MICROBIOLOGICAL STUDIES ON THE ANTIBIOTIC TOLERANCE OF NON-REPLICATING *MYCOBACTERIUM ABSCESSUS*: EFFECTS OF EFFLUX PUMP INHIBITORS AND METABOLIC ENERGY SOURCES

by

Andrea M. Funk

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THE PURDUE UNIVERSITY GRADUATE SCHOOL
STATEMENT OF COMMITTEE APPROVAL

Dr. Jaiyanth Daniel, Chair

Department of Biology

Dr. Tanya Soule

Department of Biology

Dr. Elliott Blumenthal

Department of Biology

Approved by:

Dr. Jordan M. Marshall

For my mom and others who have always encouraged my inquiring mind.

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ABBREVIATIONS

AMI = amikacin

CLR = clarithromycin

ERY = erythromycin

OFL = ofloxacin

STR = streptomycin

EPI = Efflux Pump Inhibitor

CCCP = carbonyl cyanide m-chlorophenylhydrazone

Ver = verapamil

Res = reserpine

ABSTRACT

Mycobacterium abscessus is an emerging infectious pathogen capable of causing pulmonary disease similar to tuberculosis, but has many intrinsic and extrinsic properties making it more drug-resistant than *Mycobacterium tuberculosis*. Current treatments, including those used for *M. tuberculosis* infection, have had poor results. Although *in vitro* studies have shown promise with drug treatment for this microorganism, clinical trials have been mostly unsuccessful. An *in vitro* model that mimics the physiological stresses encountered within the human body is likely to enable the discovery of mechanisms of antibiotic resistance used by *M. abscessus* during infection. Therefore, we subjected *M. abscessus* to a combination of stresses thought to be encountered by mycobacteria inside the human body. We subjected the pathogen to low oxygen, low pH, and nutrient starvation. This is the first report on subjecting *M. abscessus* to such a combination of stresses. It is also the first to investigate the effect of the combination of stresses on the tolerance of the pathogen to antibiotics, and the effect of efflux pump inhibitors under such conditions. We found that under these conditions, *M. abscessus* entered a non-replicating state. We investigated whether the multiple-stressed *M. abscessus* displayed altered tolerance to antibiotics commonly used to treat infection, and whether efflux pump inhibitors affected the antibiotic resistance under such conditions. We found that when subjected to our multiple stress model, *M. abscessus* in the reactivating phase had higher tolerance to erythromycin in combination with efflux pump inhibitors verapamil and reserpine compared to non-replicating *M. abscessus*. Reactivating phase cells had a higher tolerance to antibiotic erythromycin than non-replicating cells. Reactivating phase cells also showed antibiotic tolerance in the presence of ATP. This physiologically-relevant experimental model for *M. abscessus* could potentially be used in discovering the mechanisms of antibiotic resistance in the pathogen.

CHAPTER 1. INTRODUCTION

1.1 History and Nomenclature of *Mycobacterium abscessus*

Mycobacterium abscessus was first encountered in 1952 when it was removed from a woman's knee abscess and was named after its ability to cause abscesses (Moore et al., 1953). At this time, it was believed to be the same organism as *Mycobacterium chelonae* due to identical biochemical tests. In 1972, *M. abscessus* was then believed to be a subspecies of *M. chelonae*. In 1992, *M. abscessus* was finally determined to be its own species based on the dissimilarity of its DNA to *M. chelonae*. It wasn't until 40 years after its discovery that it was recognized to cause pulmonary infections when 82 % of 154 patients with rapidly growing mycobacteria (RGM) in their lungs were positive for *M. abscessus.* In 2013, whole genome sequencing revealed that there are three subspecies of *M. abscessus*: *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii*, and *M. abscessus* subsp. *massiliense* (Lopeman et al., 2019) .

1.2 *Mycobacterium abscessus* Pathogenicity

Mycobacterium abscessus is a multi-drug resistant emerging infectious pathogen that causes a plethora of infections, including skin and soft tissue infections (SSTIs), central nervous system infections, septicemia, and respiratory infections similar to tuberculosis. It is considered a nontuberculous mycobacterial (NTM) disease, which are not reportable diseases. As of 2010, there were 86,244 estimated cases of NTM in the United States, costing an estimated $815,098,690 annually. These numbers are likely to be underestimates due to NTM diseases not being reportable conditions as well as the nomenclature changes that occurred with this species beginning in the 1950's after its discovery (Johansen et al., 2020; Lee et al., 2015; Strollo et al., 2015). Despite not having accurate prevalence for NTM and *M. abscessus*, it is known that prevalence is increasing. In the United States, prevalence increased from 2.4 cases per 100,000 people to 15.2 cases per 100,000 people in a span of thirty years (Ratnatunga et al., 2020). It has also been reported that there is a decline in tuberculosis incidence and an increase in NTM incidence, which could be partially from better diagnostic techniques (Bento et al., 2020). *M. abscessus* is nosocomial as well as ubiquitous in the environment. It is found in drinking fountains, shower heads, dental sprays, as well as in the soil (Lee et al., 2015; Strollo et al., 2015). This organism is a major contributor to

pathogenesis in patients with cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD). Other risk factors include being on broad spectrum antibiotics, immunosuppressive therapy, and behaviors that decline lung function such as smoking (Johansen et al., 2020). Age and low body mass index (BMI) are risk factors for NTM infection in individuals with CF; 40 % of CF patients over the age of 40 are positive for NTM. Low BMI is also a risk factor in non-CF NTM positive cases, although the mechanism is not yet understood. Its ability to form biofilms and live in nutrient poor environments make it especially difficult to treat. SSTIs occur after using contaminated needles or other fomites from surgery including cosmetic surgery. *M. abscessus* was not believed to be transmitted from person to person until 2013, when it was found that *M. abscessus* subsp. *massiliense* was being transferred from patient to patient in an adult CF treatment facility. In the same year, analysis was done on 1080 isolates from 517 patients from around the world which suggested that there is high human-human transmission, with three clones with high virulence and poor outcomes circulating among those infected. This suggests that *M. abscessus*, an environmental pathogen, has evolved to be transmitted from person to person (Lopeman et al., 2019). Individuals infected with *M. abscessus* often have bronchiectasis which is thickening of the walls of the bronchi, but it is not known whether infection occurs before or after this condition occurs. Individuals with primary ciliary dyskinesia develop bronchiectasis and are susceptible to *M. abscessus* infection due to a loss of function of cilia. Another group of patients prone to *M. abscessus* are those with COPD. COPD patients have cavities in the lung predisposing them to infection. Many of these individuals also have bronchiectasis (Ryan et al., 2018).

M. abscessus has two colony morphotypes: rough and smooth that can arise from the same parental strain. In pulmonary infection, the rough strain replicates in macrophages, and forms corded colonies that invade tissue. The smooth strain does not display such characteristics. The smooth strain has glycopeptidolipid (GPL) in the cell wall which allows for sliding motility and biofilm formation. The rough phenotype, which lacks GPL, is found in patients with chronic pulmonary infection, while the smooth phenotype is found in wounds. It has been found that in patients, the smooth phenotype switches to the rough phenotype, resulting in worse severity of disease. This suggests that the smooth morphotype enters abnormal lungs and transitions to the rough morphotype. The proposed model for pathogenesis suggests that the smooth phenotype *M. abscessus* enters the lungs where it is able to form a biofilm. The presence of GPL in the smooth phenotype masks underlying lipids, blocking an immune response from occurring. The smooth

phenotype then loses GPL and becomes the rough morphotype, which the immune system recognizes. However, the host immune response is unable to eradicate the bacteria, which is poorly understood (Ryan et al., 2018).

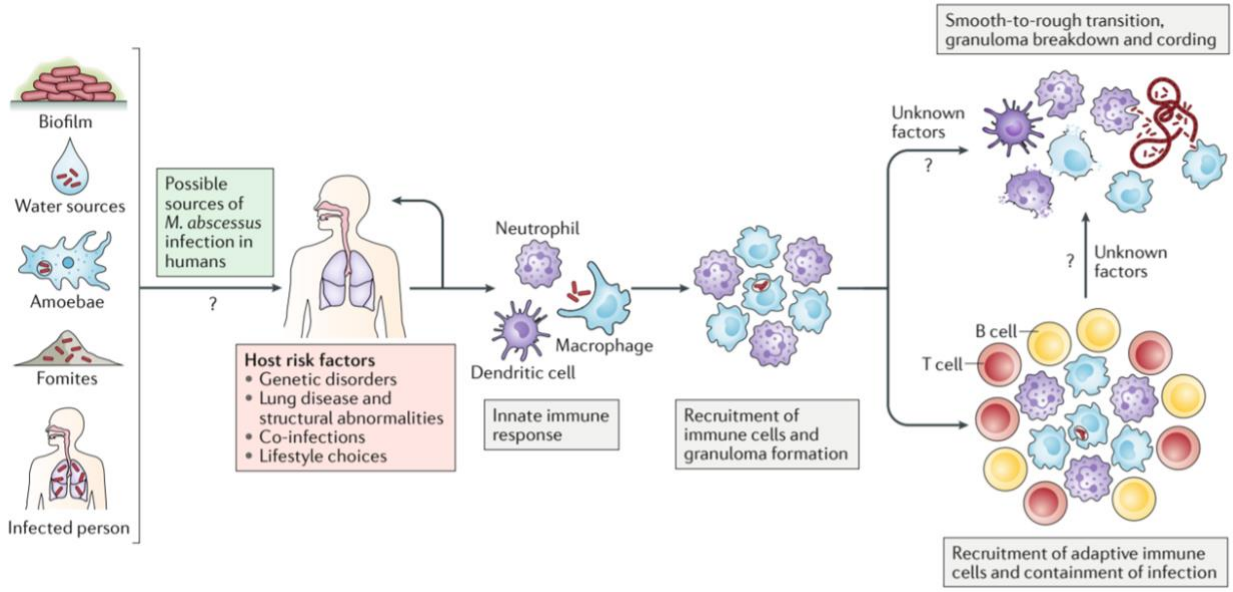

Figure 1: *M. abscessus* pulmonary infection cycle. The infection of *M. abscessus* comes from several potential sources. Once the bacteria is inside the lungs they are phagocytized and granulomas form. *M. abscessus* then has the capability, by unknown mechanisms, to switch morphotype to break out of the granuloma (Johansen et al., 2020).

In pulmonary infection (Figure 1), once the smooth variant of *M. abscessus* colonizes the lungs, it is phagocytized by macrophages and neutrophils. Inside the phagocytic cell, *M. abscessus* resists cell death. Inflammatory cytokines, such as tumor necrosis factor (TNF), are induced causing immune cells to swarm the bacterium. A granuloma forms which is an aggregation of immune cells entrapping tubercle bacteria. The immune system successfully entraps the bacteria by forming the granuloma. Unique to *M. abscessus*, it can transform from the smooth phenotype to the rough phenotype while inside the granuloma, breaking down the granuloma allowing for the newly formed bacterial cords to escape. These bacterial cords then cause tissue destruction and inflammation. The rough variant is lacking GPL, which makes the bacteria more hydrophobic increasing virulence and pathogenicity by increasing surface aggregation, biofilm formation, and sliding motility. These biofilms formed from the rough variant are able to tolerate antimicrobial agents better than planktonic cultures (Johansen et al., 2020).

1.3 *Mycobacterium abscessus* Multi-Drug Resistance

Treatment for *M. abscessus* infections consists of antibiotics in combination for lengthy periods of time and is often unsuccessful. There are many factors, intrinsic and extrinsic, which make *M. abscessus* multi-drug resistant. One of the factors is its relatively slow growth rate. *M. abscessus* is considered a rapidly growing mycobacterium (RGM), but grows slower than many other pathogens. Antibiotics target dividing cells and since *M. abscessus* grows at a slower rate than many pathogens, the antibiotics don't work as well. Mycobacteria also have a thick waxy coat made of hydrophobic mycolic acids (Figure 2).

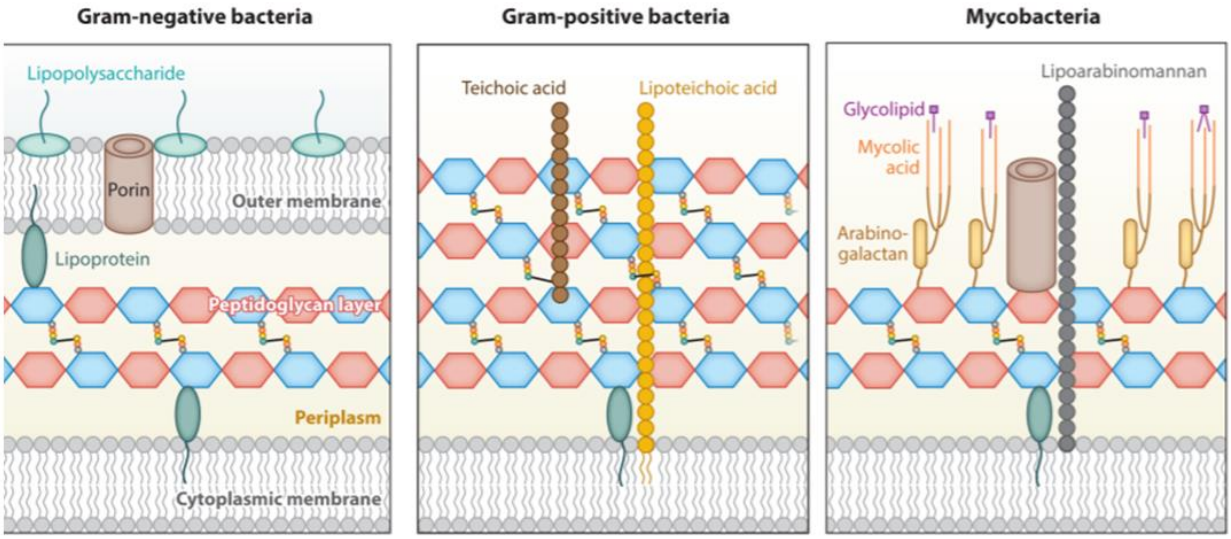

Figure 2: Mycobacterial cell wall in relation to Gram-positive and Gram-negative bacterial cell walls. Mycobacteria are unique in that they do not stain with Gram-staining. Mycobacteria have a thin layer of peptidoglycan and a layer of mycolic acids and glycopeptidolipids which give the bacteria its hydrophobicity (Radkov et al., 2018).

This thick waxy barrier is a physical and chemical barrier that can prevent the antibiotics from entering the cell. *M. abscessus* has several classes of efflux pumps, which expel antibiotics lowering drug concentrations intracellularly. Genetic polymorphisms can also occur which result from selective pressure. Since treatment can last a long time, these slowly growing cells can undergo mutations which enable them to survive antibiotic treatment (Johansen et al., 2020; Strnad et al., 2018). Other factors that contribute to antibiotic resistance of *M. abscessus* include enzymes which alter antibiotic structure and inducible macrolide resistance encoded in the gene e*rm41* (Johansen et al., 2020). In addition to these intrinsic and extrinsic factors affecting treatment,

children with CF have been found to have multiple subpopulations of *M. abscessus* in their lungs which have different antimicrobial resistance profiles (Shaw et al., 2019). Not only is this pathogen difficult to treat, but it can exist as subpopulations within a single person making it exceptionally difficult to treat.

1.4 Antibiotics Currently Used to Treat *M. abscessus* Infection

M. abscessus is one of the most drug resistant bacteria known. It is resistant to most antibiotics including those used for tuberculosis treatment. A few antibiotics, including clarithromycin and amikacin, show activity against *M. abscessus* (Hurst-Hess et al., 2017). Current guidelines for treatment of *M. abscessus* pulmonary infection include a multidrug regimen including a macrolide until the sputum is culture negative for one year. Treatment lasts months to years and often has low efficacy and high toxicity. Unfortunately, another issue in treatment is that health professionals fail to stick to these guidelines. In 2011, one survey reports that only 13 % of treatments for NTM followed the guidelines, with 64 % not including a macrolide. Treatment for *M. abscessus* is also not evidence based, as *in vitro* drug susceptibilities do not correlate with clinical studies (Bento et al., 2020).

Clarithromycin and erythromycin belong to the class of antibiotics known as macrolides. Clarithromycin is a synthetic variant of erythromycin which is a naturally occurring product of *Streptomyces erythraeus* and *Arthrobacter*. Macrolides are bacteriostatic, inhibiting protein synthesis by binding to the large ribosomal subunit, blocking the exit site on the ribosome preventing elongation of the polypeptide chain. Resistance occurs mainly from reducing binding affinity of the drug to the target and efflux from the cell (Dinos, 2017). Resistance also occurs by mutation; *M. abscessus* can undergo an A to G or C missense mutation in 23S rRNA which interferes with macrolide binding to the ribosome. The *erm* genes mentioned earlier encode rRNA methyltransferases that methylate 23S rRNA, interfering with macrolide binding and function (Fyfe et al., 2016).

Amikacin and streptomycin belong to the antibiotic class aminoglycosides. Aminoglycosides are isolated from *Streptomyces* and *Micromonospora.* Streptomycin was the first aminoglycoside discovered in 1943, and was the first antibiotic to successfully treat *M. tuberculosis*. As more aminoglycosides were discovered and clinical use grew, resistance grew as well. Derivatives of these aminoglycosides were created including amikacin in 1972. Aminoglycosides are bactericidal,

inhibiting protein synthesis by binding to the A site on the ribosome. Streptomycin binds to a different site, interfering with tRNA selection. Common resistance mechanisms to aminoglycosides include aminoglycoside modifying enzymes, target modification, changing uptake and efflux, and membrane proteases (Becker et al., 2013).

Ofloxacin belongs to the antibiotic class fluoroquinolones. Fluoroquinolones are synthetic broad-spectrum antibiotics that were created in 1962. They bind DNA gyrase and topoisomerase, interfering with DNA replication, making them bactericidal (Fan et al., 2018). In *M. tuberculosis* the main mechanism of fluoroquinolone resistance are mutations in DNA gyrase, altering the target of the fluoroquinolone (Bernard et al., 2015).

