of recombineering Msm with allelic exchange substrate and screening of mutant clones

The electrocompetent, recombineering Msm cells were transformed by electroporation with the linearized AES construct for disruption of the two ORFs *MSMEG_5008* and *MSMEG_5009* following the protocol described above. The transformation mixture was plated on Middlebrook 7H10 agar plates containing kanamycin (50 µg/ ml) and zeocin (50 µg/ ml) and plates were incubated at 30 °C and 37 °C until colonies appeared in about 3-5 days.

The colonies that grew on the dual-antibiotic plates were cultured in 3.5 ml 7H9 broth containing both antibiotics and genomic DNA was isolated from cells as described above. The clones were screened for double crossover mutants using the primers listed in Table 3.

Table 3. Primers for confirming double-crossover mutants

Primer Pairs	Sequence (5'→ 3')	Q5 Tm	Expected PCR Product (bp)
MSMEG_5009_upflank_fwd 5008DNFLrv	GGTGACCACGCTGGTGATCAACG CGTGGCGGGCGACACCAG	64 °C	WT- 1931 bp Δ- No product
5008UPFLfw Bleo129R	AGGTTGGGGTACTGCCGC CCGGACCACACCGGCG	59 °C	WT- No product Δ- 1109
Bleo264F MSMEG_5009_downflank_rev	GCCTCCGGGCCGGCC CGGGCCCCGGACCCGGCG	67 °C	WT- No product Δ- 1215

3.4 Data Analysis

The data obtained was analyzed using SigmaPlot® 14.0. The means and standard deviations of each experimental group (Cells + antibiotic of log-phase vs Cells + antibiotic of non-replicating Msm, Cells + EPI + antibiotic of both log and non-replicating Msm,) were compared by one way analysis of variance (ANOVA). Tukey's test for significance was done post-hoc to determine *p*-

values and differences between group means were considered significant when $p < 0.05$. The graphs show means ± standard deviations from duplicates within each group. Independent experimental replicates were performed (when possible) and are indicated in figure legends.

CHAPTER 4. RESULTS

4.1 *Mycobacterium smegmatis* (Msm) enters a non-replicating state under a combination of stresses

Msm cells were subjected to a combination of stresses (low O_2, low pH and low nutrients) as described under Methods. Methylene blue was used as indicator of hypoxic condition. Its blue color faded gradually in the wells containing cells. But the control wells with medium without cells remained blue (Fig 2A). The methylene blue color was darker in the medium-only control wells than the wells containing cells indicating that cells depleted the dissolved oxygen under the sealed condition.

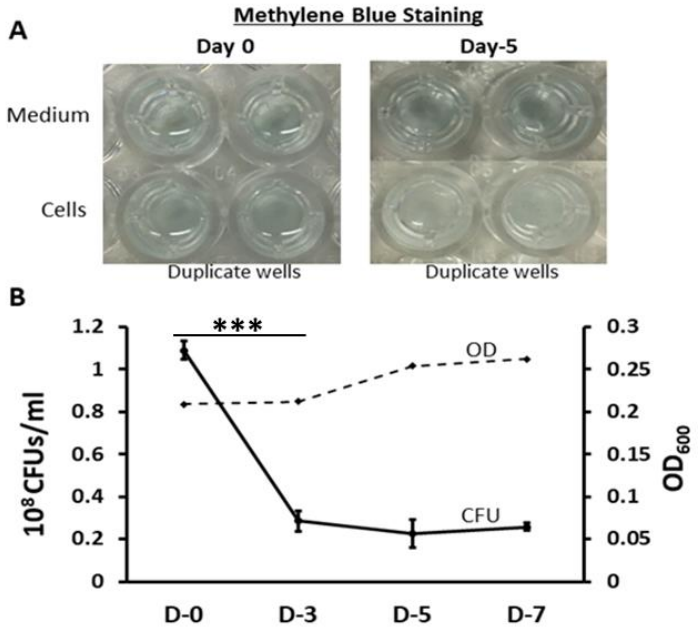

Figure 2**. Msm enters a non-replicating state as oxygen is depleted. A**, Color change of methylene blue. **B**, viable cell count (CFUs/ml) and optical density (OD_{600}) of Msm over time. In panel A, fading of methylene blue indicates depletion of oxygen by the growing cell at day-5. In panel B viable cell count drops to one-third of starting count by day 3 under the combined stress condition. At day 5 counts remain the same indicating that the cells in this condition enter non-replicating state. Cell counts were measured in 3 independent experiments. Values shown are means ± SD of duplicate measurements from a representative experiment. ***, $p<0.001$.

The initial viable cell count was set at approximately 10_8 colony-forming units (CFUs)/ml so that there were enough cells to deplete all dissolved oxygen and generate a hypoxic condition

in the sealed wells (plate was sealed in such way that all 96 wells were completely blocked and could not exchange oxygen from outside). We observed that the viable cell numbers decreased and became stable after 3 days in the multiple-stressed conditions. At day-0 viable cell count was 1.09×10^8 CFUs/ml and at day-3 it went down to 0.28×10^8 CFUs/ml. At day-5 it was 0.23×10^8 CFUs/ml and at day-7 CFU was 0.2585×10^8 CFUs/ml. Therefore, the viable cell count decreased to one- third of the starting value by day-3 and remained stable when measured at day-5 (Fig. 2B). Viable cell count was approximately the same at day-7 (data not shown) indicating that Msm cells under the combined stress condition had entered a non-replicating state by day-5. The optical density (OD_{600}) values were increasing gradually over this time period (Fig. 2B). The potential reason for the increase in OD_{600} may be due to dead cells or cell debris. Therefore, we used viable cell counting after agar plating to determine CFUs. However, viable cell count (CFU) indicates that one-third of the cells survived and became non-replicating under the combination of stresses.

4.2 Non-replicating Msm cells display increased claithromycin resistance that is inhibited by verapamil

The resistance of actively-growing (log-phase) Msm to the antibiotic clarithromycin (0.50 µg/ml) was observed to be 58.9 %. The efflux pump inhibitor verapamil (50 µg/ml) caused a decrease in

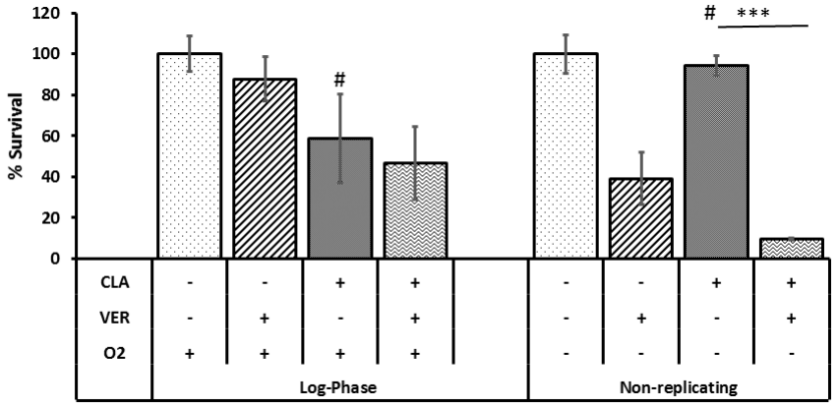

Figure 3**. Clarithromycin resistance of non-replicating Msm is significantly inhibited by verapamil.** The survival of Msm in the presence (+) of clarithromycin (CLA) and verapamil (VER) is shown in comparison to controls that lack (-) CLA and VER. Log-phase (day-0) cells were grown in the presence (+) of oxygen (O_2) but non-replicating cells (day-5) were incubated in the absence (-) of O_2. Percent survival of cells was measured in two independent experiments. Values shown are mean ± SD of duplicate measurements from a representative experiment. #, $p<0.05$; ***, $p<0.001$.

