MICROBIOLOGICAL AND BIOCHEMICAL INVESTIGATIONS OF EFFLUX AND LIPID BIOSYNTHESIS IN MYCOBACTERIUM ABSCESSUS BIOFILMS

by

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A Thesis

Submitted to the Faculty of Purdue University

In Partial Fulfillment of the Requirements for the degree of

Master of Science

Department of Biology at Purdue Fort Wayne

Fort Wayne, Indiana

May 2021

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To everyone who has encouraged and supported me on this endeavor

ACKNOWLEDGMENTS

I would first like to thank Dr. Jaiyanth Daniel. This wouldn't have been possible without you. You have been an amazing teacher to me in the classroom, research, and with writing. Thank you for your endless guidance and patience while working with me. I will forever remember and make use of what you have taught me.

To all my lab mates, both graduate and undergraduate, your presence has been invaluable during this time. The time we spent together, and our discussions were great learning experiences and will be remembered.

I would like to thank Dr. Soule and Dr. Palu for serving on my committee and offering their guidance and support. I have learned much from the both of you during my time at Purdue Fort Wayne. I would also like to thank the Biology department, your support, insight, and friendship. To the other graduate students in the department, your encouragement and advice has been invaluable.

To my family, immediate and extended, thank you for your encouragement and support.

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ABBREVIATIONS

CCCP = Carbonyl cyanide m-chlorophenylhydrazone

DTT = Dithiothreitol

FA = Fatty acid

GPL = Glycopeptidolipid

LOS = Lipooligosaccharides

PIM = Phosphatidylinositol mannnosides

PGL = Phenolic glycolipids

TAG = Triacylglycerol

TM = Trehalose mycolates

VER = Verapamil

ABSTRACT

Mycobacterium abscessus is a nontuberculous mycobacterium found in the environment that is becoming an emerging infectious pathogen capable of causing numerous types of infections. It is more antibiotic resistant than *Mycobacterium tuberculosis* and is becoming more prevalent in developed nations. Current treatments are not standardized and have poor success records and there is no definitive method in properly treating these infections. We tested an *in vitro* model that mimics the reducing environment that *M. abscessus* experiences during an infection by subjecting it to thiol-reductive stress. We observed that thiol reductive stress stabilized biofilms formed by *M. abscessus*. We found that *M. abscessus* in biofilms became tolerant to the antibiotics: clarithromycin, amikacin, and streptomycin. We postulated that efflux pumps might be involved in transport of the precursors of lipids associated with biofilm formation. Therefore, we investigated whether the efflux pump inhibitors affected biofilm formation and found that verapamil, CCCP and reserpine inhibited the formation of biofilms. We investigated the biosynthesis of lipids during biofilm formation by metabolic radiolabeling with ^{14}C-acetate, the building block of fatty acids. We found that the biosynthesis of several phospholipids were elevated during biofilm formation and that the efflux pump inhibitor verapamil and exogenous fatty acids inhibited their biosynthesis. Further studies are needed to understand the roles of these lipids in biofilm formation.

CHAPTER 1. INTRODUCTION

1.1 History and Pathogenicity of *Mycobacterium abscessus*

Mycobacterium abscessus was originally isolated in 1952 from an abscess in a chronic knee infection (Moore & Frerichs, 1953). However, it was originally believed to be another mycobacterium species due to similarities in biochemical testing. Eventually, scientists determined that *M. abscessus* was its own species in 1992. This was further expanded upon in 2013 when it was determined that there were three subspecies of *M. abscessus*: *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolleti*, and *M. abscessus* subsp. *massiliense* (Lopeman et al., 2019).