1.5 Efflux Pump Inhibitors and Their Role in Antibiotic Resistance

Efflux pumps are present in all organisms, contributing greatly to antibiotic resistance. They can expel conventional antibiotics as well as other harmful substances such as dyes and heavy metals (Blanco et al., 2016). It has been suggested that the use of antibiotics induces the genes for efflux pumps and that prolonged use of antibiotics to treat mycobacterial infections along with drug efflux leads to chromosomal mutations in genes for the drug targets (Schmalstieg et al., 2012). Recently, the use of efflux pump inhibitors to increase the drug concentration in mycobacteria has shown promise *in vitro*. Some efflux pump inhibitors are used clinically for other diseases. The efflux pump inhibitor verapamil increased killing of mycobacteria in macrophages, but had severe cytotoxic effects (Rodrigues et al., 2017). It was recently found that instead of increasing intracellular drug concentrations, verapamil disrupts the proton motive force of the membrane causing stress which indirectly disrupts efflux pumps and membrane function (Chen et al., 2018). Efflux-mediated antibiotic resistance is one of the major players in antibiotic resistance in Mycobacteria. Bacterial efflux pumps can be classified into one of the five superfamilies (Figure 3): major facilitator superfamily (MFS), ATP-binding cassette (ABC) superfamily, resistance-nodulation-cell division (RND) superfamily, small multidrug resistance (SMR) superfamily, and the multidrug and toxic compound extrusion (MATE) superfamily. The ABC transporters use ATP as an energy force to expel drugs from the inside of the cell while the other superfamilies use the proton motive force (PMF) or the sodium/hydronium ion electrochemical gradient. The increase in drug-resistance of bacteria has highlighted the need for new drugs to combat pathogens such as *M. abscessus*. Efflux pump inhibitors are potential drugs that could be used in combination with

antibiotics. Efflux pump inhibitors in addition to antibiotics would decrease the minimal inhibitory concentration (MIC) of antibiotics to successfully clear infection. There are currently no efflux pump inhibitors designed for the SMR superfamily. The efflux pump inhibitors reserpine antagonizes the efflux activity of the RND superfamily, verapamil and piperine antagonize ABC transporters, and CCCP antagonizes the MFS superfamily and the electron transport chain (ETC). The MFS superfamily is known to export antibiotics such as ofloxacin and streptomycin from the cell, ABC transporters export amikacin, streptomycin, clarithromycin, and erythromycin, and the SMR superfamily exports amikacin and streptomycin (Pule et al., 2016).

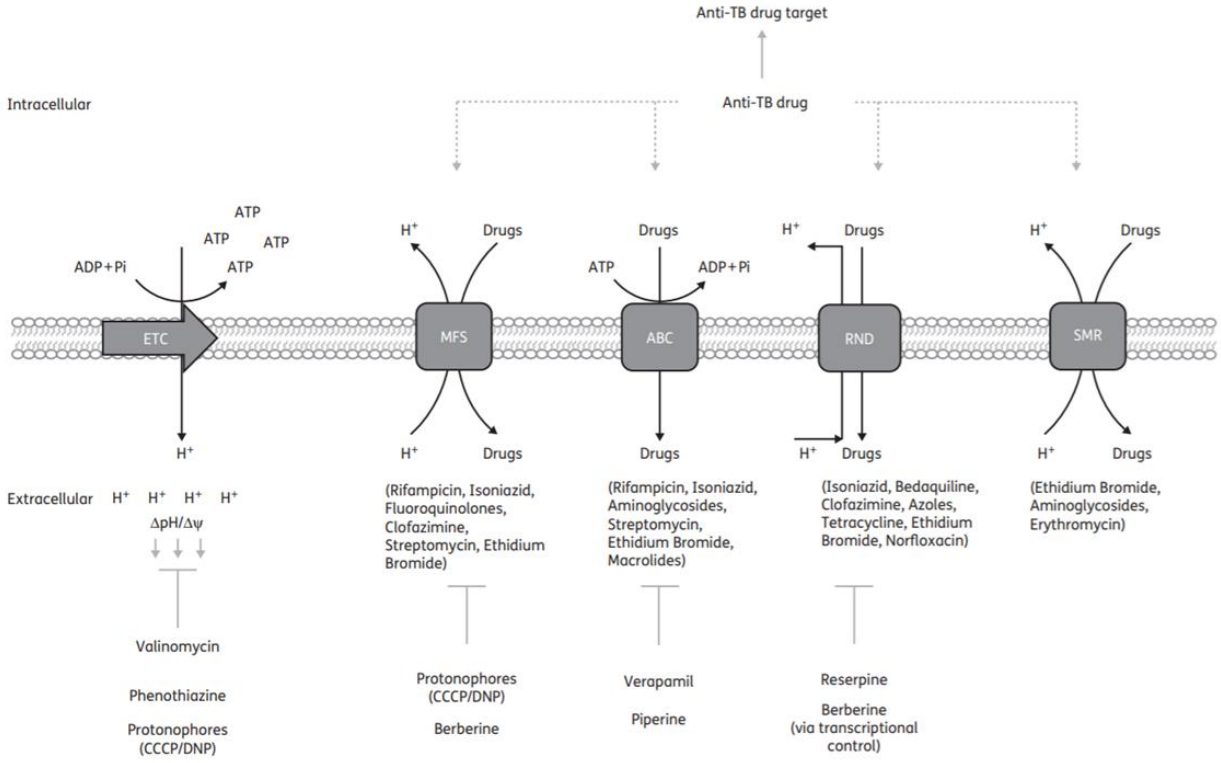

Figure 3: Efflux pump superfamilies found in Mycobacteria with known inhibitors for classes of antimicrobials. Several efflux pump inhibitors are known to directly or indirectly affect the export of antibiotics in *M. tuberculosis* (Pule et al., 2016).

1.6 Antibiotic Tolerance of *M. abscessus* in a Macrophage-Like Environment

Multiple stress models have been created to mimic the intra-alveolar macrophage environment inside the lung for *Mycobacterium tuberculosis*, however, this has not been done with

M. abscessus. Current studies on *M. abscessus* include examining transcriptomics under hypoxia and select antibiotic pressure in artificial sputum to mimic the cystic fibrosis lung (Miranda-CasoLuengo et al., 2016). Another study has examined transcriptomics of *M. abscessus* in murine macrophages without antibiotic pressure (Dubois et al., 2019). Other studies on *M. abscessus* report antibiotic resistance or the effect of efflux pump inhibitors without stressors applied (Hurst-Hess et al., 2017; Ramis et al., 2019; Vianna et al., 2019).

Inside the macrophage, tubercle bacteria become dormant and therefore phenotypically drug resistant. They experience low oxygen, high carbon dioxide, low nutrients, and acidic pH inside the granuloma (Deb et al., 2009). In this study we use stressors similar to those used in the *M. tuberculosis* study to examine the effects of multiple stressors on antibiotic tolerance.

Current treatment for *M. abscessus* infections is unsuccessful despite promise of *in vitro* studies (Lopeman et al., 2019). It is likely that the dormancy acquired from the multiple stresses inside the alveolar macrophage adds to the intrinsic antibiotic resistance and creates a pathogen that is nearly impossible to treat despite *in vitro* studies showing possible treatment plans. This is the first study to examine antibiotic tolerance under a combination stress model mimicking the macrophage environment encountered by *M. abscessus* in pulmonary infections.

1.7 ABC Transporter in Mycobacterium *abscessus* May Import Fatty Acids and Export Antibiotics

ATP binding cassette (ABC) transporters consist of at least two intracellular nucleotide-binding domains which bind ATP. ABC transporters also have two transmembrane domains. ABC transporters have been shown to be involved in antibiotic resistance, bacterial virulence, and bacterial pathogenesis. They also function as importers or exporters in cells (Lewinson et al., 2017). One study, in a related species to *M. abscessus*, shows the involvement of an ABC transporter in *Mycobacterium smegmatis* in antibiotic resistance from efflux of the transporter (Banerjee et al., 2000). Another study shows that an ABC transporter in the bacterium *Lactococcus lactis* is capable of importing and exporting ethidium across the cell membrane; it acts as a reversible ABC transporter (Balakrishnan et al., 2004). *M. tuberculosis* has been shown to have 2.5 % of its genome of 4.4 million base pairs encoding ABC transporters with 26 complete transporte rs and 11 incomplete transporters, with an equal number of exporters and importers. Many of the exporters potentially play a role in antibiotic resistance and one potentially exports a substance which

contributes to attachment to eukaryotic cells (Braibant et al., 2000). The *M. abscessus* genome encodes for many proteins, including ABC transporters, which may export drugs. *M. abscessus* contains 124 putative ABC transporters encoded in its 5 million base pair genome. Earlier studies from our laboratory showed that an ATP binding cassette (ABC) transporter protein, Rv1272c, in *M. tuberculosis* enhanced the uptake of fatty acids into surrogate bacterial cells and incorporated them into phospholipids (Martin et al., 2018). There is no current published study on ABC transporters in *M. abscessus*. Sequence similarity has revealed an ABC transporter, MAB_1414c, in *M. abscessus* with 70 % sequence identity (over a 624 amino acid stretch) to the *M. tuberculosis* transporter protein, Rv1272c. The gene encoding another putative ABC transporter, MAB_1415c, is 65 % identical (over a 582 amino acid stretch) to the ABC transporter Rv1273 in *M. tuberculosis*. The two protein transporters are 34 % identical in their amino acid sequences to each other. Of all the homologs to Rv1272c in *M. abscessus*, MAB_1414c and MAB_1415c were the most similar.

CHAPTER 2. HYPOTHESIS AND OBJECTIVES

We hypothesize that, under *in vitro* stresses (low pH, low nutrient, and hypoxia) that mimic physiological conditions, *Mycobacterium abscessus* will show altered sensitivity to antibiotics currently used to treat pulmonary infections. We also hypothesize that, under such conditions, efflux pump inhibitors will increase the pathogen's sensitivity to antibiotics. We further postulate that metabolic energy sources such as ATP and glycerol could supply energy to the bacterial efflux pumps that pump out antibiotics and modulate the antibiotic resistance observed. Since there are no studies on the antibiotic tolerance of *M. abscessus* under *in vitro* stress conditions that mimic those inside the macrophage, we aimed to evaluate the effects of antibiotics and efflux pump inhibitors on the pathogen under such conditions in our study.

We postulate that *M. abscessus* ABC transporters, MAB_1414c and MAB_1415c, which are homologous to previously studied ABC transporters in *M. tuberculosis*, could function as antibiotic efflux pumps and pump antibiotics out of the cell. Homology suggests that the *M. abscessus* transporter may import phospholipids which may incorporate into the cell wall, increasing antibiotic resistance. Therefore, we attempted to construct a gene-knockout mutant lacking those transporters.

CHAPTER 3. METHODS

3.1 Bacterial Strains and Growth Conditions

Mycobacterium abscessus ATCC 19977 (ATCC, Manassas, VA) colonies were grown on Luria-Bertani (LB) agar plates and in Dubos broth (pH 6.6 with 5 % glycerol) without enrichment (bovine albumin, dextrose, and catalase) (BD Life Sciences, Sparks, MD). *M. abscessus* was grown on LB agar plates from glycerol stocks stored at -80 °C. For the antibiotic assays, cells were inoculated into 3 mL Dubos broth and grown until saturated. The culture was vortexed and sonicated to ensure no clumping, split evenly in volume, and inoculated into two 500 mL flasks containing 75 mL Dubos broth (pH 6.6 with 5 % glycerol) and incubated at 37 °C with shaking at 100 rpm until optical density at 600 nm (OD_{600}) reached 0.15-0.3. We followed a modification of a previously reported combined multiple-stress model (Deb et al., 2009) to subject *M. abscessus* to a combination of hypoxia, low pH and nutrient starvation. Log phase cells, non-replicating phase cells, and reactivating phase cell antibiotic assays were set up in 96-well plates originating from the same culture grown to an OD_{600} of approximately 0.25 (Figure 4).

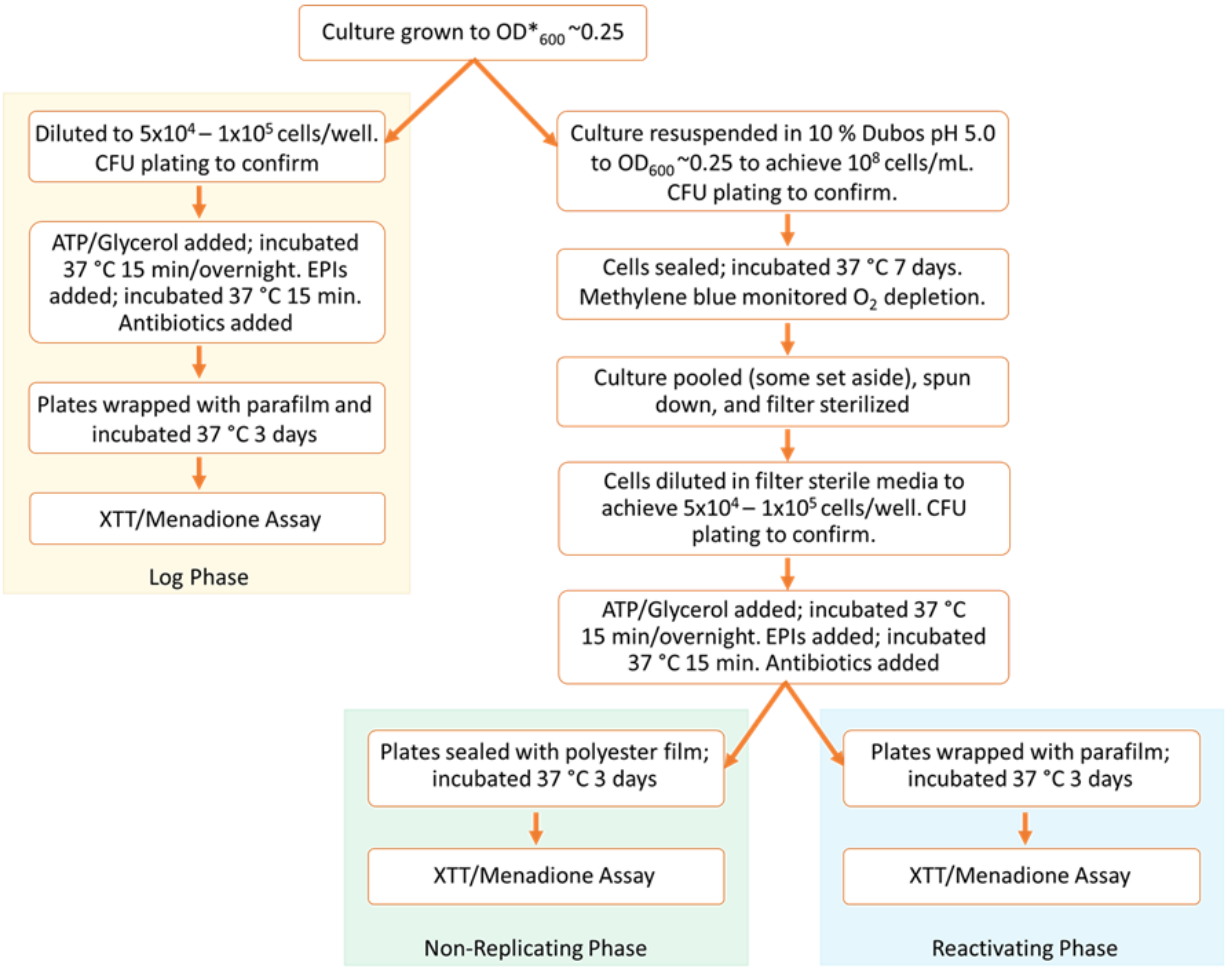

Figure 4: Protocol followed for the antibiotic assay

To set up stressed phases, the culture was spun down at 8000 X g for 20 min and resuspended in 10 % Dubos pH 5 containing 0.5 % glycerol to an OD_{600} of approximately 0.25 to achieve 10^8 cells per well. The resuspended cells were then aliquoted into 96-well plates (240 µL/ well of approximately 10^8 colony-forming-units (CFU)/mL cells) to achieve a head-space ratio of 0.5 (Wayne et al., 1996). The plates were sealed with sterile polyester film (VWR International, Radnor, PA) to prevent gas exchange and incubated at 37 °C for periods up to 7 days. For the actively dividing log phase control (100% Dubos pH 6.6), cells were incubated in plates covered with lids to allow gas exchange. Methylene blue dye was added to selected wells as an indicator of oxygen concentration (Khan et al., 2008). CFUs were determined at each time-point by agar plating.

3.2 Determination of Antibiotic Resistance and Effects of Efflux Pump Inhibitors

Log-phase cultures were diluted with Dubos (for 0-day time-point) for assessing antibiotic resistance. The multiple-stressed 7-day cultures were diluted with spent medium filtered through 0.22 µM filter to achieve 5 x 10^4 – 1 x 10^5 cells per well. CFU plating was done to confirm viable cell count at each stage. The following antibiotics were used in our assays: clarithromycin (Alfa Aesar, Ward Hill, MA), ofloxacin (Alfa Aesar, Ward Hill, MA), amikacin (MP Biomedicals, Solon, OH), erythromycin (MP Biomedicals, Solon, OH) and streptomycin (Sigma-Aldrich, St.Louis, MO). We tested the following efflux pump inhibitors in our assays: verapamil (TCI America, Portland, OR), CCCP (Alfa Aesar, Ward Hill, MA), and reserpine (Alfa Aesar, Ward Hill, MA). Antibiotics and inhibitors were dissolved in dimethyl sulfoxide or pH-adjusted buffer as appropriate. The antibiotics were used at or near their reported minimal inhibitory concentrations (MICs) for *M. abscessus* or other mycobacteria (Hurst-Hess et al., 2017; Li et al., 2013; Nessar et al., 2012; Vianna et al., 2019). Efflux pump inhibitors were used at concentrations reported in previous studies (Jin et al., 2011; Ramis et al., 2019; Viljoen et al., 2019) . Verapamil and reserpine were used at final concentrations of 50 µg/mL and 40 µg/mL, respectively (Viljoen et al., 2019). CCCP was used at final concentration 0.4 µg/mL. clarithromycin, ofloxacin, amikacin, erythromycin, and streptomycin were used at concentrations 0.5 µg/mL, 50 µg/mL, 10 µg/mL, 1 µg/mL, and 20 µg/mL respectively. The effects of ATP and glycerol on modulating antibiotic resistance and efflux pump inhibitor action were determined. Cells were incubated for 15 min at 37 °C with ATP (final concentration of 0.25 µM) or glycerol (final concentration of 10 mM) prior to the addition of efflux pump inhibitor. The efflux pump inhibitors were added, and the cells were incubated for 15 min at 37 °C before adding the antibiotics. Cells were incubated for 16 h at 37 °C with glycerol at a final concentration of 100 µM before addition of efflux pump inhibitors and antibiotics as described above. For 0-day controls, plates with lids were wrapped with parafilm and incubated for 3 days at 37 °C. After 3 days a tetrazolium salt (XTT; Biotium, Fremont, CA; final concentration of 125 µM (24 µl/well of 0.84 mg/ml, dissolved in PBS) and menadione (Alfa Aesar, Ward Hill, MA; 1.032 mg/ml, final concentration 60 µM) assay was done to measure viable cells (Singh et al., 2011). The 7-day plates were sealed with polyester film and incubated at 37°C for 3 days before doing the tetrazolium salt and menadione assay. Control wells included medium only, medium plus inhibitor, cells only, cells plus inhibitor, and cells plus inhibitor and ATP (0.25 µM).