the resistance (survival) of the log-phase cells to 46.6 % (Fig. 3), as predicted by our hypothesis. Verapamil alone caused a decrease in survival of log-phase cells to 87.8 %. Non-replicating Msm cells at day-5 showed a much higher rate of survival (94.5 %) against clarithromycin compared to log-phase cells (58.9 %). The combination of clarithromycin and verapamil caused a significant drop in the survival of non-replicating Msm to 9.8 % (Fig. 3). Thus, non-replicating Msm cells were about 10-fold more susceptible to the antibiotic clarithromycin in the presence of the efflux pump inhibitor verapamil. Non-replicating cells showed higher sensitivity to verapamil alone (39.2 % survival) compared to log-phase cells (87.8 % survival).

4.3 The efflux pump inhibitor verapamil decreases resistance of non-replicating Msm to the antibiotic rifampin

The resistance of Msm to the antibiotic rifampin increased from 73.3 % for log-phase cells to 97.7 % for non-replicating cells (Fig. 4).

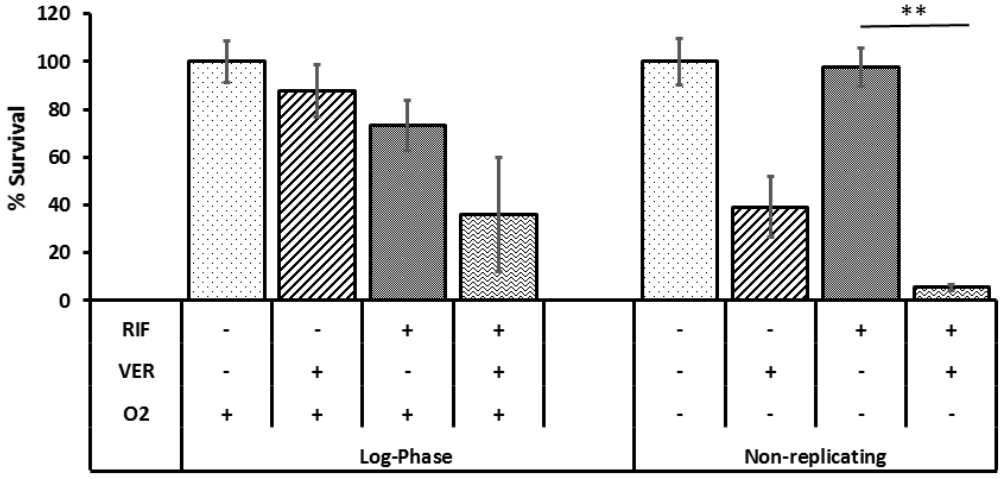

Figure 4**. Verapamil increases the susceptibility of both actively growing and non-replicating Msm to rifampin**. The resistance (% survival) of log-phase (day-0) Msm exposed (+) to oxygen (O_2) and non-replicating (day-5) Msm deprived (-) of O_2 in the presence (+) or absence (-) of rifampin (RIF) and verapamil (VER). Percent survival of cells was measured in two independent experiments. Values shown are average ± SD of duplicate measurements from a representative experiment. **, $p<0.01$.

The efflux pump inhibitor verapamil decreased the rifampin resistance to 36 % for log-phase cells and 5.7 % for non-replicating cells. Thus, in accordance with our hypothesis, the efflux pump inhibitor decreases rifampin tolerance of non-replicating Msm cells by 17-fold.

4.4 Non-replicating Msm becomes completely resistant to isoniazid and verapamil decreases this resistance

Actively growing Msm cells are completely susceptible to isoniazid. After 5 days under stressed conditions, the the non-replicating Msm cells become completely resistant (100 % survival) to isoniazid (Fig. 5). However, verapamil decreases the isoniazid resistance of non-replicating Msm to 57.4 %. Therefore, the efflux pump inhibitor decreases the isoniazid resistance of non-replicating cells by 1.7-fold, in agreement with our hypothesis.

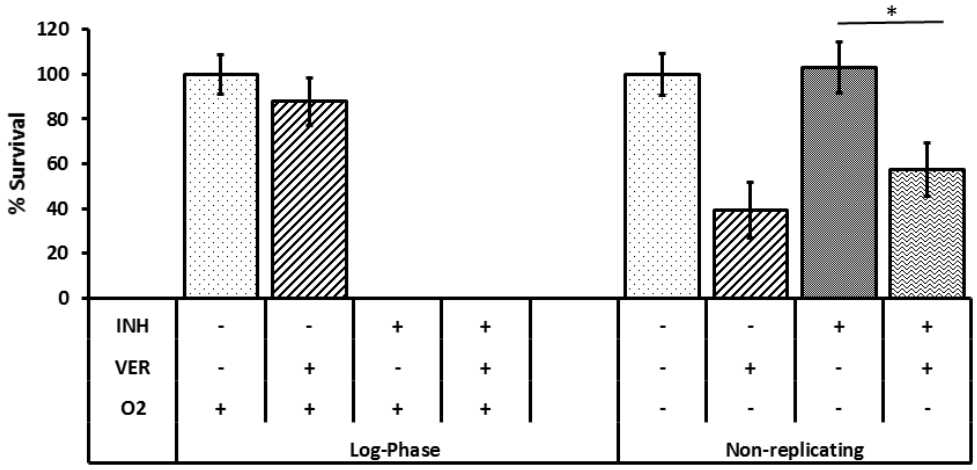

Figure 5**. Non-replicating Msm cells become completely resistant to isoniazid in contrast to actively-growing cells and verapamil inhibits this resistance.** The survival of log-phase (day-0) Msm with (+) oxygen (O_2) and non-replicating (day-5) Msm without (-) O_2 in the presence (+) or absence (-) of isoniazid (INH) and verapamil (VER) is shown. Percent survival of cells was measured in two independent experiments. Values shown are average ± SD of duplicate measurements from a representative experiment. *, $p<0.05$.

4.5 Verapamil decreases the resistance of actively growing and non-replicating Msm to erythromycin

The resistance of log-phase Msm to the antibiotic erythromycin was 70 % (% survival) and non-replicating cells showed 47 % resistance. The efflux pump inhibitor verapamil decreased the resistance of log-phase cells to 25 % and non-replicating cells to 3 % (Fig. 6). Thus, blocking the efflux pumps with the inhibitor had a significant effect on antibiotic resistance as expected by our hypothesis.