M. abscessus is a nontuberculous mycobacterium (NTM) that is normally causes skin and soft tissue infections (SSTIs), pulmonary infections, and respiratory infections. However, in rare cases *M. abscessus* is capable of causing infections in the central nervous system (Lee et al., 2015). Like other nontuberculous mycobacteria, it is a saprophyte. Due to its classification as nontuberculous mycobacteria, infections are not subject to being reported. Nomenclature has also changed in the past 20 years, which also contributes to the difficulty of determining accurate incidence numbers (Johansen et al., 2020; Lee et al., 2015; Strollo et al., 2015). However, reports have shown that the number of cases and prevalence is increasing in the United States in comparison to *Mycobacterium tuberculosis (Mtb)*, which have fallen (Bento et al., 2020). *M. abscessus* is ubiquitous in nature and household environments such as shower heads. It is also a source of nosocomial infections and is a common source of infections for patients with cystic fibrosis (CF). SSTIs usually occur from contaminated needles or through other fomites come in contact with patients during surgeries (Lee et al., 2015; Lopeman et al., 2019; Shaw et al., 2019; Strollo et al., 2015). It was originally thought that *M. abscessus* had limited potential for person to person infection until it was discovered that subspecies *bolleti* and *massiliense* had been transferred from patient to patient during treatments.

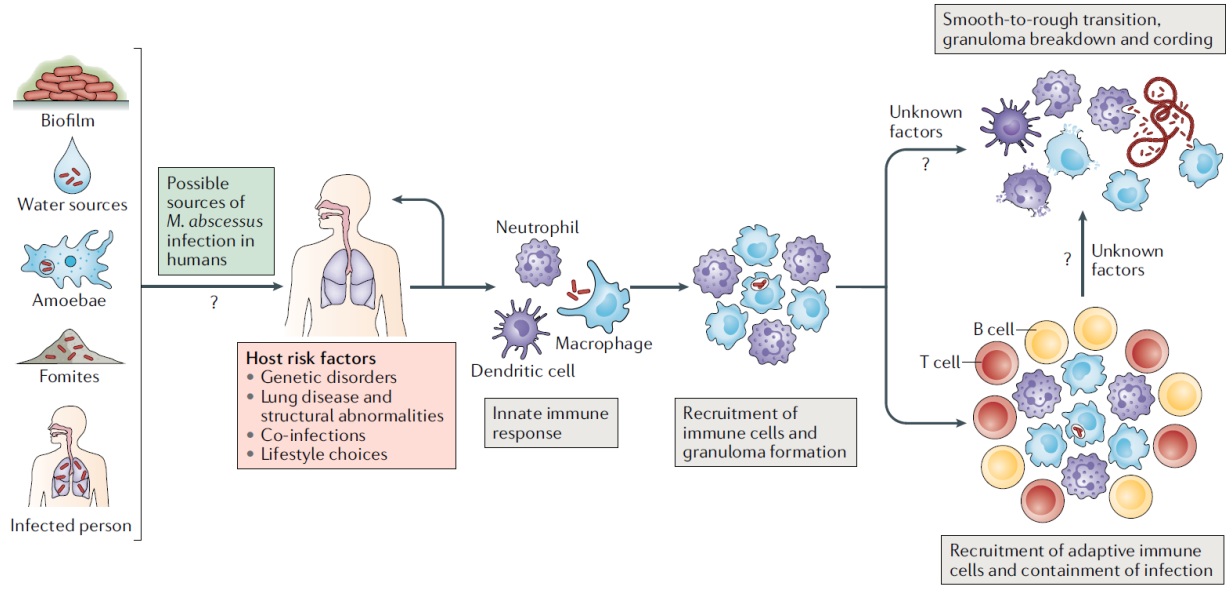

Figure 1. Route of *M. abscessus* infections. *M. abscessus* can infect the human body through several different ways. Once an infection has occurred, they are phagocytized, and granuloma formation occurs. These granulomas are broken apart when *M. abscessus* changes morphotype (Johansen et al., 2020).

M. abscessus has two colony morphotypes, which are commonly referred to as rough and smooth. They can originate from the same parent strain and are capable of reverting to another morphotype by an unknown mechanism (Howard et al., 2006; Rüger et al., 2014). Each morphotype has different characteristics and appearances. The rough morphotype is able to invade tissue, causing active infections. The smooth morphotype has glycopeptidolipids (GPL), which are located in the cell walls and colonies have a smooth and waxy appearance. The presence of GPL contributes to biofilm formation and prevent the immune system from recognizing the bacteria. The waxy surface allows the bacteria to resist cell death after being phagocytized by macrophages and neutrophils (Clary et al., 2018; Greendyke & Byrd, 2008). These macrophages and neutrophils are then entrapped by other components of the immune system, which causes a granuloma to develop. This process also happens during *Mtb* infections, and do not cause an immune response to occur. An active infection and immune response will occur if *M. abscessus* changes to a rough morphotype (Figure 1). The rough morphotype will break down the surrounding granuloma to spread, causing tissue damage and inflammation (Ryan & Byrd, 2018).