To set up day 7 plates, culture subjected to multiple stress for 7 days was pooled, leaving out wells which lacked condensation on the film, potentially indicating a break in the seal. Some culture was saved for setting up day 7 plates. The rest of the culture spun down at 8000xg for 5 minutes and filter sterilized to collect spent medium. Dilution was done to achieve the $5 \times 10^4 - 1 \times 10^5$ cells per well before adding ATP, efflux pump inhibitor, and antibiotics as described above. SigmaPlot was used for analysis. One-way ANOVA comparing all groups together was performed with post-hoc Tukey's test. Shapiro-Wilk test for normality was done.

3.3 Genomic DNA Isolation

We followed a procedure modified from a previously published report to isolate genomic DNA from *M. abscessus* (Medjahed et al., 2010). Briefly, *M. abscessus* was 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.

3.4 Construction of Mab Mutant Lacking *MAB_1415c-1414c*

To test whether the putative ABC transporters MAB_1414c and MAB_1415c act as antibiotic efflux pumps as well as importing fatty acids into the cell and incorporating them into the cell wall, contributing to antibiotic resistance, the genes needed to be knocked out.

3.4.1 Preparation of MAB1415c-*1414c* Allelic Exchange Substrate

We used a recombineering approach towards disruption of the *MAB1415c-1414c* open reading frames following modifications of previously reported procedures (Medjahed et al., 2010; van Kessel et al., 2008a). 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. abscessus*. The Q5 Hot Start High-Fidelity DNA polymerase (New England BioLabs, Ipswich, MA) was used for the PCR amplification. The zeocin 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 *MAB1415c-1414c* AES construct

Primer	Sequence (5'-3')	Optimal Annealing Temperature
1415UP_fwd 1415UP_rev	AGTTGAAGGAAAAATGCGGAC ACTTGGCCATTACAGCGAAGCCAGGGTG	62 °C
1415Bleo_fwd 1415Bleo_rev	CTTCGCTGTAATGGCCAAGTTGACCAGTG CAGCGTCGCGTCAGTCCTGCTCCTCGGC	64 °C
1414DN_fwd 1414DN_rev	GCAGGACTGACGCGACGCTGACCGCATC CCGTCATCGGCCGGCTGA	70 °C

The PCR products were purified from agarose gels using the Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA) 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 1414c UP_fwd and 1414c DN_rev at an annealing temperature of 71 °C. The AES product was purified from the agarose gel as described above.

3.4.2 Preparation of Electrocompetent *M. abscessus*

Electrocompetent cells were prepared by following a previously published protocol (van Kessel et al., 2008a). Wild type *M. abscessus* 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. *M. abscessus* 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 cells were dissolved and then 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.

3.4.3 Transformation of Electrocompetent *M. abscessus* Cells with pJV53

Wild type *M. abscessus* cells were transformed with the pJV53 plasmid (Figure 5) expressing the mycobacterial recombination proteins Che9c 60-61 (Addgene, Ipswich, MA) by electroporation to generate the recombineering strain. Briefly, electrocompetent *M. abscessus* cells were transformed following a previously published protocol (van Kessel et al., 2008a) using a BioRad MicroPulser electroporation unit (Bio-Rad Laboratories, Hercules, CA).The pJV53 plasmid (100ng) was added to the 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 *M. abscessus* transformants were

screened for the presence of the pJV53 kanamycin resistance gene using the primers listed below in Table 2.

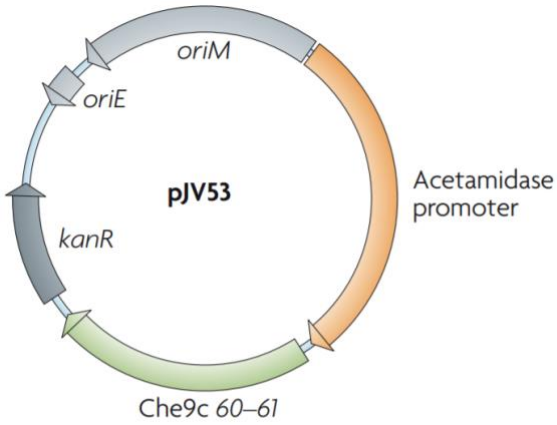

Figure 5: Plasmid pJV53 diagram. pJV53 contains a kanamycin cassette to select for as well as *Che9c 60-61* which codes for two proteins that aid in the recombination of the allelic exchange substrate as depicted with the arrow and AES (van Kessel et al., 2008b).

Table 2: Primers used for screening pJV53-containing *M. abscessus*

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

3.4.4 Generation of *M. abscessus* Recombineering Strain

M. abscessus cells containing pJV53 were grown in 5 ml of 7H9 + ADC broth containing kanamycin and Tween 80) at 37 °C for five 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 for four days. 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 and then placed on ice for 2 h. These recombineering *M. abscessus* were made electrocompetent following the procedure described above for wild type *M. abscessus*, aliquoting 200 µl per sterile microcentrifuge tube.

3.4.5 Transformation of Recombineering *M. abscessus* with Allelic Exchange Substrate and Screening of Mutant Clones

The electrocompetent, recombineering *M. abscessus* cells were transformed by electroporation with the linearized AES construct for disruption of the two ORFs *MAB1415c-1414c* following the protocol described above. The transformation mixture was plated on LB 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 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. Figure 6 shows the insertion of the AES into the open reading frames and the location of the primers listed in Table 3.

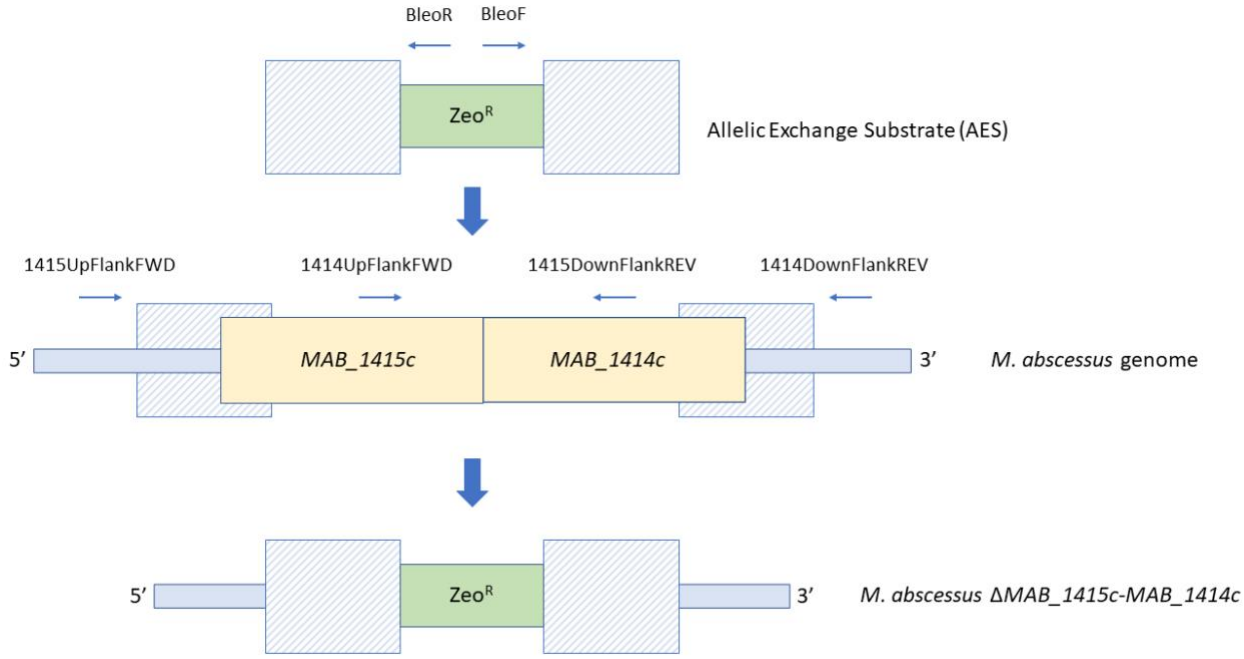

Figure 6: Schematic for AES insertion into *MAB_1415c-MAB_1414c* open reading frames. The AES is composed of Zeor flanked by homologous upstream and downstream flanking regions complimentary to the open reading frames. Once transformed, the AES will recombine with the genomic DNA, knocking out genes *MAB_1415c* and *MAB_1414c*. Blue arrows above the DNA sequence represents the primers listed in Table 3.

Table 3: Primers for screening mutants

Primer Pairs	Sequence (5'-3')	Optimal Q5 Polymerase Annealing Temperature	Expected PCR Products (bp)
MAB_1414c_upflank_fwd 1415DNFLrv	GGGCCGGTGGCAGGCTCT CGACCACTGCGACCGCCA	63 °C	WT- 1968 Δ - None
1415UPFLfw Bleo129R	CGACCAGCCCACCGCTCT CCGGACCACACCGGCG	62 °C	WT- None Δ - 1188
Bleo272F MAB_1414c-downflank_rev	GCCGGCCATGACCGA CGCGGACGCAATCAGTCTG	57 °C	WT- None Δ - 1189

CHAPTER 4. RESULTS

We cultured wild-type *M. abscessus* to early log-phase and then subjected the cells to a stress combination (low O_2, low pH and low nutrients) thought to exist inside human macrophages. In 5-days, the cells entered a non-replicating phase as described above. After 7-days under the stress conditions, the cells were allowed access to normal oxygen levels which allows re-growth of cells from the non-replicating phase. This mimics the reactivation of dormant cells inside the human body. The cells were exposed to antibiotics in the presence or absence of efflux pump inhibitors and their survival was measured using a high-throughput cell viability assay as described under Methods.

4.1 *M. abscessus* Enters a Non-Replicating State under a Multiple Stress Combination that Mimics *In Vivo* Conditions

The conditions that tubercle bacteria are hypothesized to encounter in the macrophage in the human body (hypoxia, low nutrients, acidity) were mimicked *in vitro* in a combination of the three stress factors. Hypoxia was achieved by sealing the 96 well plates with an air-tight sealing film. Low nutrient and acidic conditions were achieved by using diluted (10 %) Dubos broth (with 0.5 % glycerol) at pH 5. Depletion of oxygen was monitored by methylene blue. The mycobacterial cells in the sealed wells depleted the oxygen in the medium which was indicated by the decolorization of methylene blue. The fading of the blue color was lesser in the medium-only control wells (Figure 7A). Agar plating and colony forming unit counting of cells after 5 and 7 days under the combined stress conditions showed that *M. abscessus* enters a non-replicating state under the combination of stress conditions (Figure 7B).

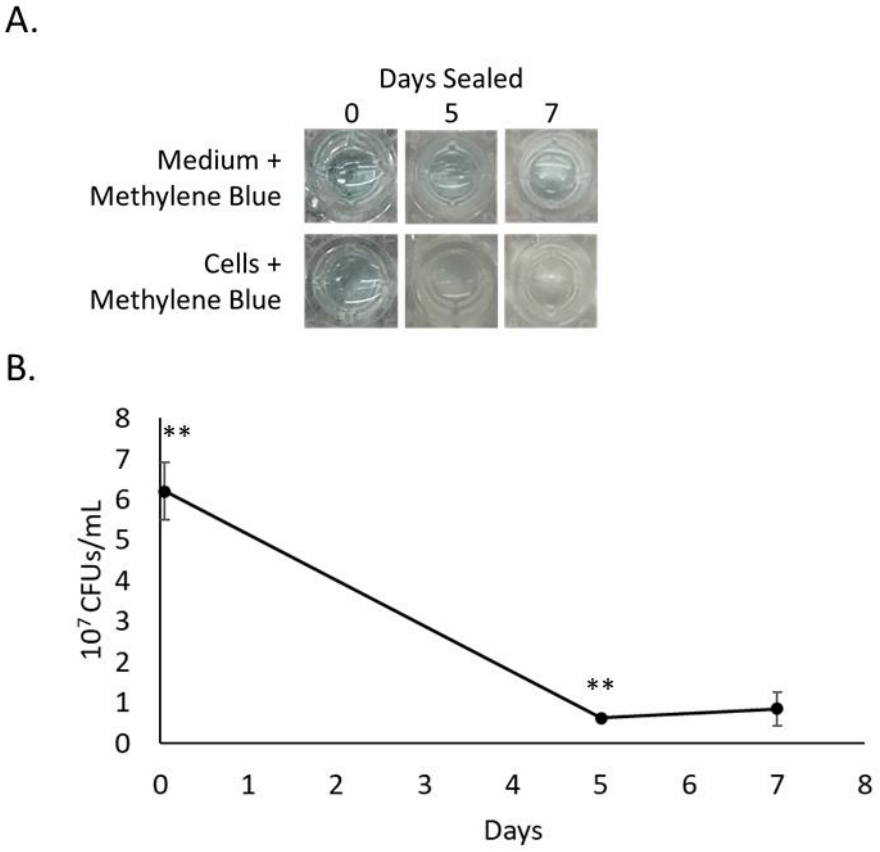

Figure 7: *Mycobacterium abscessus* enters a non-replicating state under multiple stress conditions. (A) Oxygen depletion is indicated with the indicator methylene blue. As oxygen is consumed by the cells in the bottom wells the blue color fades to clear. The top wells are medium controls and with a lack of cells there is no decolorization. (B) Non-replication is indicated by the decrease in colony forming units as cells experience combination stress. At day 5 and 7 colony forming units level off indicating cells are not dying nor growing at these time points. Experiments were performed independently three times. Average ± SD from a representative experiment is shown in panel B. Student's T-test was performed: **(Day 0 – Day 5) P-value 0.0079; *(Day 5 – Day 7) P-value 0.5198.

4.2 The Clarithromycin Resistance of Actively Growing *M. abscessus* Is Inhibited by Verapamil

We examined the resistance of the *M. abscessus* cells in these phases to the antibiotic clarithromycin and the effect of the efflux pump inhibitor verapamil on the antibiotic resistance. We observed that nearly a quarter (24.5 %) of the cells were resistant to clarithromycin during log-phase (Figure 8). Verapamil significantly decreased the resistance (survival) of these cells to about 4 %. Cells in the non-replicating and reactivating phases did not show significant resistance to clarithromycin.

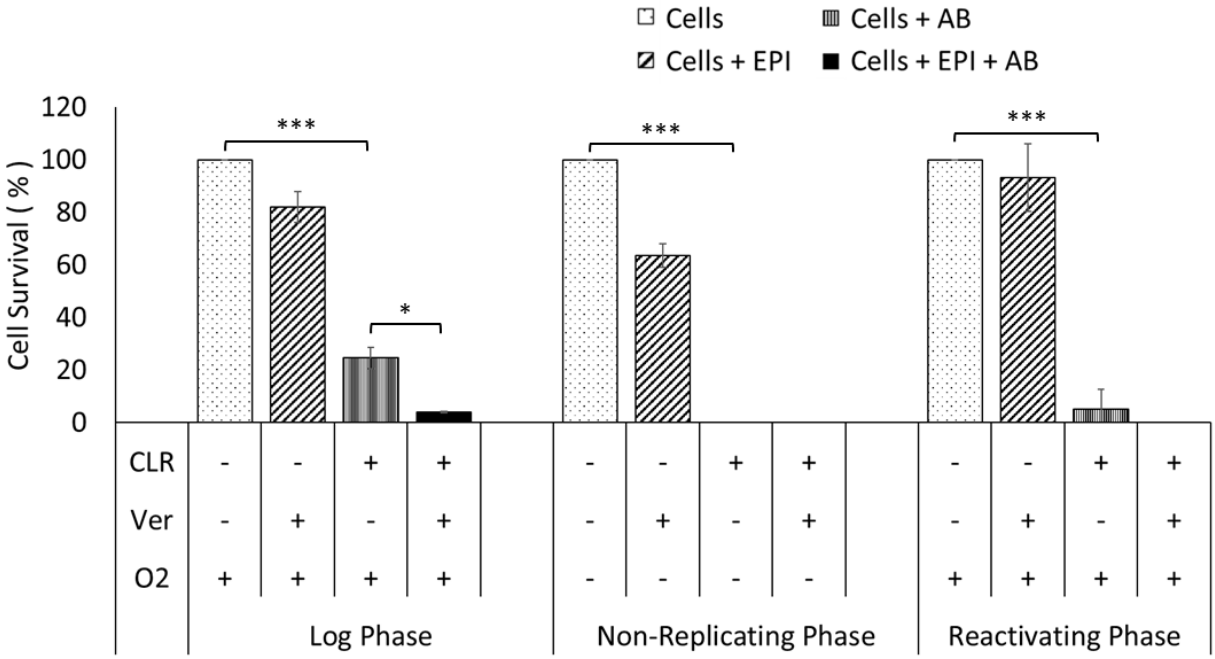

Figure 8: Verapamil inhibits the clarithromycin resistance of actively growing *M. abscessus*. *M. abscessus* cells in log-, non-replicating and reactivating phases were cultured as described under Methods and incubated in the presence (+) or absence (-) of clarithromycin (CLR) and/ or verapamil (Ver). The survival of *M. abscessus* exposed to the drugs is shown relative to respective untreated controls. EPI = efflux pump inhibitor. Percent survival of cells was measured in three independent experiments. Values shown are average ± SD of duplicate measurements from a representative experiment. ANOVA was performed followed by post-hoc Tukey's test: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

4.3 The Erythromycin Resistance of Actively Growing *M. abscessus* Is Inhibited by Verapamil.

We examined the resistance of *M. abscessus* cells in the phases previously described to the antibiotic erythromycin and the efflux pump inhibitor verapamil. We observed an almost 2-fold significant decrease in survival (to 55 %) of log-phase cells upon exposure to erythromycin. Only one-tenth of the cells survived after exposure to erythromycin in combination with verapamil. In the reactivating phase, only one-fourth (24 %) of the cells were resistant to erythromycin. The addition of verapamil did not significantly decrease survival of the cells in this phase. Non-replicating cells showed very low (1.8 %) resistance to erythromycin (Figure 9).