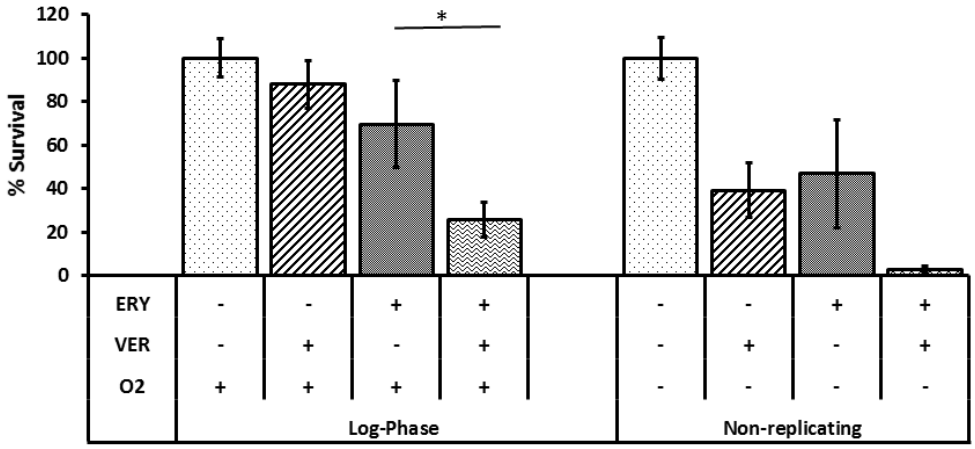

Figure 6**. Verapamil decreases erythromycin resistance of log-phase and non-replicating Msm.** Log-phase (day-0) cells were grown in the presence (+) of oxygen (O_2) and non-replicating (day-5) cells were incubated in the absence (-) of O_2. Cells were exposed (+) to erythromycin (ERY) and verapamil (VER) and survival was compared to controls not exposed (-) to the drugs. Percent survival of cells was measured in two independent experiments. Values shown are average ± SD of duplicate measurements from a representative experiment. *, $p<0.05$.

***(NOTE:** The following experiments were performed once and showed very interesting results. Unfortunately, the ongoing repeat experiments had to be abandoned due to unexpected closure of Purdue University Fort Wayne due to COVID-19.)*

4.6 Reserpine decreases the clarithromycin resistance of Msm

The efflux pump inhibitor reserpine decreases the resistance of actively growing, non-replicating Msm to clarithromycin. Reserpine decreased the clarithromycin resistance of log-phase cells by 2-fold and non-replicating Msm by 4-fold (Fig. 7). Thus, the efflux pump inhibitor reserpine decreased the resistance of Msm to the antibiotic clarithromycin as expected by our hypothesis.

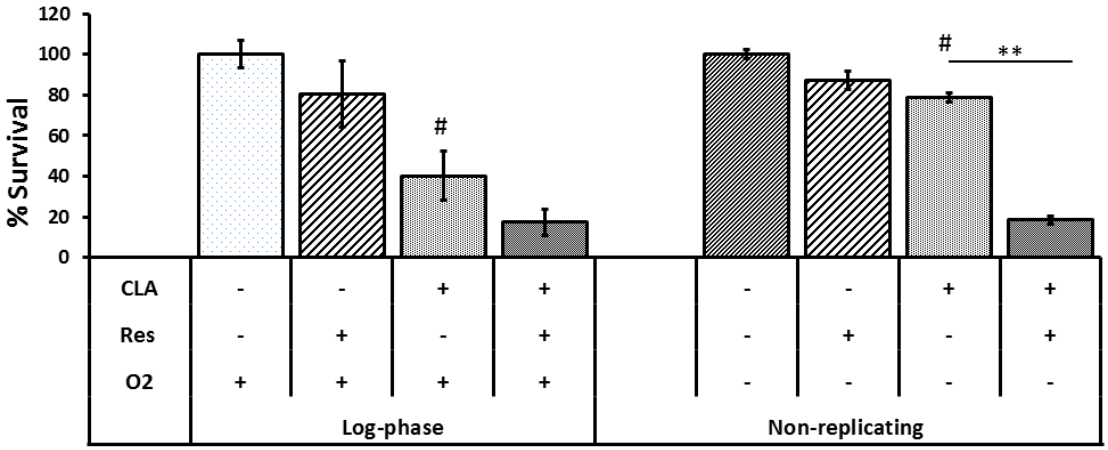

Figure 7**. Reserpine decreases clarithromycin resistance of log-phase and non-replicating Msm.** The survival of log-phase (day-0) Msm with (+) oxygen (O_2) and non-replicating (day-5) Msm without (-) O_2 in the presence (+) or absence (-) of clarithromycin (CLA) and reserpine (RES) is shown. Percent survival of cells shown from one experiment. Values shown are average ± SD of duplicate measurements. #, $p<0.05$; **, $p<0.01$.

4.7 The rifampin resistance of non-replicating Msm is inhibited by reserpine

The efflux pump inhibitor reserpine did not appear to affect the resistance of log-phase Msm to the antibiotic rifampin. However, the resistance of non-replicating Msm was inhibited by approximately 30 % (Fig. 8).

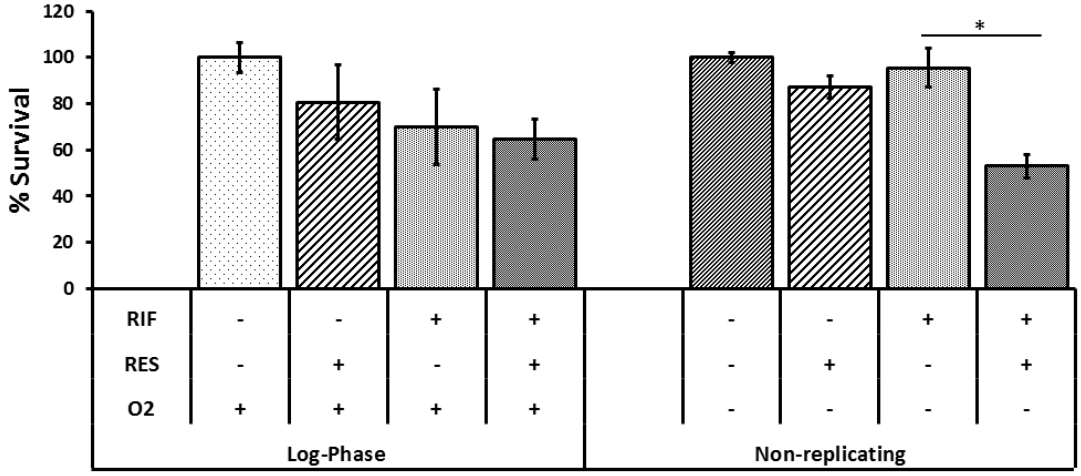

Figure 8**. The resistance of non-replicating Msm to rifampin is diminished by reserpine.** Log-phase (day-0) Msm with (+) oxygen (O_2) and non-replicating (day-5) Msm without (-) O_2 were incubated with (+) or without (-) of rifampin (RIF) and reserpine (RES). Percent survival of cells shown from one experiment. Values shown are average ± SD of duplicate measurements. *, $p<0.05$.

4.8 Isoniazid resistance is not affected by reserpine

Our preliminary observations suggest that isoniazid resistance does not appear to be affected by the efflux pump inhibitor reserpine (Fig. 9).