1.2 Antibiotic Resistance

Antibiotic resistance has become an issue in our society today. Since their discovery in the early twentieth century, antibiotics have been relied on to kill bacterial infections, prevent the spread of disease, and prevent surgical complications resulting from infection. The constant use of antibiotics has allowed for bacteria to develop resistance to many antibiotics, causing them to lose effectiveness (Becker & Cooper, 2013; Bento et al., 2020; Fyfe et al., 2016). This has made it necessary to develop new antibiotics in order to maintain the ability to limit infections. However, exorbitant research and development costs have limited these endeavors. This is further complicated by each generation of antibiotics having less time to be effective. For example, penicillin and methicillin were effective antibiotics for many years while the current generation, which was developed recently is already experiencing limited effectiveness (Towse et al., 2017).

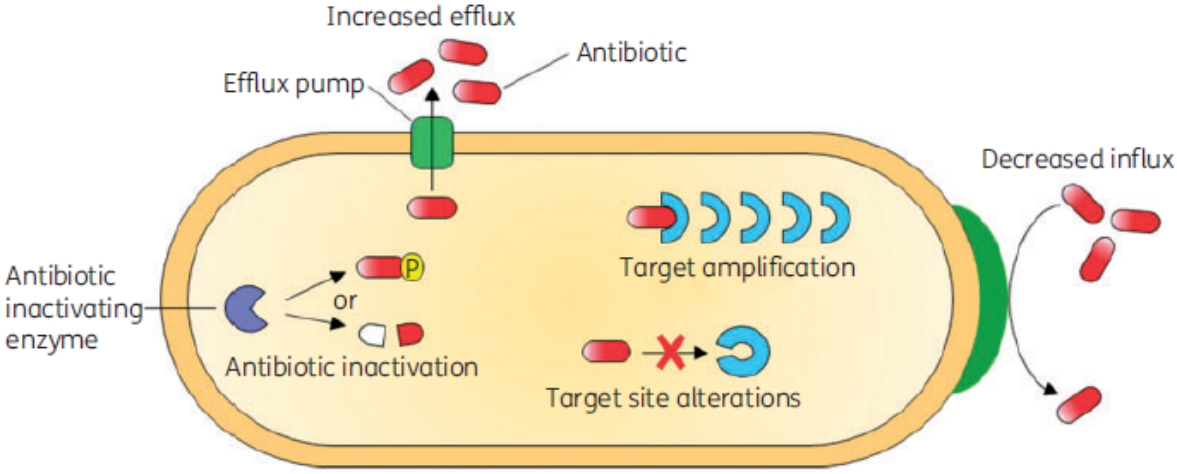

Figure 2. Mechanisms of Antibiotic Resistance. There are multiple mechanisms that promote antibiotic resistance and bacteria are capable of utilizing more than one at a time to generate antibiotic resistance (Alav et al., 2018).

Bacteria have multiple mechanisms to limit the effectiveness of antibiotics, such as gene upregulation, site inhibitors, increased efflux, decreased influx, site cleavage, and modifying enzymes. Bacteria are not limited to one single mechanism and can often utilize multiple mechanisms at the same time to increase resistance (Figure 2) (Alav et al., 2018; Briffotaux et al., 2017; Greendyke & Byrd, 2008).

1.3 Antibiotic Resistance in *Mycobacterium* species

Antibiotic resistance is a major problem for bacteria but it is exacerbated with mycobacteria. Mycobacteria are slow growing bacteria compared to many other pathogens and can also enter a dormant state. This limits the effectiveness of antibiotics, which require actively growing bacteria to work effectively (Daniel et al., 2014; Deb et al., 2009). To compensate for dormancy, treatment regimens for mycobacteria infections generally last several months and require patients to consume a cocktail of antibiotics. The time requirement is to help ensure that the bacterial infection has been completely cleared. The cocktail of antibiotics is to help limit the ability for the infection to develop resistance to the antibiotics or increase the level of resistance that might already exist. It is imperative that patients complete their full treatment regimen for both their own health and to also help limit the development of antibiotic resistant bacteria. To make sure this happens, it has become common for treatments to be dispensed and patients observed until antibiotics have been consumed at treatment centers (Bento et al., 2020; Johansen et al., 2020).