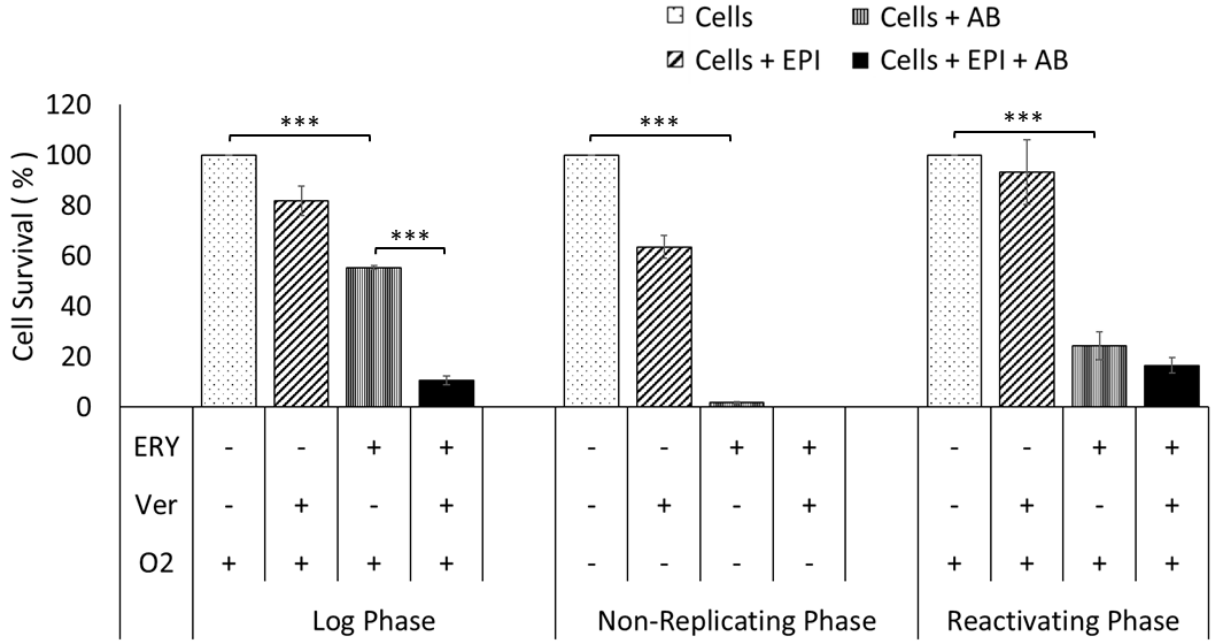

Figure 9: Verapamil inhibits the erythromycin resistance of actively growing *M. abscessus*. *M. abscessus* cells in log-, non-replicating and reactivating phases were cultured as described under Methods and incubated in the presence (+) or absence (-) of erythromycin (ERY) and/ or verapamil (Ver). The survival of *M. abscessus* exposed to the drugs is shown relative to respective untreated controls. Percent survival of cells was measured in three independent experiments. Values shown are average ± SD of duplicate measurements from a representative experiment. ANOVA was performed followed by post-hoc Tukey's test: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

4.4 Clarithromycin Resistance of Actively Growing *M. abscessus* Is Not Inhibited by CCCP

We examined the resistance of *M. abscessus* cells in the three phases to clarithromycin and efflux pump inhibitor CCCP. We observed that only 25 % of log-phase cells were resistant to clarithromycin. The addition of CCCP to clarithromycin had no significant effect on the resistance. Cells in the non-replicating and reactivating phases showed no significant tolerance to clarithromycin (Figure 10).

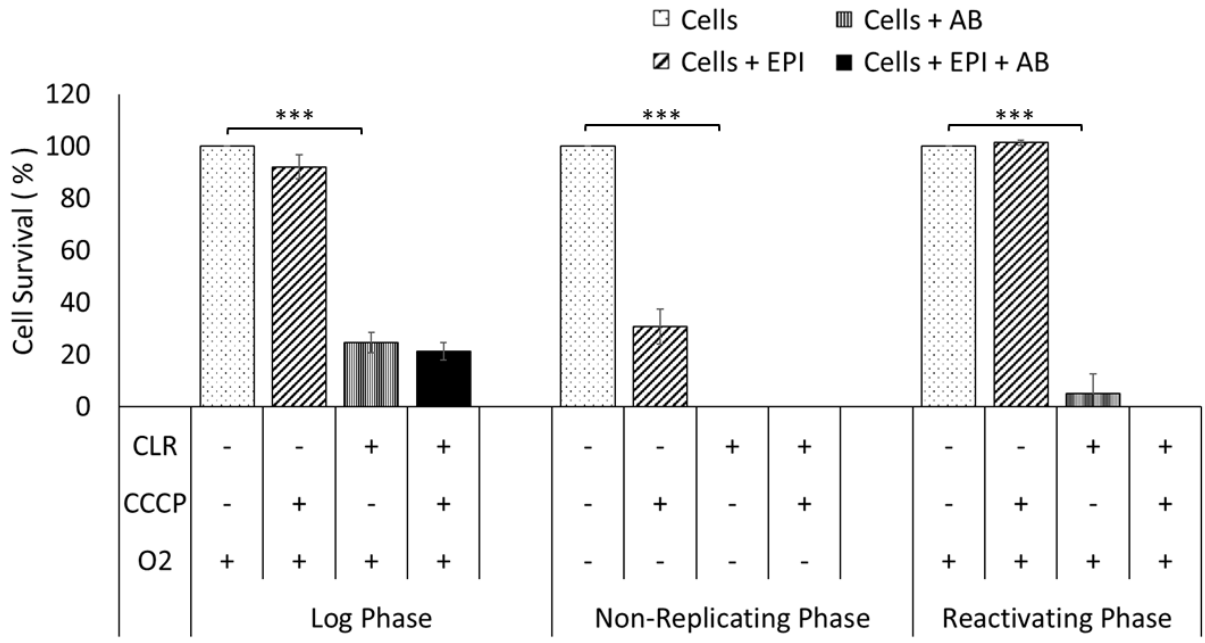

Figure 10: CCCP does not inhibit the clarithromycin resistance of actively growing *M. abscessus.* *M. abscessus* cells in log-, non-replicating and reactivating phases were cultured as described under Methods and incubated in the presence (+) or absence (-) of clarithromycin (CLR) and/ or CCCP. The survival of *M. abscessus* exposed to the drugs is shown relative to respective untreated controls. Percent survival of cells was measured in two independent experiments. Values shown are average ± SD of duplicate measurements from a representative experiment. ANOVA was performed followed by post-hoc Tukey's test: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

4.5 Erythromycin Resistance of Actively Growing *M. abscessus* Does Not Show a Significant Decrease in the Presence of CCCP

We examined the resistance of the previously described three phases of *M. abscessus* cells to erythromycin and the efflux pump inhibitor CCCP. We observed that about 55 % of log-phase cells were resistant to erythromycin. The addition of CCCP to these cells did not cause a significant difference in resistance. Non-replicating phase cells showed very little tolerance (1.8 %) to erythromycin, while reactivating phase cells showed almost 25 % survival. The addition of CCCP to reactivating phase cells with erythromycin showed to significant difference in resistance (Figure 11).

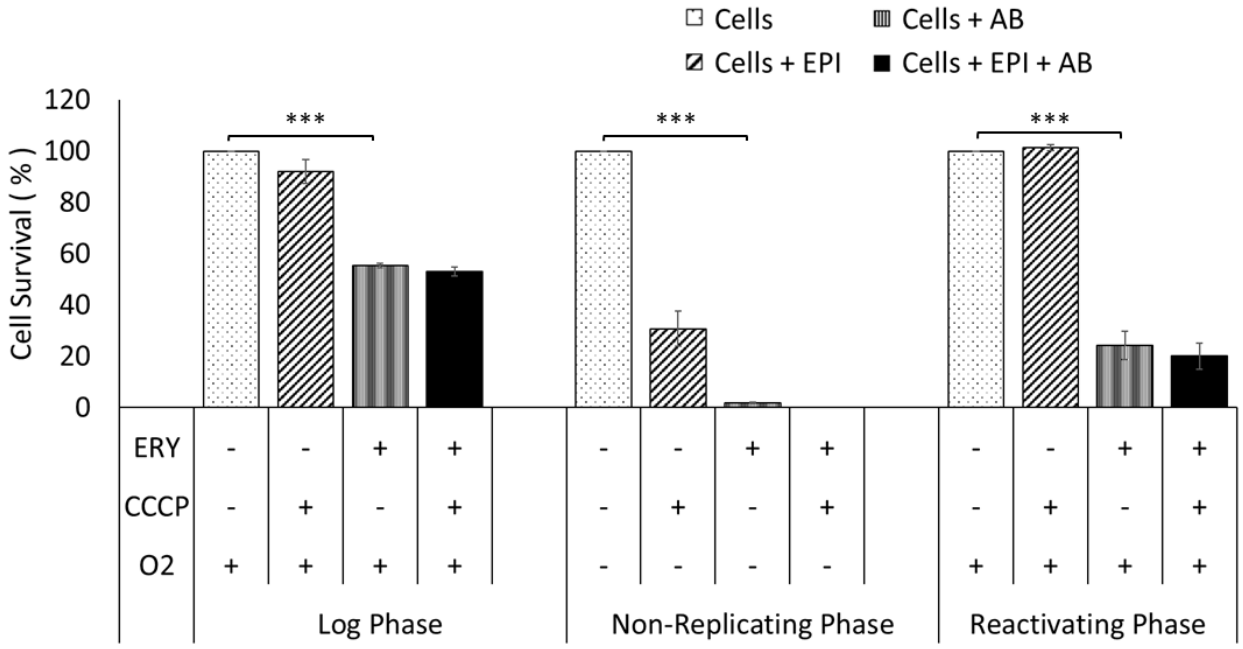

Figure 11: CCCP does not inhibit the erythromycin resistance of actively growing *M. abscessus*. *M. abscessus* cells in log-, non-replicating and reactivating phases were cultured as described under Methods and incubated in the presence (+) or absence (-) of erythromycin (ERY) and/ or CCCP. The survival of *M. abscessus* exposed to the drugs is shown relative to respective untreated controls. Percent survival of cells was measured in two independent experiments. Values shown are average ± SD of duplicate measurements from a representative experiment. ANOVA was performed followed by post-hoc Tukey's test: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

4.6 The Clarithromycin Resistance of Actively Growing *M. abscessus* Is Inhibited by Reserpine

We examined the resistance of cells in the three phases described previously to clarithromycin and the effect of the efflux pump inhibitor reserpine. We observed that one-fourth (24.5 %) of the log-phase cells were resistant to clarithromycin. The addition of reserpine to these cells did not significantly alter survival (resistance). Non-replicating cells showed no significant survival while reactivating cells showed a low level of resistance (6 %) to clarithromycin (Figure 12).

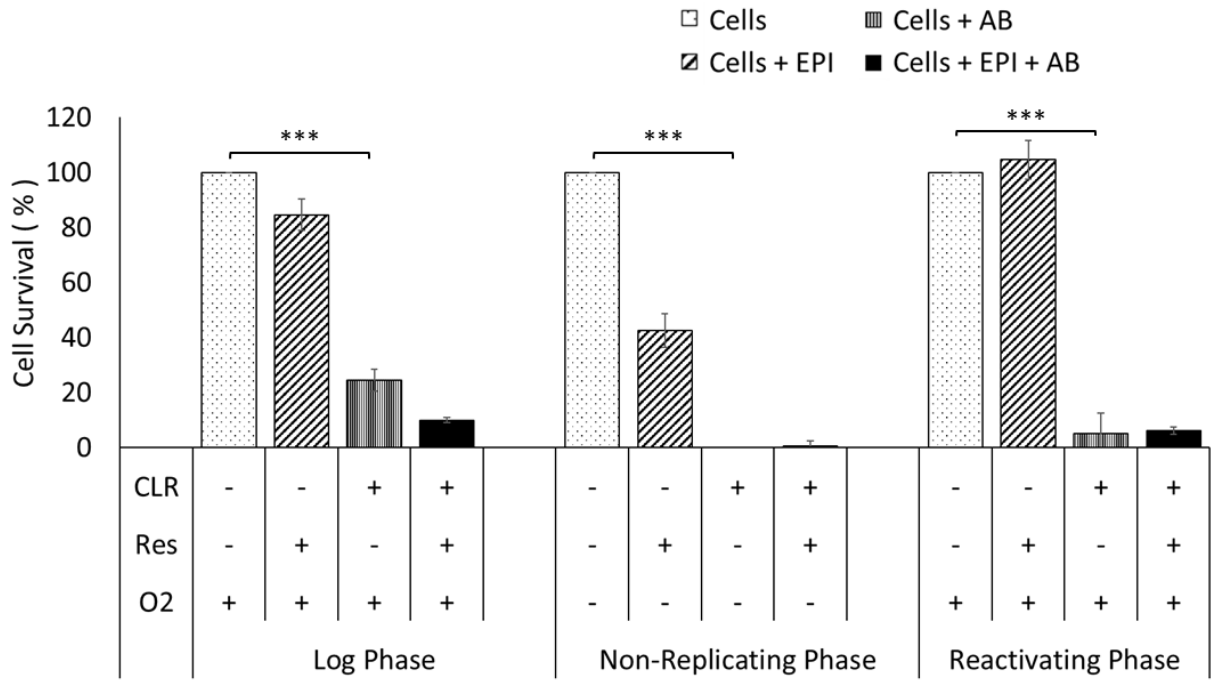

Figure 12: Reserpine inhibits the clarithromycin resistance of actively growing *M. abscessus.* *M. abscessus* cells in log-, non-replicating and reactivating phases were cultured as described under Methods and incubated in the presence (+) or absence (-) of clarithromycin (CLR) and/ or reserpine (Res). The survival of *M. abscessus* exposed to the drugs is shown relative to respective untreated controls. Percent survival of cells was measured in two independent experiments. Values shown are average ± SD of duplicate measurements from a representative experiment. ANOVA was performed followed by post-hoc Tukey's test: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

4.7 Reserpine Decreases Erythromycin Resistance of Actively Dividing *M. abscessus* and Increases Resistance with Reactivating Cells

We examined the resistance of cells in the three phases to erythromycin and analyzed the effect of the efflux pump inhibitor reserpine. We observed slightly more than 55 % of log-phase cells were resistant to erythromycin (Figure 13). The addition of reserpine to these cells caused the resistance to decrease to 26 %. Non-replicating phase cells showed negligible resistance (1.8 %) to erythromycin while reactivating phase cells showed almost 25 % resistance. The addition of reserpine to reactivating phase cells significantly increased resistance to 42 %.

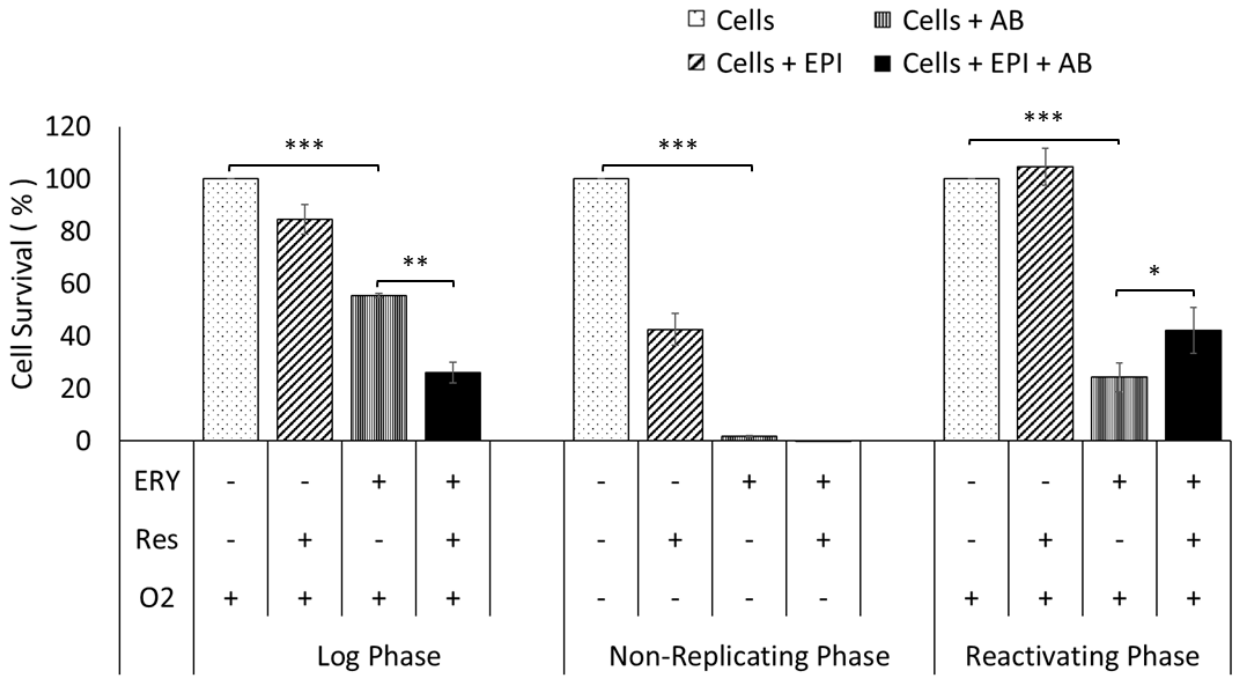

Figure 13: Reserpine in the presence of erythromycin decreases survival of actively growing *M. abscessus*. *M. abscessus* cells in log-, non-replicating and reactivating phases were cultured as described under Methods and incubated in the presence (+) or absence (-) of erythromycin (AMI) and/ or reserpine (Res). The survival of *M. abscessus* exposed to the drugs is shown relative to respective untreated controls. Percent survival of cells was measured in two independent experiments. Values shown are average ± SD of duplicate measurements from a representative experiment. ANOVA was performed followed by post-hoc Tukey's test: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Note: The following antibiotic assays were completed once. Due to the COVID-19 pandemic, repeats were abandoned.

4.8 Actively Growing *M. abscessus* Is Resistant to Amikacin and Is Not Inhibited by Verapamil

We examined the resistance of *M. abscessus* cells under the phases described above to the antibiotic amikacin and the efflux pump inhibitor verapamil. We observed that log-phase cells were resistant to amikacin and that verapamil did not significantly decrease resistance. Cells in the non-replicating and reactivating phases did not show resistance to amikacin or verapamil (Figure 14).