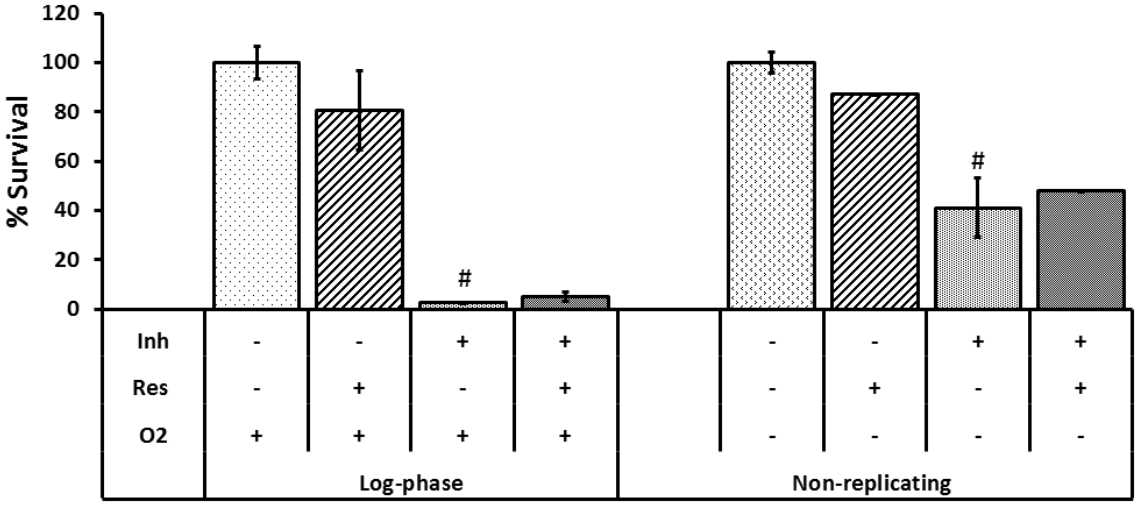

Figure 9**. Reserpine does not affect the isoniazid resistance of log-phase and non-replicating Msm.** Log-phase (day-0) Msm cells with (+) oxygen (O_2) and non-replicating (day-5) Msm without (-) O_2 were incubated with (+) or without (-) of isoniazid (INH) and reserpine (RES). Percent survival of cells shown from one experiment. Values shown are average ± SD of duplicate measurements. #, $p<0.05$.

4.9 Erythromycin resistance of non-replicating cells is diminished by reserpine

Our results suggest that the resistance of actively-growing log-phase Msm cells to erythromycin is not affected by reserpine. However, there is a clear indication that the erythromycin resistance of non-replicating cells is decreased by more than 60 % (Fig. 10).

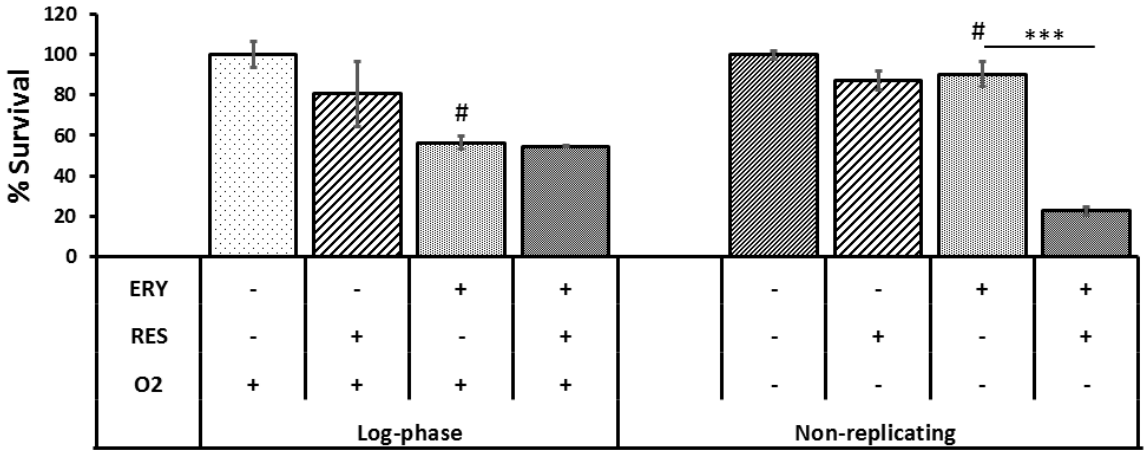

Figure 10**. Erythromycin resistance of non-replicating Msm is inhibited by reserpine.** The survival of log-phase (day-0) Msm with (+) oxygen (O_2) and non-replicating (day-5) Msm without (-) O_2 in the presence (+) or absence (-) of erythromycin (ERY) and reserpine (RES) is shown. Percent survival of cells shown from one experiment. Values shown are average ± SD of duplicate measurements. #, $p<0.05$; ***, $p<0.001$.

4.10 Glycerol does not affect the role of reserpine in decreasing antibiotic resistance of non-replicating Msm

Our results suggest that there is a 4-fold reduction of clarithromycin resistance by reserpine of the non-replicating Msm. We investigated whether the availability of a carbon-source such as glycerol would affect the observed antibiotic resistance of non-replicating cells. We observed that glycerol does not have any effect on clarithromycin resistance and does not affect the role of reserpine in decreasing resistance to clarithromycin (Fig. 11A).

Data from our experiments show that there is 50 % decrease of the resistance to rifampicin of non-replicating Msm. However, there is no effect of glycerol on the resistance of rifampicin with or without reserpine (Fig. 11B).

Our preliminary observations suggest that reserpine decreases resistance to erythromycin of non-replicating Msm by 4 folds. In the absence of reserpine non-replicating Msm is 90 % resistant to erythromycin. In the presence of reserpine, only 22 % non-replicating Msm can survive. In the presence of glycerol resistance of non-replicating Msm to erythromycin or erythromycin with reserpine remains the same (Fig. 11C).

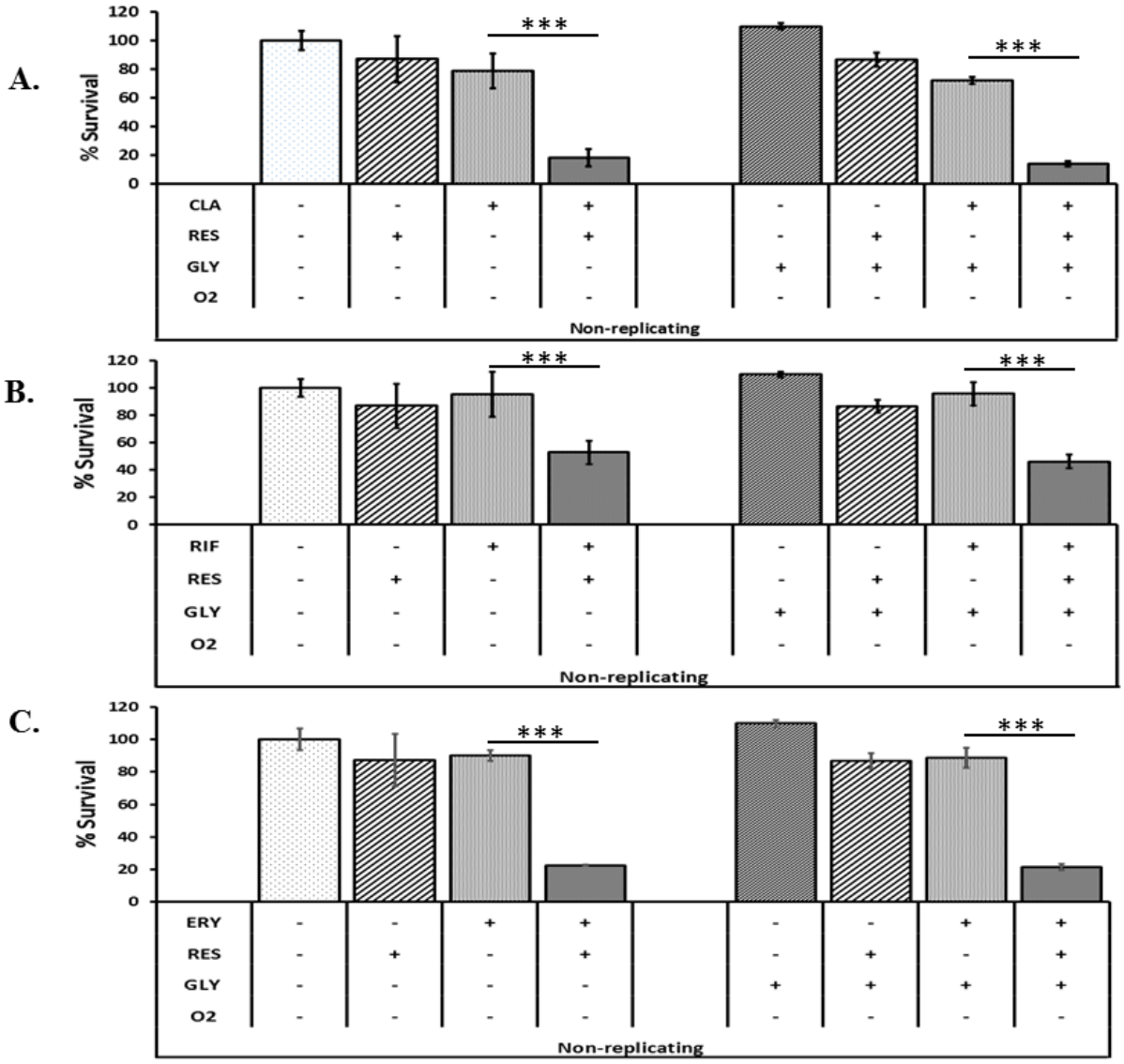

Figure 11**. Reserpine decreases antibiotic resistance of non-replicating Msm and glycerol has no effect. A.** Glycerol does not affect clarithromycin resistance of non-replicating Msm and its inhibition by reserpine. The survival of non-replicating (day-5) Msm without (-) O_2 in the presence (+) or absence (-) of clarithromycin (CLA), reserpine (RES) and glycerol (GLY) is shown. Percent survival of cells shown from one experiment. **B.** Reserpine decreases resistance to rifampicin by 2 fold and is not affected by glycerol. The survival of non-replicating (day-5) Msm without (-) O_2 in the presence (+) or absence (-) of rifampicin (RIF), reserpine (RES) and glycerol (GLY) is shown. **C.** Reserpine decreases resistance to erythromycin by 4 fold and is not affected by glycerol. The survival of non-replicating (day-5) Msm without (-) O_2 in the presence (+) or absence (-) of erythromycin (ERY), reserpine (RES) and glycerol (GLY) is shown. ***, $p<0.001$.

4.11 Antibiotic resistance of non-replicating Msm is not affected by oleic acid

The resistance of non-replicating Msm to rifampicin was observed to be 97%. Verapamil decreases resistance by 2 fold. 73 % of non-replicating Msm can survive against rifampin in the presence of oleic acid. However, survival of non-replicating Msm to rifampicin in the presence of verapamil was not affected by oleic acid (Fig. 12A). Non-replicating Msm is completely resistant to isoniazid which is decreased by 3 fold by verapamil.

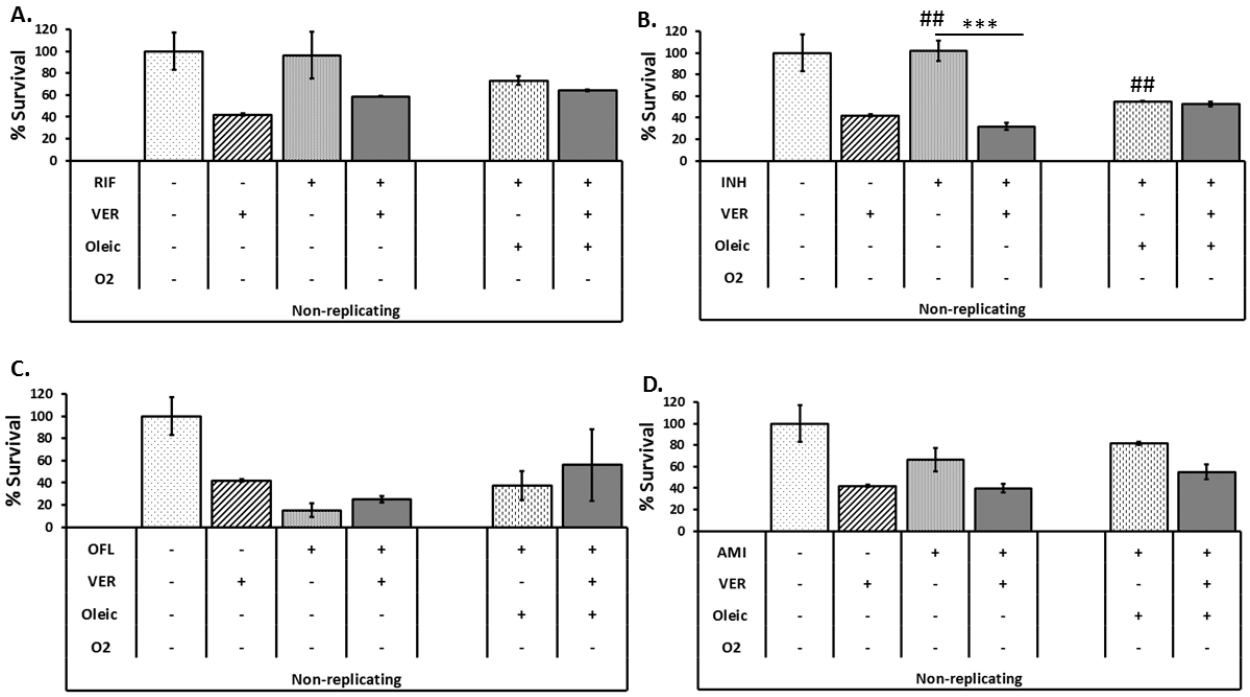

Figure 12**. Oleic acid does not affect the resistance of non-replicating Msm to antibiotics.** The survival of non-replicating (day-5) Msm without (-) O_2 in the presence (+) or absence (-) of rifampicin (RIF), isoniazid (INH), ofloxacin (OFL), amikacin (AMI) and oleic acid is shown in figure 14 panel A, B, C and D respectively. Percent survival of cells shown from one experiment. Values shown are average ± SD of duplicate measurements. ##, $p<0.01$; ***, $p<0.001$.