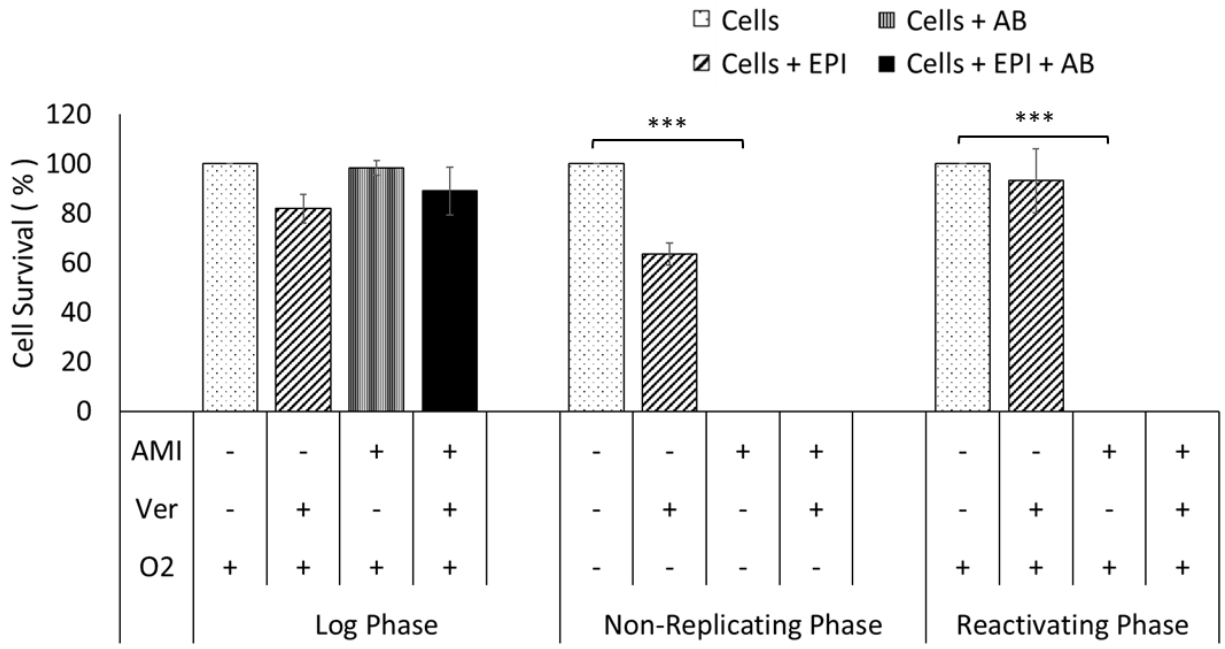

Figure 14: Verapamil does not inhibit the amikacin resistance of actively growing *M. abscessus.* *M. abscessus* cells in log-, non-replicating and reactivating phases were cultured as described under Methods and incubated in the presence (+) or absence (-) of amikacin (AMI) and/ or verapamil (Ver). The survival of *M. abscessus* exposed to the drugs is shown relative to respective untreated controls. Percent survival of cells was measured in one experiment. Values shown are average ± SD of duplicate measurements. ANOVA was performed followed by post-hoc Tukey's test: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

4.9 Actively Growing M. abscessus Is Resistant to Streptomycin and Verapamil Does Not Decrease Resistance

We examined the resistance of *M. abscessus* cells in log, non-replicating and reactivating phases to streptomycin and efflux pump inhibitor verapamil. Log-phase cells were not resistant to streptomycin (Figure 15), and verapamil did not alter resistance. Cells in the non-replicating phase and reactivating phase showed no resistance to streptomycin.

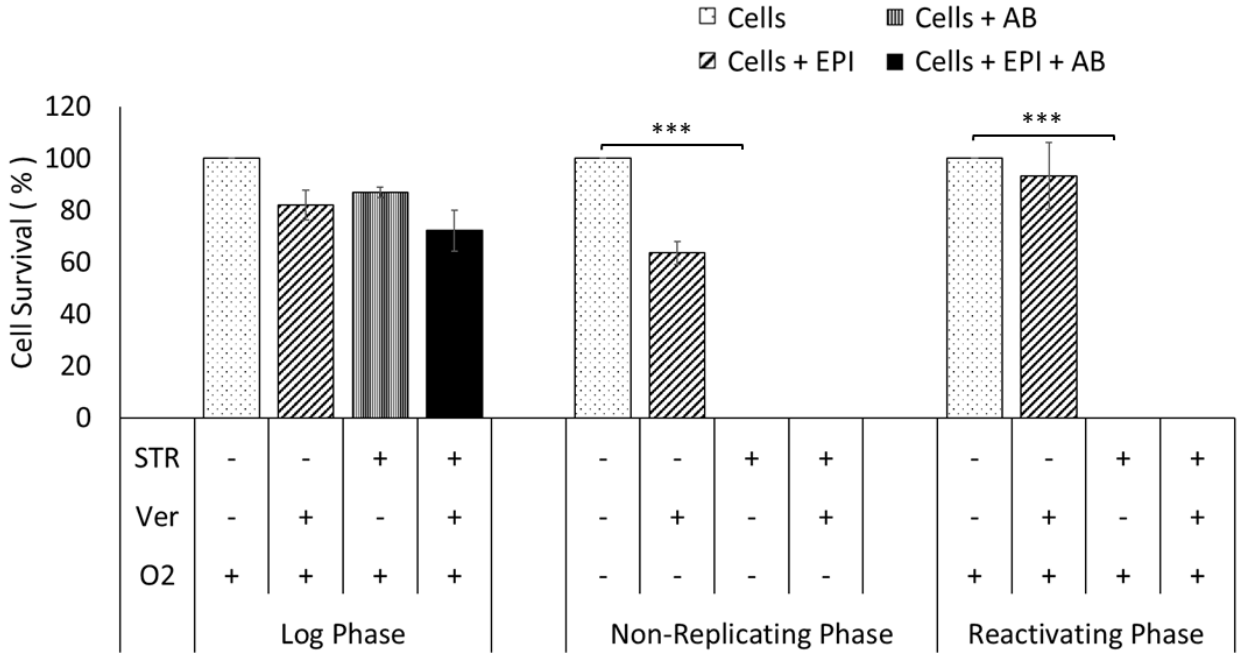

Figure 15: Verapamil does not inhibit streptomycin resistance of actively growing *M. abscessus*. *M. abscessus* cells in log-, non-replicating and reactivating phases were cultured as described under Methods and incubated in the presence (+) or absence (-) of streptomycin (STR) and/ or verapamil (Ver). The survival of *M. abscessus* exposed to the drugs is shown relative to respective untreated controls. Percent survival of cells was measured in one experiment. Values shown are average ± SD of duplicate measurements. ANOVA was performed followed by post-hoc Tukey's test: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

4.10 CCCP Does Not Decrease Amikacin Resistance of Actively Growing *M. abscessus*

We examined the resistance of log-, non-replicating and reactivating phase *M. abscessus* cells to amikacin and CCCP. Log-phase cells were completely resistant to amikacin and CCCP did not decrease resistance. Non-replicating and reactivating phase cells showed no significant tolerance to amikacin (Figure 16).

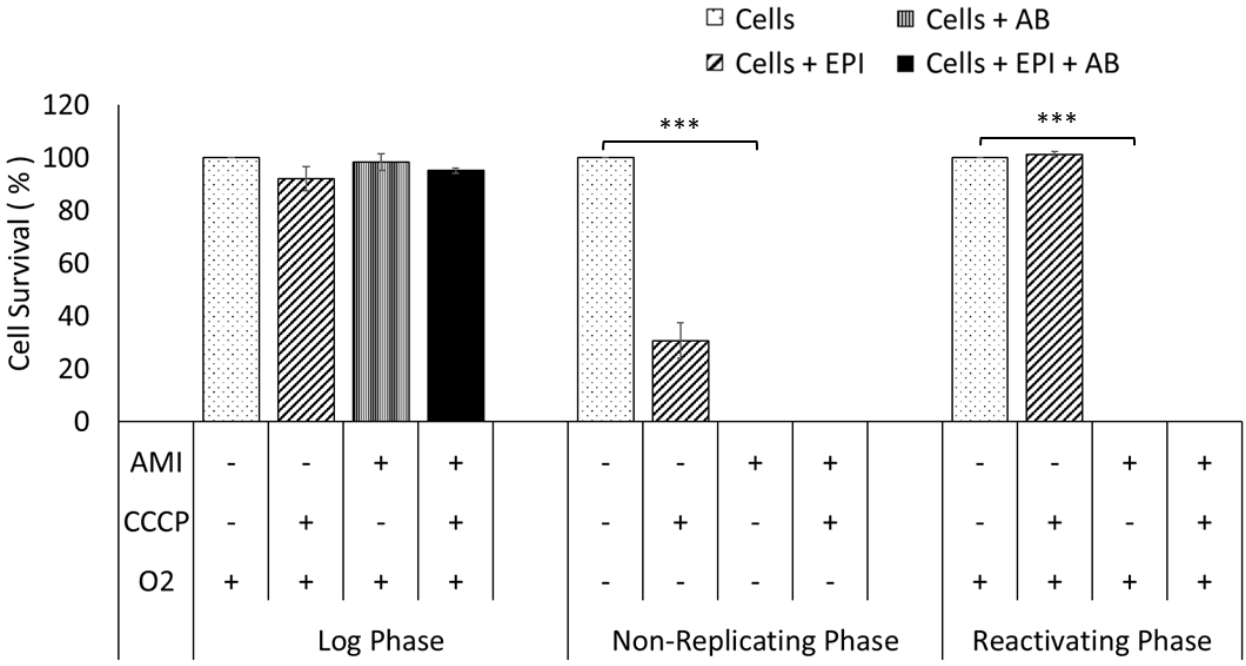

Figure 16: CCCP does not decrease amikacin resistance of actively growing *M. abscessus*. *M. abscessus* cells in log-, non-replicating and reactivating phases were cultured as described under Methods and incubated in the presence (+) or absence (-) of amikacin (AMI) and/ or CCCP. The survival of *M. abscessus* exposed to the drugs is shown relative to respective untreated controls. Percent survival of cells was measured in one experiment. Values shown are average ± SD of duplicate measurements. ANOVA was performed followed by post-hoc Tukey's test: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

4.11 The Streptomycin Resistance of Actively Growing *M. abscessus* Is Not Affected by CCCP

We examined the resistance of log-phase, non-replicating, and reactivating phase cells to streptomycin and efflux pump inhibitor CCCP. Streptomycin did not cause a significant decrease in log phase cell survival. The addition of CCCP to the antibiotic did not significantly increase resistance of log phase cells. Non-replicating and reactivating phase cells showed no significant tolerance to streptomycin or streptomycin with CCCP (Figure 17).

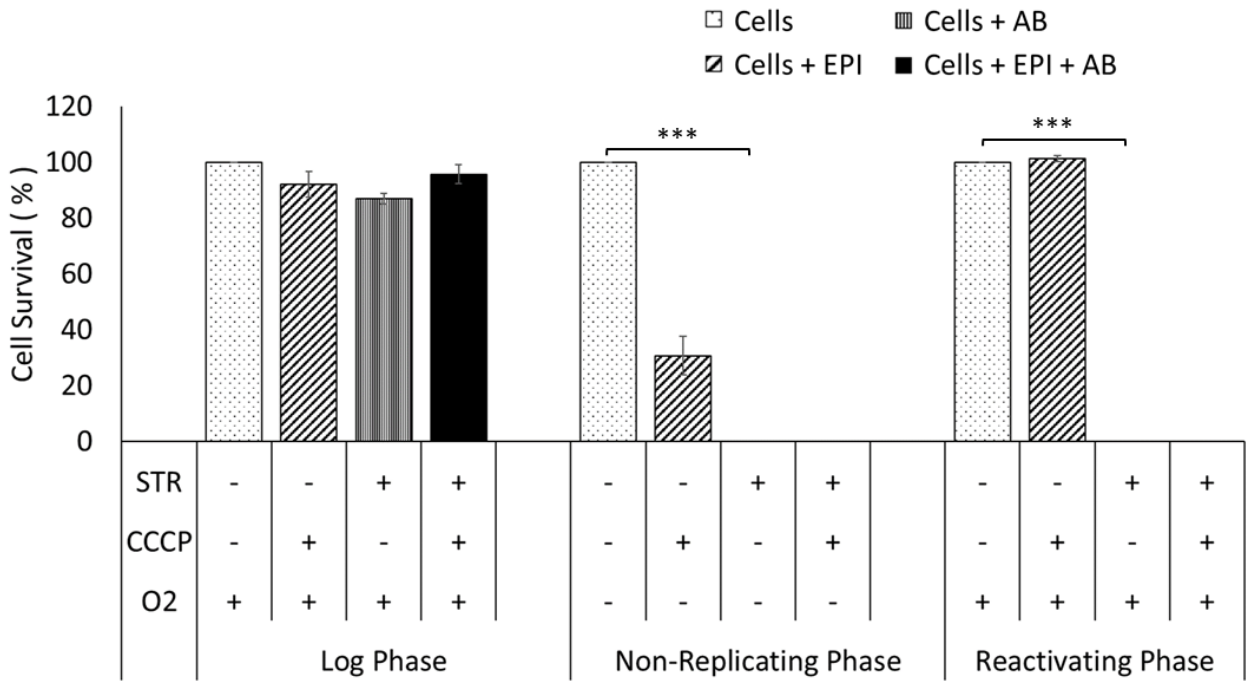

Figure 17: CCCP does not alter tolerance to streptomycin with actively growing *M. abscessus.* *M. abscessus* cells in log-, non-replicating and reactivating phases were cultured as described under Methods and incubated in the presence (+) or absence (-) of streptomycin (STR) and/ or CCCP. The survival of *M. abscessus* exposed to the drugs is shown relative to respective untreated controls. Percent survival of cells was measured in one experiment. Values shown are average ± SD of duplicate measurements. ANOVA was performed followed by post-hoc Tukey's test: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

4.12 The Amikacin Resistance of Actively Growing *M. abscessus* Is Not Inhibited by Reserpine

We examined the resistance of cells under the three phases to amikacin and tested the effect of the efflux pump inhibitor reserpine. We observed that there was no significant decrease in survival of log-phase cells with amikacin. Log-phase cells showed nearly total resistance (98.7 %) to amikacin. Non-replicating and reactivating phase cells showed no tolerance to amikacin (Figure 18).

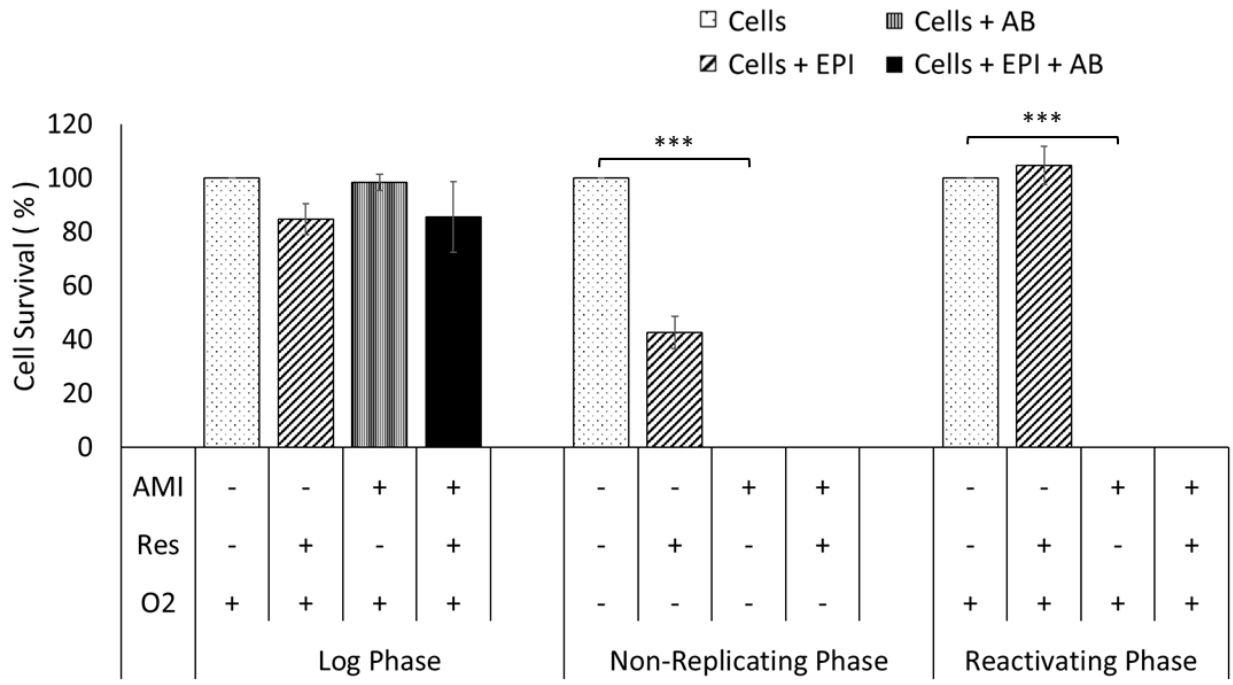

Figure 18: Reserpine in the presence of amikacin does not alter survival of actively growing *M. abscessus.* *M. abscessus* cells in log-, non-replicating and reactivating phases were cultured as described under Methods and incubated in the presence (+) or absence (-) of amikacin (AMI) and/ or reserpine (Res). The survival of *M. abscessus* exposed to the drugs is shown relative to respective untreated controls. Percent survival of cells was measured in one experiment. Values shown are average ± SD of duplicate measurements. ANOVA was performed followed by post-hoc Tukey's test: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

4.13 The Streptomycin Resistance of Actively Growing *M. abscessus* Is Inhibited by Reserpine

We examined the resistance of cells in log-phase, non-replicating phase, and reactivating phase to streptomycin and efflux pump inhibitor reserpine. We observed no significant decrease in survival of log phase cells with streptomycin. The addition of reserpine to the cells significantly decreased resistance by almost 20 %. Non-replicating and reactivating phase cells showed no significant resistance to streptomycin (Figure 19).