In the presence of oleic acid, non-replicating Msm shows 55 % resistance to isoniazid which remains same in the presence of verapamil and was not affected by oleic acid (Fig. 12. B).

In the absence of oleic acid only 15 % non-replicating Msm can survive on ofloxacin which is increased to 25 % in the presence of verapamil. In the presence of oleic acid, resistance of non-replicating Msm is 37.5 % without verapamil which is increased to 56 % in the presence of verapamil (Fig.12C). Non-replicating Msm is 65 % resistant to amikacin in absence of verapamil

and 40 % in the presence of verapamil which is not affected by oleic acid. Our preliminary data (not shown here) also suggest that glucose does not affect the resistance of non-replicating Msm to clarithromycin, ofloxacin, rifampicin, isoniazid, amikacin and erythromycin. We also investigated the effect of ATP on the antibiotic resistance but could not get data because ATP itself killed all the cells because of high concentration (1 µM) in the preliminary experiment.

4.12 Construction of *M. smegmatis* mutants lacking ABC transporters MSMEG_5008 and MSMEG_5009PCR amplification of upstream and downstream flanking regions of *MSMEG_5008* and *MSMEG_5009* genetic locus.

We attempted to construct targeted gene knockout mutants of Msm of two ABC transporters by double-crossover recombination. We used the recombineering approach that has been widely used (Julia C. van Kessel & Hatfull, 2006; J. C. van Kessel & Hatfull, 2007; J. C. van Kessel, Marinelli, & Hatfull, 2008). Briefly, we transformed Msm with plasmid pJV53 and induced expression of recombination-promoting proteins Che9c, gp60 and gp61. Then, electrocompetent Msm expressing the recombination-promoting proteins were transformed with allelic exchange substrate (AES) which contained upstream and downstream flanking regions on either side of zeocin cassette to knock out the downstream ABC transporter protein. The clones that grew on selective plates were screened for homologous double-crossover mutants.

We used the genomic DNA of wild-type *M. smegmatis* as template for amplifying the DNA fragments for Gibson assembly of the allelic exchange substrate (AES). A 520 base pair (bp) region upstream including the first 100 bp of the *MSMEG_5008* open reading frame (ORF) was amplified by PCR using the Q5 high fidelity DNA polymerase. Likewise a 521 bp region downstream including the last 100 bp of the *MSMEG5009* ORF was amplified. The 395 bp bleomycin (zeocin) resistance cassette was amplified using the pMSG360zeo plasmid as template. The amplified fragments had overlapping ends for facilitating Gibson assembly. Agarose gel electrophoresis indicated that the fragments of expected size were amplified (Fig. 13). The DNA fragments were purified from the agarose gel for use in Gibson assembly of AES.

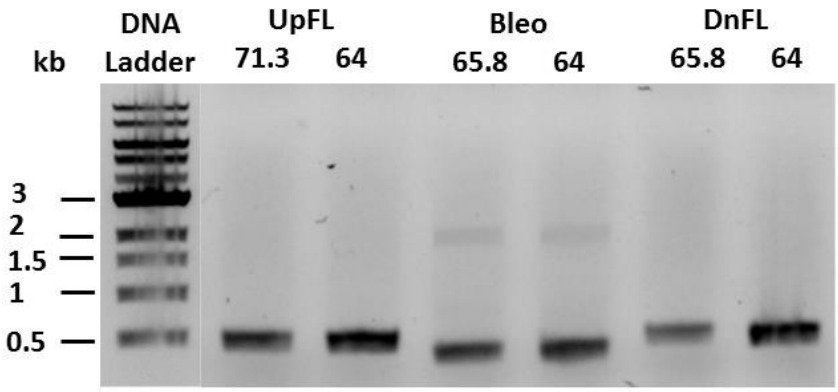

Figure 13**. PCR amplification of DNA fragments for Gibson assembly of AES.** The overlapping DNA components for the upstream flanking region (UpFL – 520 bp), bleomycin resistance cassette (Bleo – 395 bp) and downstream flanking region (DnFL – 521 bp) were amplified using the Q5 high fidelity DNA polymerase as described under Methods. Annealing temperatures used are indicated in °C above each lane.

4.13 PCR amplification of AES

The DNA fragments were assembled using HiFi DNA assembly reaction to construct the Msm AES. The HiFi DNA assembly product was used as template for PCR amplification of the 1396 bp AES. The AES was purified after gel electrophoresis (Fig. 14).

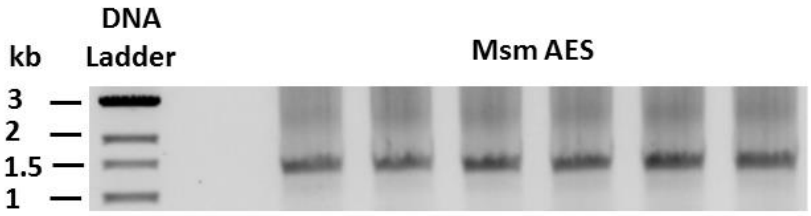

Figure 14**. PCR amplification of Msm AES.** The 1396 bp AES was amplified using the Q5 high-fidelity polymerase with the HiFi DNA assembly product as template as described under Methods.

4.14 Confirmation of Msm wild type transformed with plasmid pJV53

Msm transformed with plasmid pJV53 which contains the recombination-promoting proteins was selected on kanamycin agar plates and positive clones were confirmed by PCR amplification of the kanamycin resistance cassette. In the panel A of the figure 15, shows the colony PCR product of kanamycin resistance cassette. The expected PCR product size is 800 bp.

All the colonies showed a band at the expected size indicating all the transformed colonies contained pJV53. For further confirmation we isolated pJV53 from a selected colony and tested for the presence of the kanamycin resistance cassette by PCR. Panel B shows the confirmation of pJV53 containing Msm by amplification of isolated pJV53 from selected colony.

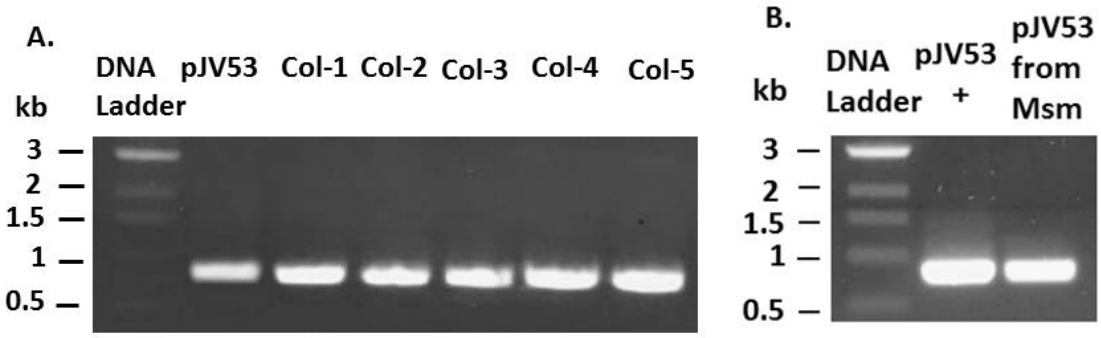

Figure 15**. Screening of pJV53 containing Msm by PCR.** Kanamycin resistance gene was amplified by Q5 hot start DNA polymerase as describe under Methods. Panel A shows the result from colony PCR screening. All the clones showed the PCR product at 800 bp. For further confirmation pJV53 was isolated from a selected colony and amplified by PCR. Panel B shows the amplification of the kanamycin resistance cassette in the isolated pJV53.