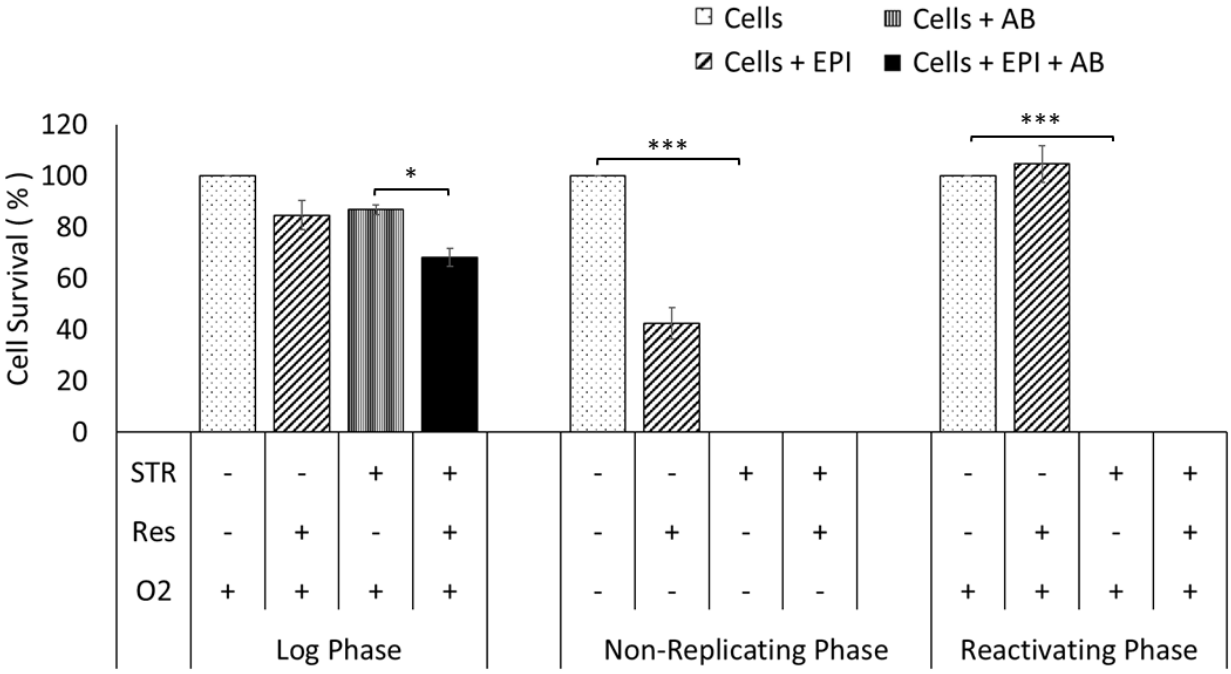

Figure 19: Reserpine in the presence of streptomycin decreases survival of actively growing *M. abscessus.* *M. abscessus* cells in log-, non-replicating and reactivating phases were cultured as described under Methods and incubated in the presence (+) or absence (-) of streptomycin (STR) and/ or reserpine (Res). The survival of *M. abscessus* exposed to the drugs is shown relative to respective untreated controls. Percent survival of cells was measured in one experiment. Values shown are average ± SD of duplicate measurements. ANOVA was performed followed by post-hoc Tukey's test: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

4.14 The Ofloxacin Resistance of Non-Replicating and Reactivating Phase *M. abscessus* Is Increased by ATP; Verapamil and Reserpine Decrease Resistance

We examined the resistance of cells in log-phase, non-replicating phase, and reactivating phase to ofloxacin and efflux pump inhibitors verapamil (Figure 20), CCCP (Figure 21), and reserpine (Figure 22). We added ATP as a carbon energy source for the cells. We observed no resistance to ofloxacin in the non-replicating and reactivating phases without ATP. The addition of ATP to the cells increased resistance significantly of both cells with antibiotic and cells with antibiotic plus inhibitor in the reactivating phase. In the non-replicating phase, ATP increased resistance to ofloxacin. Log-phase cells showed no significant difference upon addition of ATP. Verapamil and reserpine decreased ofloxacin resistance of reactivating phase cells in the presence of ATP.

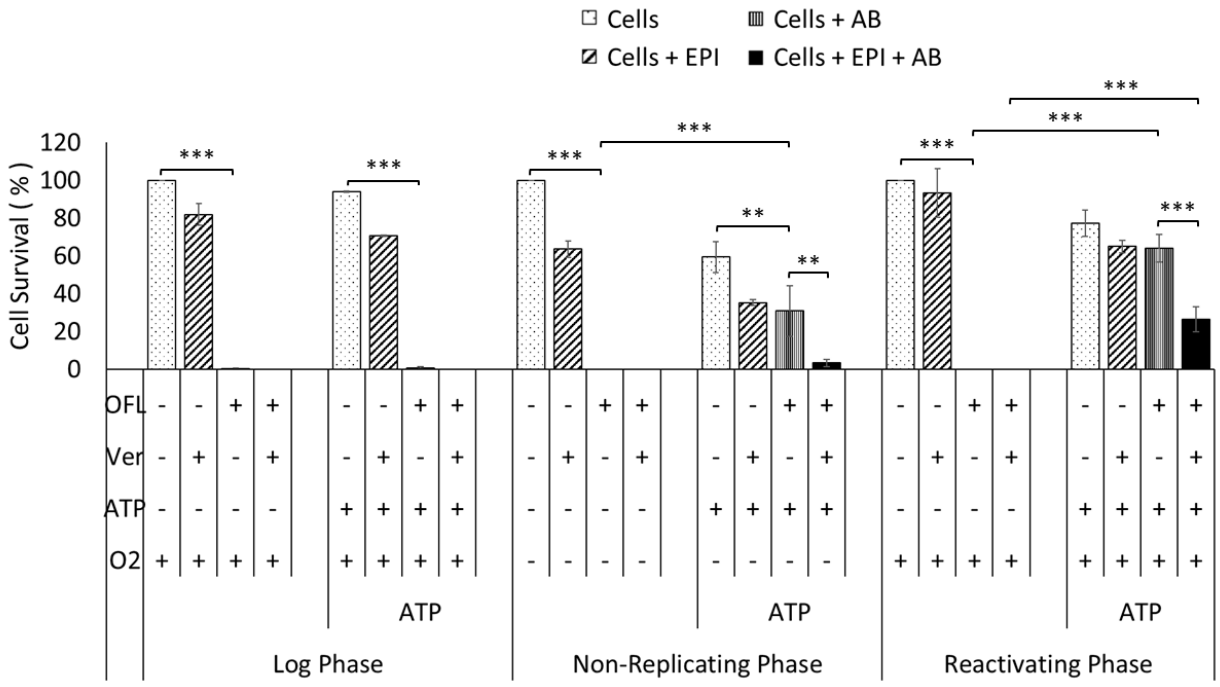

Figure 20: Ofloxacin resistance occurs in reactivating phase cells in the presence of ATP and verapamil decreases resistance. *M. abscessus* reactivating phase was cultured as described under Methods and incubated in the presence (+) or absence (-) of ofloxacin (OFL), verapamil (Ver), and/or ATP. The survival of *M. abscessus* exposed to the drugs is shown relative to respective untreated controls. Percent survival of cells was measured in one experiment. Values shown are average ± SD of duplicate measurements. ANOVA was performed followed by post-hoc Tukey's test: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

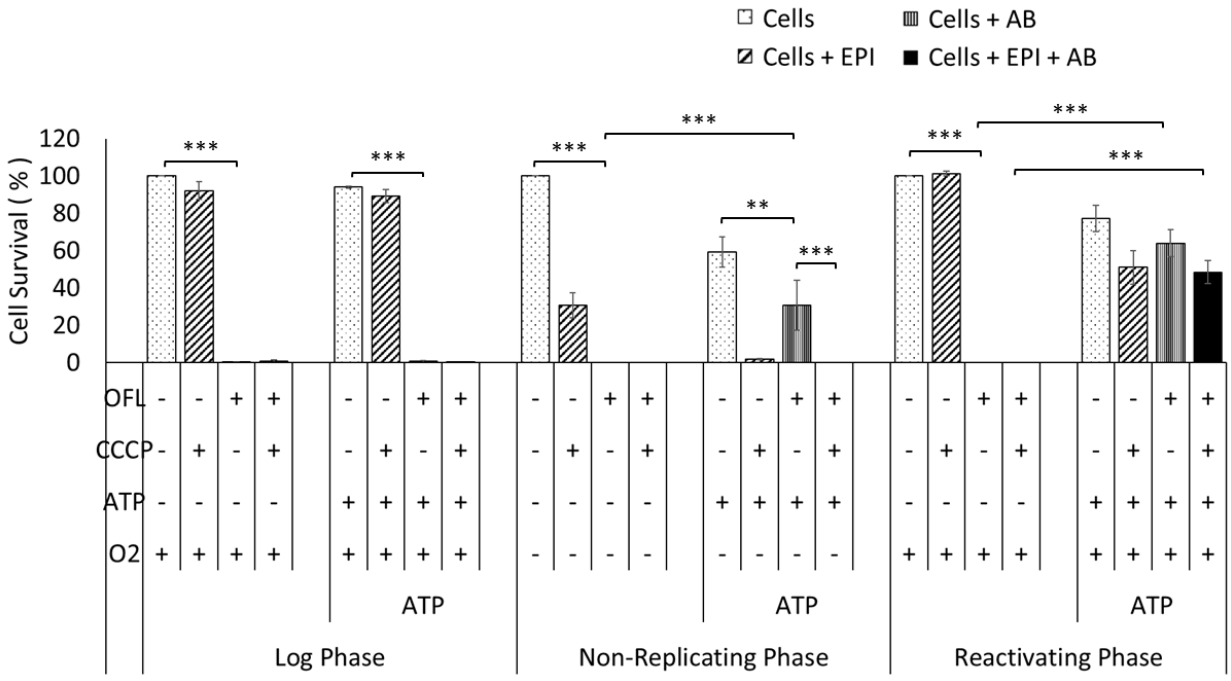

Figure 21: CCCP does not decrease ofloxacin resistance of reactivating phase cells in the presence of ATP. *M. abscessus* reactivating phase was cultured as described under Methods and incubated in the presence (+) or absence (-) of ofloxacin (OFL), CCCP, and/or ATP. The survival of *M. abscessus* exposed to the drugs is shown relative to respective untreated controls. Percent survival of cells was measured in one experiment. Values shown are average ± SD of duplicate measurements. ANOVA was performed followed by post-hoc Tukey's test: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

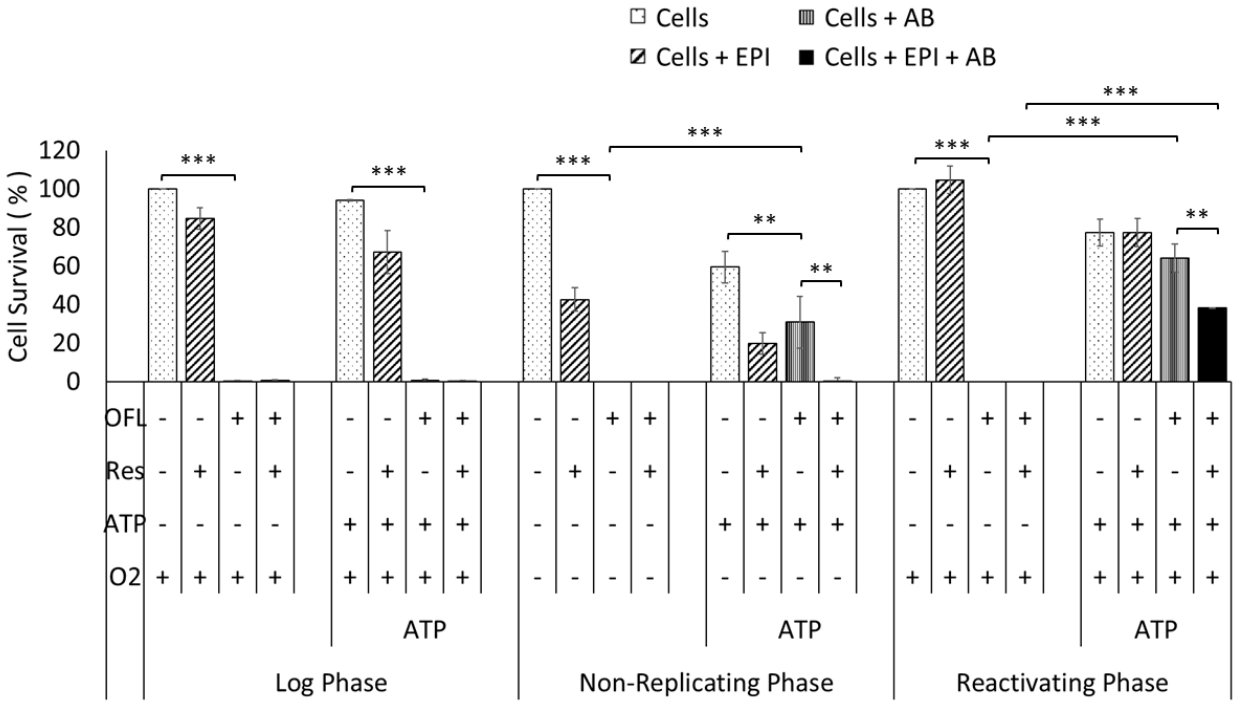

Figure 22: Reserpine decreases ofloxacin resistance of reactivating cells in the presence of ATP. *M. abscessus* reactivating phase was cultured as described under Methods and incubated in the presence (+) or absence (-) of ofloxacin (OFL), reserpine (Res), and/or ATP. The survival of *M. abscessus* exposed to the drugs is shown relative to respective untreated controls. Percent survival of cells was measured in one experiment. Values shown are ave rage ± SD of duplicate measurements. ANOVA was performed followed by post-hoc Tukey's test: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

4.15 ATP Increased Streptomycin Resistance of Reactivating Cells.

We examined the resistance of cells in log-phase, non-replicating phase, and reactivating phase to streptomycin and efflux pump inhibitors verapamil (Figure 23), CCCP (Figure 24), and reserpine (Figure 25). We added ATP as a carbon energy source for the cells. The addition of ATP to reactivating phase cells increased resistance to streptomycin. Verapamil, CCCP, and reserpine decreased this resistance.

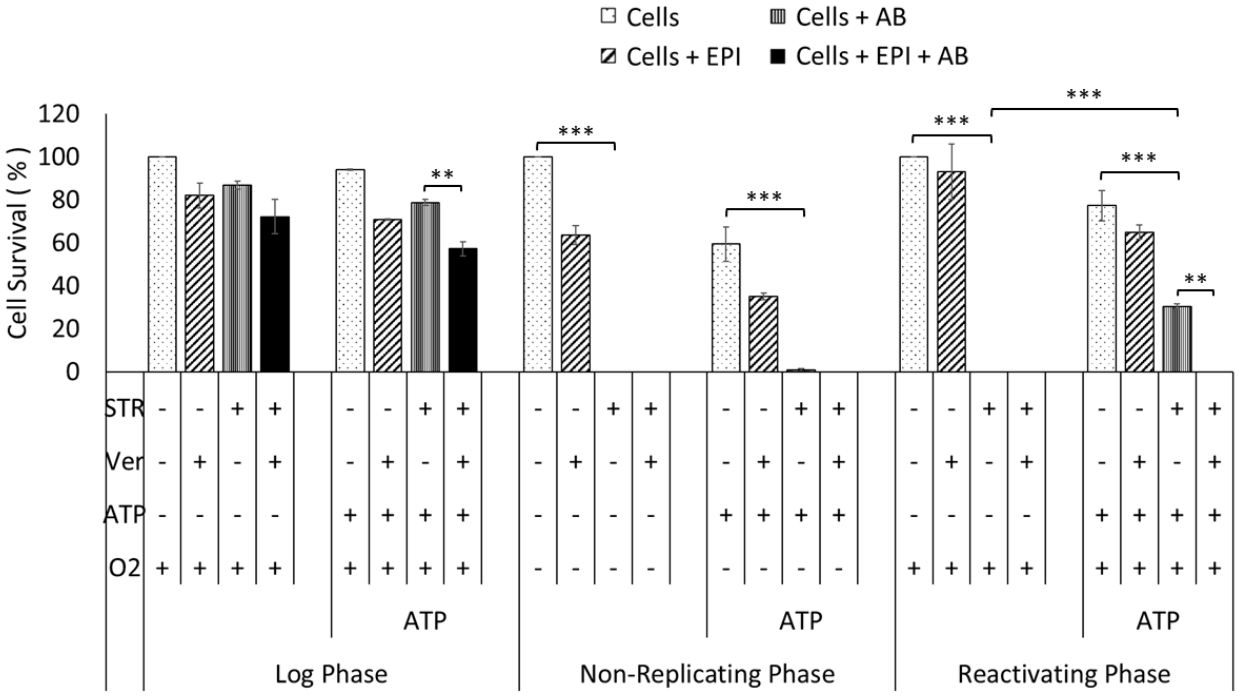

Figure 23: ATP increased streptomycin resistance of reactivating *M. abscessus*. Verapamil decreased reactivating cell resistance. *M. abscessus* cells in log- and reactivating phases were cultured as described under Methods and incubated in the presence (+) or absence (-) of streptomycin (STR), verapamil (Ver), and/or ATP. The survival of *M. abscessus* exposed to the drugs is shown relative to respective untreated controls. Percent survival of cells was measured in one experiment. Values shown are average ± SD of duplicate measurements. ANOVA was performed followed by post-hoc Tukey's test: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

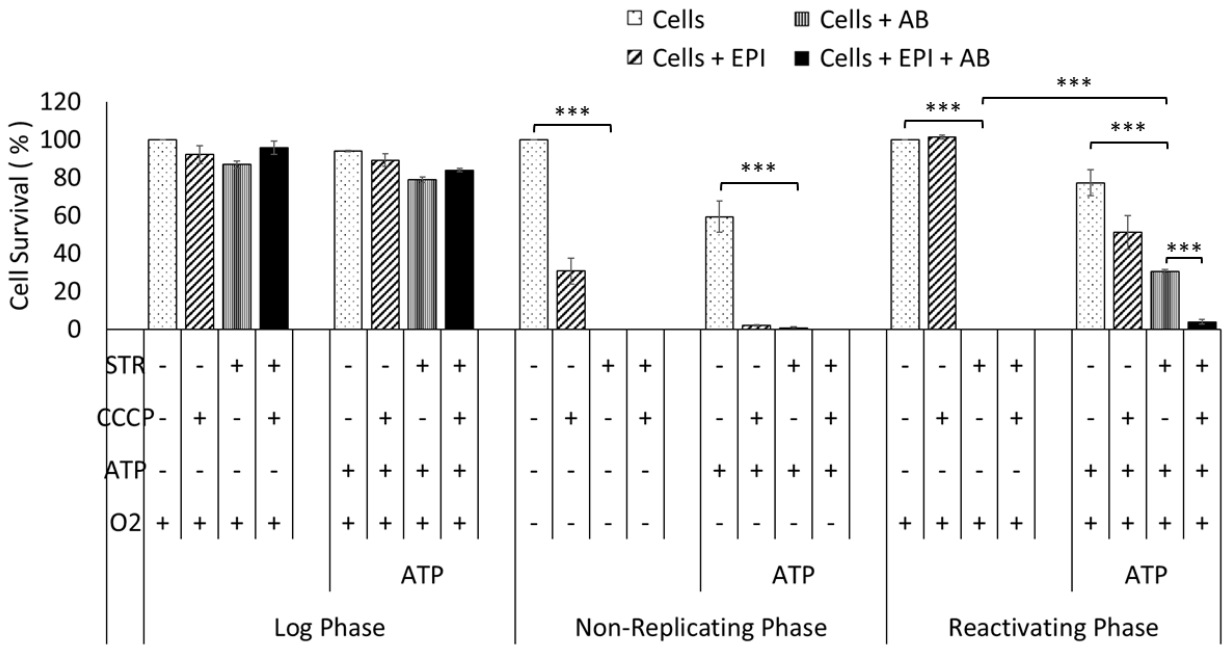

Figure 24: Streptomycin resistance of reactivating phase *M. abscessus* increases in the presence of ATP. CCCP decreased reactivating cell resistance. *M. abscessus* cells in log- and reactivating phases were cultured as described under Methods and incubated in the presence (+) or absence (-) of streptomycin (STR), CCCP, and/or ATP. The survival of *M. abscessus* exposed to the drugs is shown relative to respective untreated controls. Percent survival of cells was measured in one experiment. Values shown are average ± SD of duplicate measurements. ANOVA was performed followed by post-hoc Tukey's test: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

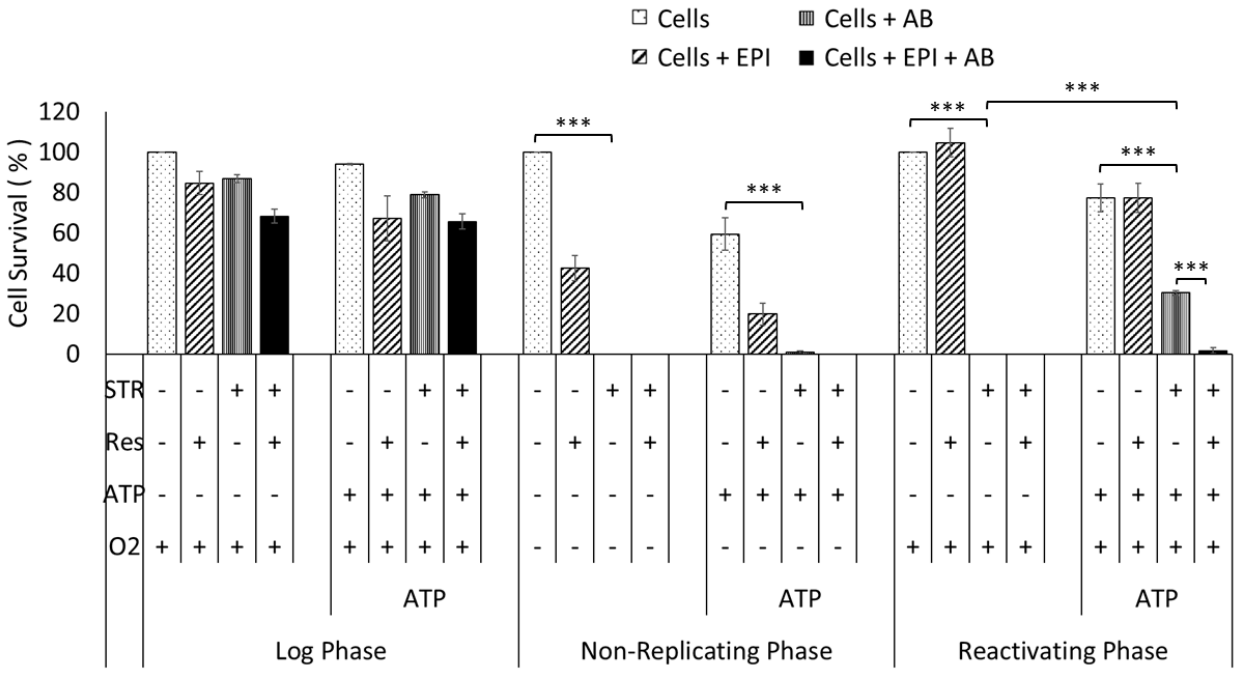

Figure 25: ATP increases streptomycin resistance in reactivating cells. Reserpine decreased reactivating cell resistance. *M. abscessus* cells in log- and reactivating phases were cultured as described under Methods and incubated in the presence (+) or absence (-) of streptomycin (STR), reserpine (Res), and/or ATP. The survival of *M. abscessus* exposed to the drugs is shown relative to respective untreated controls. Percent survival of cells was measured in one experiment. Values shown are average ± SD of duplicate measurements. ANOVA was performed followed by post-hoc Tukey's test: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

4.16 Glycerol Does Not Alter Amikacin Resistance of Actively Dividing *M. abscessus*

We examined the resistance of amikacin and the three efflux pump inhibitors in the presence of glycerol. We did not observe a significant change in resistance to actively dividing cells in the presence of amikacin when glycerol was added (Figure 26). Amikacin killed non-replicating and reactivating phase cells (not pictured) and caused no significant decrease in combination with efflux pump inhibitors in the presence of glycerol (not pictured).

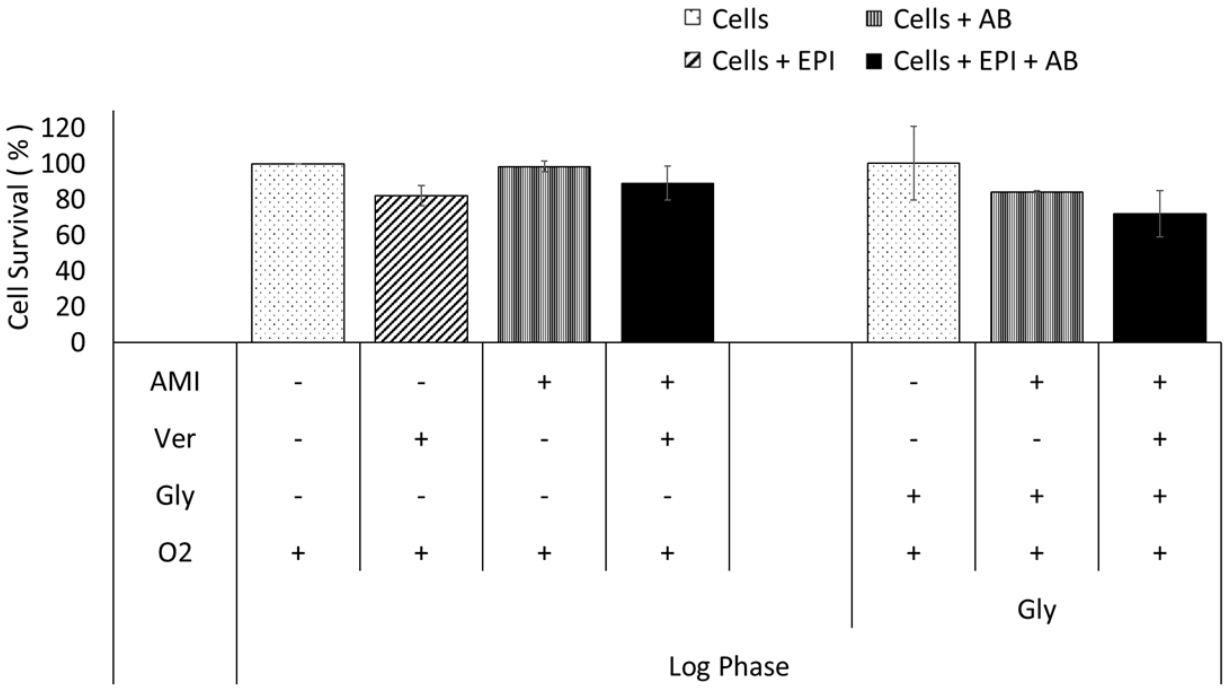

Figure 26: The addition of glycerol to actively dividing *M. abscessus* did not alter resistance to amikacin. *M. abscessus* cells in log-phase were cultured as described under Methods and incubated in the presence (+) or absence (-) of amikacin (AMI) and/or glycerol. The survival of *M. abscessus* exposed to the drugs is shown relative to respective untreated controls. Percent survival of cells was measured in one experiment. Values shown are average ± SD of duplicate measurements. ANOVA was performed followed by post-hoc Tukey's test: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

4.17 Glycerol Does Not Alter Resistance of Actively Dividing Cells to Erythromycin and CCCP

We examined resistance of erythromycin and the three efflux pump inhibitors in the presence of glycerol. We did not observe a significant change in resistance to actively dividing cells in the presence of erythromycin and CCCP when glycerol was added (Figure 27). Amikacin killed non-replicating and reactivating phase cells (not pictured) and caused no significant decrease in combination with efflux pump inhibitors in the presence of glycerol (not pictured).

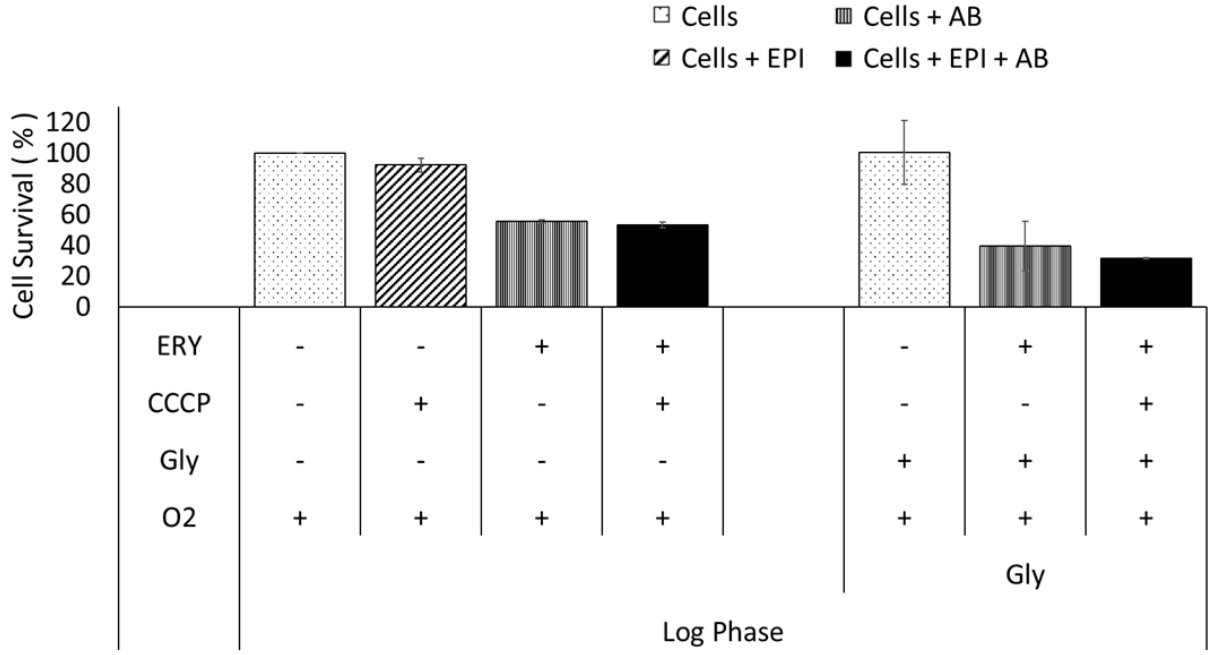

Figure 27: Glycerol did not alter erythromycin resistance when added to actively dividing *M. abscessus*. *M. abscessus* cells in log-phase were cultured as described under Methods and incubated in the presence (+) or absence (-) of erythromycin (ERY), CCCP, and/or glycerol. The survival of *M. abscessus* exposed to the drugs is shown relative to respective untreated controls. Percent survival of cells was measured in one experiment. Values shown are average ± SD of duplicate measurements. ANOVA was performed followed by post-hoc Tukey's test: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

4.18 Construction of the Mab 1415c-1414c AES and the Transformation Into Recombineering-Ready *M. abscessus*

The allelic exchange substrate (AES) consists of an up flank and down flank that are complementary to the gene of interest, and a resistance cassette in the center which makes the gene target non-functional and allows for selection of cells transformed with the AES. Figure 28 shows the PCR amplification of the three fragments that make up the AES. After performing Gibson assembly, the AES was PCR amplified (Figure 29). In parallel with AES construction, elecrocompetent *M. abscessus* was transformed with pJV53 which codes for proteins that will aid in the recombination of the AES into the mycobacterial genome. pJV53 transformed colonies were selected for using kanamycin and were PCR screened using the kanamycin cassette (Figure 30). Glycerol stocks were prepared of *M. abscessus* pJV53 and the plasmid was extracted as described in Methods and was subjected to PCR for the kanamycin cassette as a way to check that the cells were transformed (Figure 31). *M. abscessus* pJV53 cells were made electrocompetent as described in Methods and were transformed with the AES. Screening for the mutants was put on hold due to the COVID-19 pandemic.

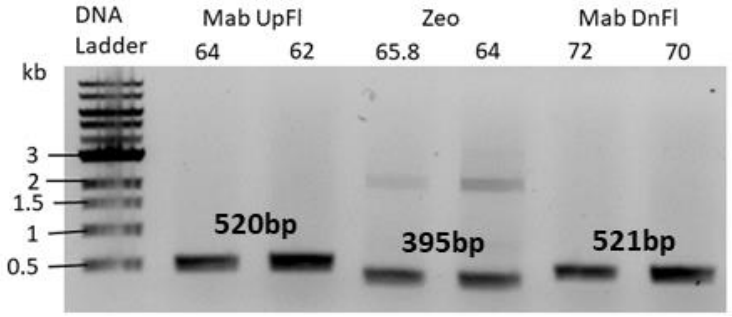

Figure 28: Amplification by PCR of MAB_1415c-1414c AES components. The overlapping DNA components for the upstream flanking region (Mab UpFL – 520 bp), bleomycin resistance cassette (Zeo – 395 bp) and downstream flanking region (Mab DnFL – 521 bp) were amplified as described under Methods. Annealing temperatures are indicated in °C above each lane.

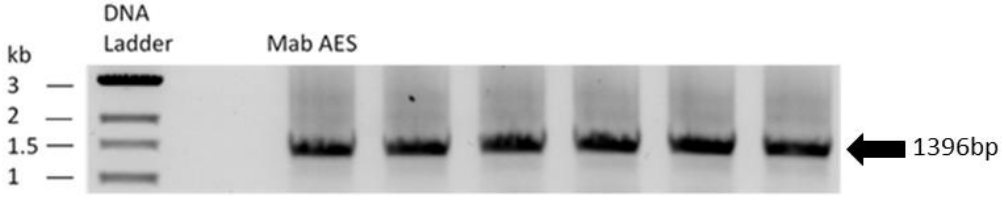

Figure 29: Large-scale PCR amplification of Mab AES. The 1396 bp AES was amplified using the Q5 high-fidelity polymerase with the HiFi DNA assembly product as template as described under Methods.

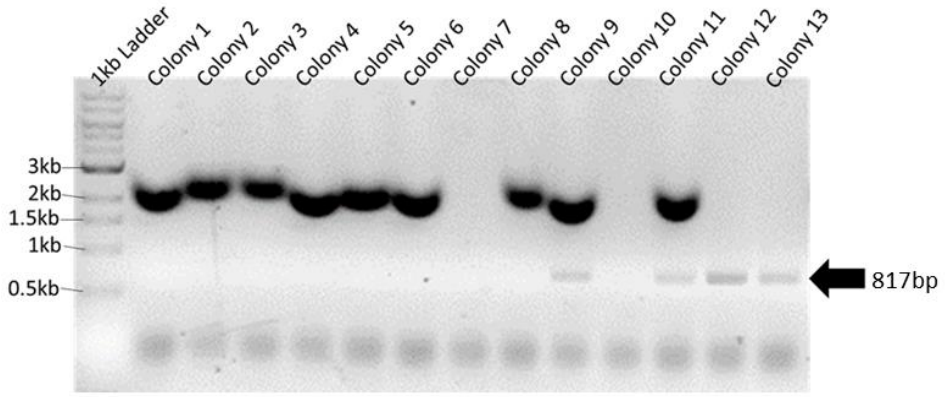

Figure 30: Screening *M. abscessus* colonies for plasmid pJV53. Electrocompetent *M. abscessus* was transformed with pJV53 as described in Methods. The PCR was carried out using One-Taq polymerase to screen for the 817bp kanamycin cassette in pJV53.

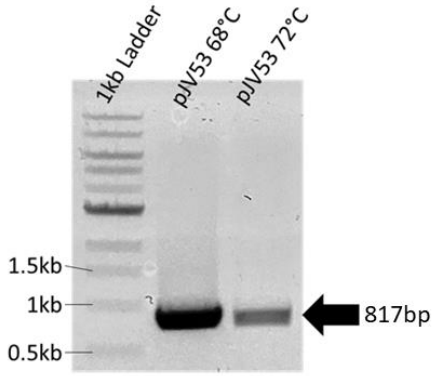

Figure 31: PCR amplification of pJV53 following plasmid extraction from glycerol stock. The PCR used I-5 polymerase to amplify the 817bp kanamycin cassette found on pJV53

CHAPTER 5. DISCUSSION

M. abscessus is known to cause pulmonary disease similar to tuberculosis in which granulomas entrap the bacteria engulfed in macrophages (Johansen et al., 2020). In the granuloma, *M. tuberculosis* encounters low oxygen, high carbon dioxide, low nutrient, and acidic conditions which make it enter a non-replicating state (Deb et al., 2009). It was hypothesized that *M. abscessus* also enters a non-replicating state due to the similarity in pulmonary infections. It was also hypothesized that under these stress conditions, *M. abscessus* resistance to antibiotics and efflux pump inhibitors would be altered, explaining the low treatment success for disease.