4.15 Illegitimate recombination of Msm AES of *MSMEG_5008* and *MSMEG_5009*:

After induction of the recombination-promoting proteins from pJV53 in Msm, the cells were made electrocompetent and transformed with the AES. More than 200 clones that grew on selective plates containing zeocin and kanamycin were screened by PCR using specific primer pairs for double crossover mutants. However, none of the clones showed the appropriate PCR product for a double-crossover mutant (Fig. 16).

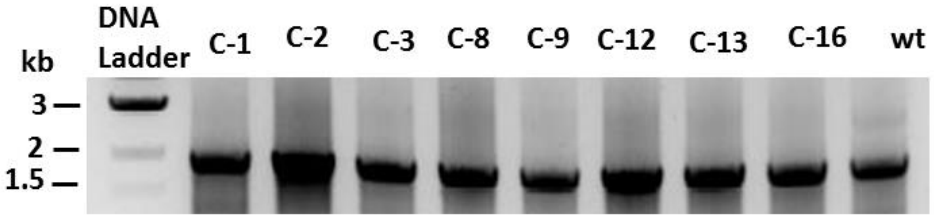

Figure 16**. Screening of Msm clones for double crossover mutants.** Genomic DNA isolated from each clone was used as template for PCR using the primer pairs described in the Table 3. The PCR product size for wild type Msm is 1931 bp while mutant should not give any product. PCR product of potential colonies are indicated above each lane. All the potential colonies show band at the 1931 bp as expected in wild type Msm indicating illegitimate recombination of Msm AES.

CHAPTER 5. DISCUSSION

Antibiotic resistance of microorganisms has become the major source of morbidity and mortality from infectious diseases worldwide. The ability of microorganisms to develop antibiotic resistance has turned back the progress in mankind's battle against infectious diseases (Reygaert, 2018). TB is caused by Mtb that remains a major public health concern. Although the rate of infection is declining in recent years, there are more than a million deaths each year worldwide because of TB. Multidrug resistance and extensive drug resistance in Mtb are a major public health concern (Miotto, Zhang, Cirillo, & Yam, 2018).

Mtb can remain persistent for a long time in the human body. Commonly used TB drugs cannot kill dormant Mtb leading to a lengthy chemotherapy which contributes to drug resistance due to poor compliance. Latent Mtb can survive for decades without showing any symptoms and can be reactivated when patient's immune system becomes weak (Zhang, 2004). When Mtb experiences low oxygen, low pH, scarcity of nutrients, and high CO_2, it enters non-replicating persistent state. This combination of stresses was reproduced in an *in vitro* combined multiple stress model to produce non-replicating Mtb in earlier studies (Deb et al., 2009). We used a modified version of this multiple-stress model as described in Methods and report here that Msm enters a non-replicating state under such conditions. We confirmed oxygen depletion (Fig. 2A) using methylene blue which is decolorized under hypoxic conditions. We also discovered that Msm enters a nonreplicating state after 3 days under the combined multiple stress conditions (Fig. 2B).

It has been shown in a previous study that mimicking the conditions that are thought to be encountered by Mtb inside the macrophage (low O_2, high CO_2, low pH and low nutrients) cause it to stop replication, lose acid-fastness (probably due to shut down of mycolic acid synthesis), accumulate TAG and acquire phenotypic antibiotic tolerance (Deb et al., 2009). We report here that drug resistance in Msm increases in the non-replicating state induced by the combination of low O_2, low pH and low nutrients (Figs. 3-13).

We studied the effects of efflux pump inhibitors on the antibiotic resistance of actively growing and non-replicating Msm. Verapamil is a calcium (Ca^{2+}) channel blocker. It inhibits efflux pump activity by reducing the transmembrane potential. Verapamil interferes with the generation of the proton motif force and inhibits ATP-dependent multidrug transporters and multidrug

resistant pumps in prokaryotes (Poelarends, Mazurkiewicz, & Konings, 2002). A study showed that verapamil induces a membrane stress like other membrane-active agents in Mtb leading to disruption of membrane functions. (Chen et al., 2018). Verapamil shows significant inhibitory activity of mycobacterial efflux pumps (Pule et al., 2016).

Clarithromycin is a commonly used anti-TB drug which inhibits protein synthesis in actively growing bacteria. Bacteria can survive by changing the target site or pumping out the clarithromycin (Gaynor & Mankin, 2003). Figure 3 shows that log phase Msm or actively growing Msm is 60 % resistant to clarithromycin. If actively growing Msm is treated with verapamil, the resistance decreases by 1.5-fold. In the non-replicating state, Msm shows more than 95 % resistance to clarithromycin. This increased resistance might be due to the halting of translation during non-replicating state. Non-replicating Msm is thought to be metabolically active at a minimal level and maintains metabolic activity just necessary for survival (Gengenbacher, Rao, Pethe, & Dick, 2010). Verapamil decreases the clarithromycin resistance to 10 %. Potentially, verapamil could inhibit efflux pumps belonging to the ABC transporter superfamily involved in clarithromycin export leading to increased retention of clarithromycin inside the cell. This could explain the decreased resistance to clarithromycin in the presence of verapamil.

Ofloxacin is a quinolone which acts as a bactericidal antibiotic by inhibiting topoisomerase IV and DNA gyrase activity leading to cell death (Bhagwat et al., 2019). Our data suggests that ofloxacin can kill all the actively growing Msm at the concentrations we tested. The resistance of non-replicating Msm is about 10 % against ofloxacin and this resistance is not affected by verapamil (Fig. 12. C). The decreased DNA replication in non-replicating persistent Msm may be a potential reason for ofloxacin resistance.

As an anti-TB drug, rifampin was first introduced in 1972 and it is bactericidal for both growing and non-growing Mtb (still metabolically active to a minimal extent). By inhibiting bacterial RNA polymerase, it stops elongation of messenger RNA. Mtb is resistant to rifampin to some extent is due to structural change of the β subunit of RNA polymerase by mutations (Miotto et al., 2018; Wehrli, 1983). According to Figure 4, actively growing Msm is 73% resistant to rifampin. If the actively growing Msm is treated with verapamil, it can decrease rifampin resistance by 1.3-fold. In non-replicating state, Msm is 98 % resistant to rifampin and if it is treated with verapamil, the resistance decreases down to 9 % which is a 10-fold decrease in resistance to

rifampin. Therefore, it appears that ABC transporter proteins, which are a target of verapamil, are involved in the efflux of rifampin when Msm is in a non-replicating state.

Isoniazid is a prodrug that needs to be activated by bacterial enzyme KatG and binds with enoyl-acyl carrier protein reductase leading to mycolic acid synthesis inhibition (Miotto et al., 2018; Timmins & Deretic, 2006). Actively growing Msm is completely susceptible to isoniazid as shown in Figure 5. In the non-replicating state, Msm becomes completely resistant to isoniazid. If non-replicating Msm is treated with the combination of verapamil and isoniazid, the isoniazid susceptibility is increased by 2-fold suggesting that verapamil blocks ABC transporter-mediated efflux of isoniazid in non-replicating Msm.