It was revealed that *M. abscessus* reaches a non-replicating phase by the fifth day under combination stress in our model (Figure 7). After CFU-plating it is evident that *M. abscessus* cells stop replicating but are not killed by the combination of stresses described in methods. *M. tuberculosis* in the non-replicating phase enters a dormancy like state where it is phenotypically resistant to antibiotics. Inside this dormancy state, *M. tuberculosis* imports fatty acids from host triacylglycerol to store its own triacylglycerol for an energy source while in dormancy (Daniel et al., 2011). The dormant state entered by *M. tuberculosis* is reversible, as latent infections turn into active infections once there is a decline in the immune system. In this dormant state, the bacteria are phenotypically drug-resistant because most available drugs target dividing cells (Batyrshina et al., 2019). Macrolides such as clarithromycin and erythromycin are bacteriostatic, inhibiting protein synthesis; they will inhibit the bacteria from multiplying. Tubercle bacteria in the non-replicating phase already have stopped dividing so they would be phenotypically resistant to macrolides (Dinos, 2017). Aminoglycosides, such as amikacin and streptomycin, as well as ofloxacin, a fluoroquinolone, are bactericidal, inhibiting protein synthesis and DNA replication, respectively (Becker et al., 2013; Fan et al., 2018).

When under stress conditions such as low nutrients, bacteria enter a stationary phase. Transition into a stationary phase affects expression of genes and proteins within the cell. Protein Y interacts with the 70S ribosome in bacteria, preventing subunit dissociation under low magnesium conditions created under low nutrient environments. Under favorable growth conditions, Protein Y dissociates from the ribosome, allowing for the continuation of translation. Another protein upregulated during stationary phase by low nutrients is the energy-dependent translational throttle A (EttA) protein. The EttA protein is an ABC transporter protein that gives

cells higher fitness when transitioning back to an actively dividing state when given nutrients. EttA binds to the ribosome and only dissociates allowing for translation when there is a high enough level of ATP in the cell These proteins aid the bacterial cell in survival in a non-replicating state (Starosta et al., 2014).

Although OD_{600} is often used to understand bacterial growth, it does not take into account dead cells or cellular debris. The XTT/menadione assay allowed for an accurate quantitative measurement of viable cells. Menadione caused viable cells to produce a superoxide which reduced the yellow tetrazolium salt XTT into formazan which is an orange color. The formazan color is read at OD_{470}. This provided a linear relationship between the number of viable cells and the orange pigment that can be read at a specific optical density value (Koban et al., 2012; Singh et al., 2011).

We also found that the efflux pump inhibitor verapamil significantly decreases log phase survival when used in combination with macrolides clarithromycin and erythromycin, but did not decrease resistance of macrolides in the non-replicating phase or reactivating phase. The efflux pump inhibitor verapamil is a calcium channel blocker commonly used to treat cardiac disorders and migraines. In prokaryotes, it interferes with the proton motive force, indirectly inhibiting ABC transporters. Verapamil has been used with *M. tuberculosis* and has been found to have an inhibitory effect on mycobacterial efflux pumps. When added to macrophages infected with *M. tuberculosis*, there was an increase in intracellular killing of the tubercle bacteria by the macrophages (Pule et al., 2016). Verapamil has been used in combination with antibiotics against *M. abscessus, in vitro* and in macrophages, and was found to improve the efficacy of the antibiotics (Ramis et al., 2019; Vianna et al., 2019; Viljoen et al., 2019). In *M. tuberculosis*, verapamil is known to inhibit ABC transporter-mediated efflux of macrolides such as clarithromycin and erythromycin as well as aminoglycosides such as amikacin and streptomycin (Pule et al., 2016). In this study, verapamil significantly decreased the survival of log-phase cells in the presence of macrolides clarithromycin and erythromycin as expected (Figures 8 and 9). Clarithromycin killed both non-replicating and reactivating phase cells; there was no significant resistance to the antibiotic. Combination stressed cells did show resistance to erythromycin. However, there was not a decrease in survival upon the addition of verapamil, suggesting that the efflux pump inhibitor may only have an inhibitory effect on log-phase cells when used in combination with clarithromycin or erythromycin. The non-replicating phase cells were nearly or completely killed

by erythromycin and clarithromycin, respectively, so the effect of verapamil can't be concluded. Macrolides are bacteriostatic protein synthesis inhibitors which prevent bacteria from multiplying (Dinos, 2017). Interestingly, the macrolides killed non-replicating cells the best, despite its mode of action. It would be expected that since non-replicating cells are not dividing, macrolides would have little effect. However, we are seeing the opposite, which means there may be an unknown mechanism which allows for macrolides to inhibit survival of already non-replicating cells.

When verapamil was used in combination with aminoglycosides amikacin and streptomycin, cells only survived with either drug when in log phase (Figures 14 and 15). Aminoglycosides are bactericidal, inhibiting protein synthesis by blocking the A site in translation (Becker et al., 2013). Log-phase cell survival did not decrease in the presence of amikacin or streptomycin at the concentrations reported, and the addition of verapamil had no effect as well. The lack of survival in the combination stressed phases suggests that amikacin and streptomycin do a better job of decreasing survival in combination stressed cells. Verapamil had no significant effect on log-phase cells which suggests that the efflux pump inhibitor does not inhibit efflux of amikacin or streptomycin.

CCCP was not found to decrease resistance to antibiotics in actively dividing cells or in reactivating phase cells. CCCP is a protonophore, which inhibits efflux pumps by reducing the transmembrane potential. In *M. tuberculosis*, CCCP blocks efflux from the MFS superfamily which is known to export streptomycin (Figure 3). CCCP also indirectly acts upon the ABC superfamily by inhibiting oxidative phosphorylation, inhibiting ATP synthase. Without ATP synthesis, the ABC transporters can't export drugs such as aminoglycosides and macrolides described earlier (Pule et al., 2016). Despite being known to inhibit ABC transporters and disrupt transmembrane potential in *M. tuberculosis*, we show that CCCP does not cause a significant effect when used in combination with macrolides (clarithromycin and erythromycin) or aminoglycosides (amikacin and streptomycin). Clarithromycin, amikacin, and streptomycin all inhibited survival of combination stressed cells, so the effect of CCCP with these antibiotics to stressed cells can't be determined. However, with these antibiotics, the addition of CCCP to actively dividing cells had no significant difference. This suggests CCCP has no effect on replicating cells at the concentration used. With erythromycin, which did not completely inhibit cell survival for stressed cells, the addition of CCCP still had no significant difference for actively replicating or non-replicating cells. One study using the same concentration of CCCP with actively replicating *M. abscessus* and

several antibiotics including clarithromycin and amikacin, showed that CCCP increases the inhibitory effect of antibiotics (Ramis et al., 2019). Our results do not show a significant difference.

We found that reserpine caused a significant decrease in actively dividing cells when used in combination with erythromycin or streptomycin. The naturally occurring efflux pump inhibitor, reserpine, is isolated from the roots of the plant *Rauwolfia vomitoria*. Reserpine irreversibly blocks uptake of dopamine in synaptic vesicles and is therefore used to treat psychiatric disorders at low doses. It blocks the RND superfamily by interacting with amino acids in the transporters. The RND family is not known to be involved in the efflux of macrolides or aminoglycosides in *M. tuberculosis* (Pule et al., 2016). One study has used reserpine in combination with an antibiotic to measure the synergistic effect; it was observed reserpine had a synergistic effect when in combination with bedaquiline and clofazimine, but at a lower extent than with verapamil (Viljoen et al., 2019). Despite not being known to inhibit efflux of macrolides or aminoglycosides, we found that reserpine inhibited survival of actively dividing cells when used with an antibiotic of each class (Figures 12, 13, and 19). Actively replicating cell survival decreased with the use of macrolides. Reserpine may be acting directly or indirectly with the ABC superfamily which is known to export macrolides in *M. tuberculosis*. There may also be a synergistic effect of reserpine with these antibiotics that are not related to the efflux pumps. Reserpine also decreased resistance to ofloxacin (Figure 22) and streptomycin (Figure 25) only in the reactivating phase when ATP was present.

The macrolides and aminoglycosides were able to inhibit survival of stressed cells to a higher degree than actively dividing cells (Figures 8-19). It is likely that while under a combination of stresses, the cells are at their most vulnerable and are easier to be killed. However, literature shows that smooth cells can switch morphotype to become rough phenotypically, which are more hydrophobic and better at forming biofilms, which is an advantageous trait in regards to being able to colonize and remain in the human body (Johansen et al., 2020). It is plausible that under our model, the phenotypically smooth cells had not had sufficient time to transition to the rough morphotype or that the transition to the rough morphotype was not favored as the cells were forced to live in the combination stressed environment unlike in the human body where the rough corded cells can burst through the granuloma. Treatment very early on in the reactivating stage or in the non-replicating stage could be ideal to treat current infection before the rough morphotype has the chance to arise. The increase of cell survival with erythromycin in the reactivating phase can be

explained by the nature of the macrolide. Being bacteriostatic, bacterial cells are not being killed, allowing for cell division if the concentration of antibiotic is not high enough. The reason this is not seen with the macrolide clarithromycin could be based on the percent decrease of survival the antibiotic causes. Clarithromycin caused more of a decrease in survival of log-phase cells than erythromycin, and a decrease in clarithromycin concentration could lead to a higher survival when under combination stress.

Interestingly, as seen in Figures 20-22, reactivating-phase cells were able to survive ofloxacin when ATP was present. In this study, reactivating-phase cells mimic the breakdown of granulomas and the exposure of the bacteria to oxygen. The addition of ATP mimics a resupply of energy to the bacteria once the granuloma breaks down. This suggests that the bacteria may be able to survive ofloxacin treatment during reactivation which is important to know when trying to treat the different stages of *M. abscessus* disease. It is also important to note that even in the presence of all three efflux pump inhibitors tested, one-quarter to one-half of cells were able to survive ofloxacin. Similarly, reactivating cells are able to survive streptomycin in the presence of ATP. All three efflux pump inhibitors tested decreased this resistance. Streptomycin in combination with an EPI may be beneficial in treating reactivating phase infection such as when the bacteria break out of the granuloma.

Glycerol did not alter resistance to antibiotics in the log phase, as seen in Figures 26 and 27. This could potentially mean that glycerol is not being used by the bacteria as an energy source as it appears to do with ATP. In *M. tuberculosis*, the bacterium takes up fatty acids from host triacylglycerol and not the glycerol backbone (Daniel et al., 2011). *M. abscessus* may follow this same mechanism.

We hypothesized that stressed cells may show increased antibiotic tolerance compared to actively growing cells, however, we saw the opposite. Both non-replicating and reactivating cells had less resistance than actively growing cells. One possible reason for this is the formation of stress granules. Stress granules consist of non-translating messenger ribonucleoproteins (mRNPs) which are stalled in translation initiation. In otherwise healthy cells, these complexes are beneficial as they modulate the stress response, viral infection, and signaling pathways (Figure 32). Constant or incorrect stress granule formation can be bad for the cell, leading to neurodegenerative disease and cancer in eukaryotic cells (Protter et al., 2016).

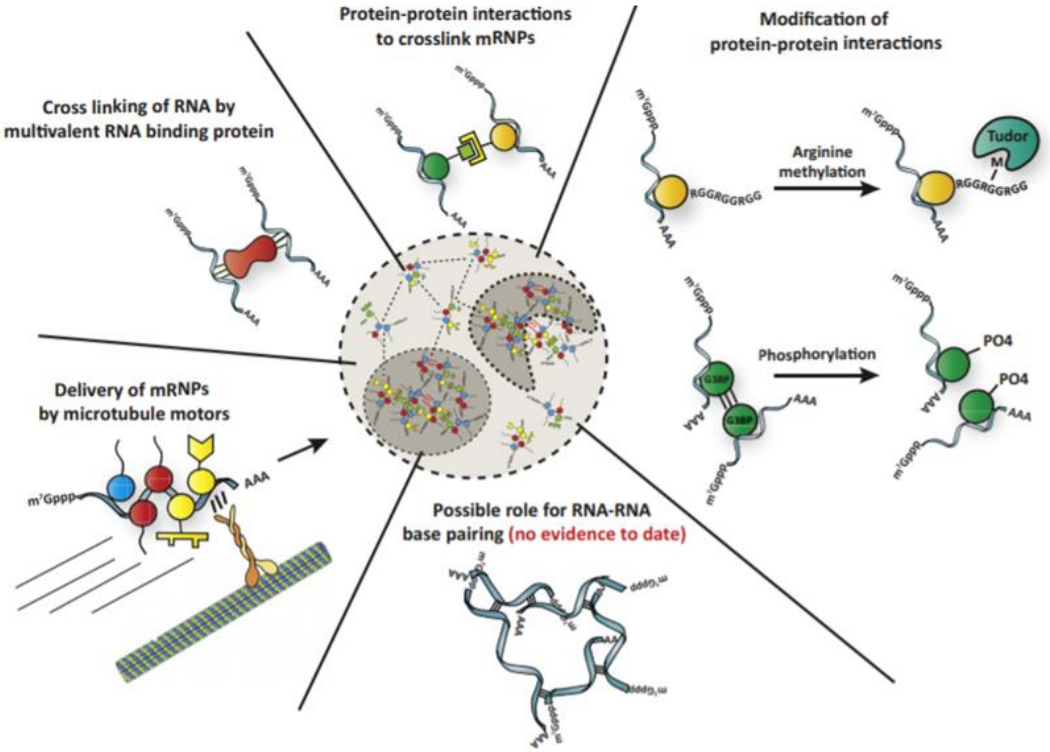

Figure 32: Stress granules contain stalled translational complexes with ribosomes. Stress granules in eukaryotes are known to carry out beneficial roles which are noted in the image (Protter et al., 2016).

Ribosomes are also known to become stalled in bacteria as well. In bacteria, ribosomes stall when there are problems with the mRNA sequence such as a missing stop codon. Bacteria use approximately 50 % of their energy to produce proteins and accumulations of these stalled complexes can be toxic to bacteria. Over 99 % of bacteria encode sequences for transfer-messenger RNA (tmRNA) and small protein B (SmpB) which mimic tRNA, bind with alanine, and interact with the stalled ribosome allowing for translation to resume. The nascent polypeptide and the mRNA that caused the stall to occur during translation are then signaled for destruction. In the absence of tmRNA, there are two backup mechanisms that will free the stalled ribosomes (Buskirk et al., 2017; Keiler, 2015). If too many of these stalled complexes accumulate in bacteria, the cells suffer from a lack of available ribosomes (Buskirk et al., 2017). We postulate that when *M. abscessus* encounters the multiple stresses in our experimental conditions, the stalled translational complexes that formed were probably not completely rescued by the three mechanisms mentioned above. The non-replicating cells that lack access to oxygen and nutrients and are in a low pH environment may have experienced increased sensitivity to antibiotics as the initiation complexes

stalled, especially to the antibiotics that inhibit the ribosome from functioning, despite efforts that may have been made to rescue the ribosomes.

Similarly, reactivating cells may have experienced a shortage of available ribosomes when they were exposed to oxygen and as the cells tried to replicate the antibiotics that interfere with ribosome function inhibited replication. Reactivating cells survived with ofloxacin and streptomycin in the presence of ATP (Figures 20-25). It is possible that reactivating phase cells have less stalled complexes as the stressors begin to be lessened and the presence of ATP gives a boost of energy to the cell to fix the stalled complexes and return to its actively dividing state. Another reason that may explain the low survival of stressed cells to antibiotics involve the 70S ribosome. In bacteria, ribosome protection proteins bind to ribosomes, displacing tetracycline antibiotics. Although tetracyclines were not tested in this study, it is possible there are similar resistance mechanisms to the antibiotics tested that involve proteins that bind to the ribosome and displace or deter antibiotics (Starosta et al., 2014).

Our data suggests that different drug combinations may be needed at different stages of *M. abscessus* disease. Actively dividing cells such as in immunocompromised SSTI infections may be best treated with macrolides with verapamil, erythromycin with reserpine, or ofloxacin. All drug combinations suggest to be effective in treating latent infection in the granuloma. Reactivating cells after emergence from the granuloma may be best treated with all combinations tested, except for ofloxacin or streptomycin alone, and erythromycin with reserpine.

This study allows for examination of *M. abscessus in vitro,* mimicking the macrophage environment, with the effect of efflux pump inhibitors and antibiotics. Until now there has not been a study that examines the effects of antibiotics and efflux pump inhibitors under a stress combination that replicates the macrophage environment experienced by tubercle bacteria once they are phagocytized. With current treatment for *M. abscessus* being ineffective, a novel combination treatment is needed. Before this can happen, it is crucial to understand how *M. abscessus* may interact while inside the macrophage under non-replicating conditions as well as what it may experience while emerging from the granuloma.

CHAPTER 6. FUTURE RESEARCH

The potential involvement of stress granules in the observed low levels of antibiotic resistance in non-replicating *M. abscessus* needs to be investigated further. Western blot and microscopy could be the main tools used to test for stress granule formation in *M. abscessus*. Current research on stress granules have been done on cells such as mammalian cells and yeast cells. In one study, stress granule cores are purified by lysing followed by a series of centrifugation. After centrifugation, the stress granules were affinity purified using antibodies and beads. This could be done, but would require knowledge on what antibodies or type of beads to use. After purification, they separated protein by mass using non-denaturing polyacrylamide gel and did a mass spectrometry analysis as well which fragments proteins and allows for identification. Microscopy was also completed using probes that would bind to the stress granules (Jain et al., 2016). With modification of these techniques, the three phases in this study could be tested for stress granule formation. The Western blot would show what proteins are upregulated or downregulated when stress was applied to the cell or removed from the cell and could be compared back to the actively dividing control. In addition, different time points within each stressed phase could be examined to see the fluctuation in protein synthesis and to see which proteins are produced more quickly in response to stress. Such studies could potentially increase our understanding of the physiological mechanisms of antibiotic resistance in *M. abscessus*.

Previous studies have demonstrated promise in using various efflux pump inhibitors in combination with various antibiotics for decreased survival of actively growing *Mycobacterium abscessus* (Ramis et al., 2019; Vianna et al., 2019; Viljoen et al., 2019). With our current data stressed cell survival was significantly decreased in the presence of antibiotics, efflux pump inhibitors did not cause a significant difference to stressed cells, and reactivating-phase cells had a higher percent survival compared to non-replicating phase cells. We have not examined what phenotype is present in the reactivating phase. It is plausible that a switch to the rough phenotype would have a higher resistance profile compared to if it were in the smooth morphotype. It has been shown that latent *Mycobacterium tuberculosis* use host derived fatty acids from triacylglycerol as an energy source while in dormancy (Daniel et al., 2011). Future studies include supplementing stressed cells with oleic acid as a fatty acid energy source to see if the cells develop more tolerance to treatment. Further experiments with ATP need repeated as well for significance

and to better understand how the bacteria may act under antibiotic pressure in different disease stages. For mutant construction, the double knock-out mutant needs to be obtained before further experiments are carried out.

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