Amikacin binds to the 16S rRNA in the 30S ribosomal subunit leading to inhibition of protein synthesis. The strains of Mtb that are resistance to amikacin produce aminoglycoside-modifying enzymes which can modify amikacin (Ramirez & Tolmasky, 2017). Actively growing Msm is completely susceptible to amikacin. In the non-replicating state, the resistance to amikacin is only 60 % (Fig. 12D). Verapamil decreases resistance to 40 %.

Erythromycin is a common macrolide that bind to the 50S ribosomal subunit inside the nascent peptide exit tunnel that is located near to peptidyl transferase center leading to inhibit peptidyl transferase activity. During translation and protein assembly process, erythromycin interferes with amino acid translocation and subsequently inhibits protein synthesis (Gaynor & Mankin, 2003). The resistance of actively growing Msm to erythromycin is 70 %. Verapamil treatment decreases the resistance to 25 % which is a nearly 3-fold reduction in resistance to erythromycin. Interestingly, non-replicating Msm showed lower resistance (47%) to erythromycin. However, verapamil decreased this resistance to 3 %, a 16-fold reduction (Fig. 6).

Another naturally found efflux pump inhibitor is reserpine which is isolated from root of *Rauwolfia vomitoria* Afz. Reserpine targets the RND efflux pump family (Pule et al., 2016). Reserpine decreases the resistance of actively growing and non-replicating Msm to clarithromycin. Reserpine decreases the clarithromycin resistance of log-phage cells by 2-fold and non-replicating Msm by 4-fold (Fig. 7). Reserpine did not appear to affect the resistance of log-phage Msm to antibiotic rifampin. However, the resistance of non-replicating Msm was inhibited approximately 30% (Fig. 8.). Our data suggest that reserpine does not affect the isoniazid resistance of log-phage and non-replicating Msm. We also found that the resistance of actively growing Mms to erythromycin is not affected by reserpine. However, there is a clear indication that the

erythromycin resistance of non-replicating Msm is decreased by more than 60% (Fig. 10.). Reserpine could potentially inhibit resistance modulation efflux pump family involved in drugs pump out leading to increased retention of clarithromycin inside cell.

We examined the effect of glycerol as a carbon source on the antibiotic resistance of non-replicating Msm. Resistance to clarithromycin of Non-replicating Msm is decreased 3-4-fold by reserpine (Fig. 11A). Reserpine decreases resistance to rifampin and erythromycin by 2-fold and 4-fold respectively (Fig. 11B, 11C). Reserpine decreases antibiotic resistance of non-replicating Msm which in not affected by glycerol. Potentially, the non-replicating Msm does not use carbon source from medium. Therefore, addition of glycerol in the medium does not affect inhibition of antibiotic resistance of reserpine.

We examined the effect of exogenously supplied oleic acid and found that the antibiotic resistance is not affected. Our data suggest that verapamil decreases the antibiotic resistance of non-replicating Msm. We tested effects of oleic acid on antibiotic resistance and found no significant effect on rifampin, isoniazid, ofloxacin and amikacin (Figs. 12A, 12B, 12C and 12D respectively). Latent Mtb stores fatty acid as triacylglycerol (TAG) which can act as an energy source during latent state (Daniel et al., 2004). Study also suggests that the composition of Mtb TAG and host TAG was nearly identical. The incorporated fatty acid is used to synthesize TAG that potentially could serve as an energy source during latent period (Daniel et al., 2011). Verapamil could potentially block ABC transporter that is involved in importing other energy source and cells could not synthesize TAG from fatty acid because of lack of energy for metabolic activity. Therefore, there is no effect of oleic acid in the presence of verapamil on antibiotic resistance.

Bacteria can develop drug resistance by pumping out the drug molecules through ABC transporter proteins that are embedded on the membrane of the bacterium. Some ABC transporters such as Rv2686c-Rv2687c-Rv2688c act as a fluoroquinolone efflux pump in Mtb. ABC transporters are involved in drug resistance such as ciprofloxacin, norfloxacin, moxifloxacin and sparfloxacin by pumping out the drug molecules (Pasca et al., 2004). Therefore, we have identified two potential ABC transporter gene *MSMEG_5008* and *MSMEG_5009* and decided to construct a gene knockout mutant lacking these two genes.

Msm AES was constructed according to published protocol described in methods. Then electrocompetent pJV53 containing Msm was transformed by the AES which was designed to

disrupt the targeted double-gene ORF *MSMEG_5008* and *MSMEG_5009.* The clones that grew on kanamycin and zeocin containing 7H10 plate were isolated. Genomic DNA from the selected clones was isolated using protocols described in methods. We screened more than two hundred potential mutants by PCR using primers specific for the homologous recombination mutant but all of them showed a band expected in wild type. This suggests that the targeted genes are not being knocked out but an illegitimate recombination event occurs every time we attempted the mutation of the ORF. The 1396 bp Msm AES is very short in length in compare to the whole length of the complete genome (6,988,269 bp) for Msm. Since the clones grew on the double antibiotic containing 7H10 medium, the strain should contain pJV53 and bleomycin resistance cassette. Therefore, Msm AES is present in the genome but not in the targeted site indicating illegitimate recombination of Msm AES. We conclude that the targeted ORFs may be critical for survival of Msm and therefore, knockout mutants may not be viable and those that survive on our selective plates are not legitimate mutants.

Because of the long treatment regimen and non-compliance of drugs, Mtb develops drug resistance. Multidrug resistance Mtb is a major source of morbidity and mortality of TB (WHO, 2019). Some efflux pump inhibitors such as verapamil, piperine, reserpine and berberine decrease drug resistance of *Mycobacterium* (Jin et al., 2011; Menichini et al., 2020; Sharma et al., 2010). Therefore, efflux pump inhibitors are potential adjunctive therapies along withanti-TB drugs.

A previous study showed that Mtb enters a non-replicating state under multiple stress conditions that mimic conditions thought to be encountered inside macrophages. In the non-replicating state, Mtb showed increased resistance to anti-TB drugs rifampin and isoniazid (Deb et al., 2009). The dormant form of Msm showed distinct morphology and increased sensitivity to stress like temperature and antibiotic like doxycycline and hygromycin (Anuchin et al., 2009). However, there is scarcity of knowledge on the effect of efflux pump inhibitors on antibiotic resistance of non-replicating Msm.

A previous finding showed that verapamil inhibits intracellular Mtb growth. The same study showed that verapamil inhibits tolerance to isoniazid, rifampin, bedaquiline and moxifloxacin (Adams, Szumowski, & Ramakrishnan, 2014). Our study suggests that verapamil and reserpine decrease the antibiotic resistance of non-replicating Msm (in multiple stress conditions). The treatment regimen for drug resistant TB is 20-30 months. As efflux pump inhibitors decrease drug resistance, addition of efflux pump inhibitors to TB therapy would

possibly enable antibiotics to kill Mtb in a shorter time. Further studies on efflux pump inhibitors and drug efficacy, toxicity and bioavailability could potentially help to minimize the TB-treatment regimens by developing novel therapy.

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