1.4 Antibiotics used to treat *Mycobacterium abscessus*

M. abscessus is resistant to many antibiotics, which include those normally used to treat *Mtb* infections. Some antibiotics used currently to treat *M. abscessus* are clarithromycin, amikacin, erythromycin, streptomycin, and ofloxacin. Treatments using these antibiotics will last several months and have demonstrated low efficacy and also have toxic side effects. This also coincides with health professionals and patients struggling to follow guidelines to ensure a successful treatment as much possible when treating *M. abscessus*. There is also no definitive method to successfully treat the infections. A successful treatment regimen is declared after sputum samples test negative for the period of one year after treatment (Bento et al., 2020).

Clarithromycin and erythromycin are part of a series of antibiotics classified as macrolides. Clarithromycin specifically is a synthetic variant, while erythromycin is a naturally occurring version from *Streptomyces erythraeus* and *Arthrobacter*. This class of antibiotics are bacteriostatic, meaning they prevent bacteria from reproducing, which is accomplished by inhibiting protein synthesis. Drug resistance for this antibiotic is typically accomplished by changing the binding sites configuration, which interferes with the antibiotics ability to bind properly (Dinos, 2017; Fyfe et al., 2016).

Amikacin and streptomycin are part of a class of antibiotics known as aminoglycosides, which are isolated from various *Streptomyces* and *Micromonospora* species. This class of antibiotics is bactericidal, meaning they actively kill bacterial infections. This is accomplished by inhibiting protein synthesis and interfering with tRNA selection. Antibiotic resistance mechanisms for aminoglycosides include modifying enzymes, target modification, changing uptake and efflux, and membrane proteases (Becker & Cooper, 2013; Dal Molin et al., 2017). They are also some of the first drugs successfully used to treat tuberculosis infections. Since then, we have discovered more aminoglycosides with varied clinical uses. This has all contributed to increased antibiotic resistance.

Ofloxacin is a fluoroquinolone, which is a series of synthetic broad-spectrum antibiotics. They are bactericidal, which occurs by binding to DNA gyrase and interfering with replication. Antibiotic resistance is usually developed by mutations to DNA gyrase to alter the target site, which prevents binding (Bernard et al., 2015).

1.5 Biofilms

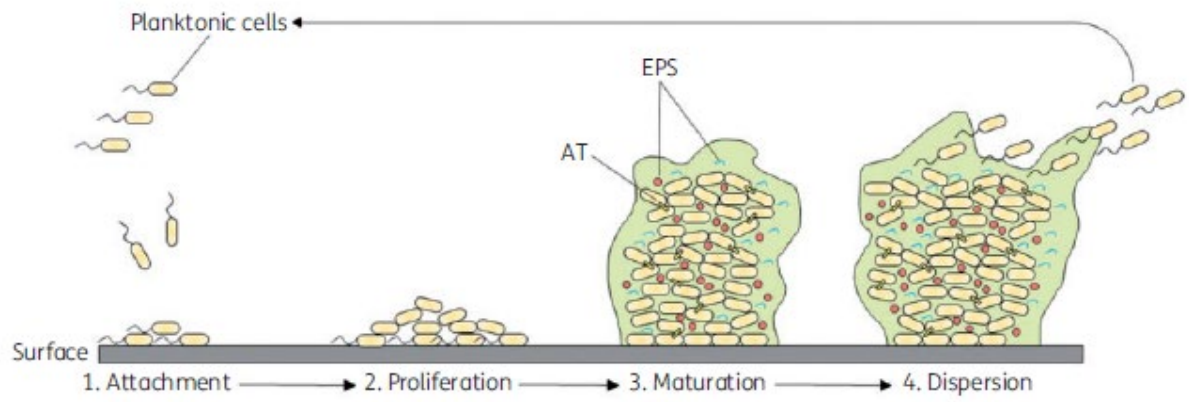

Figure 3. Formation of a Mature Biofilm. Biofilms form in four stages after bacteria experience stress. These four stages are: attachment, proliferation, maturation, and dispersion. Dispersion of planktonic cells is a sign that the biofilm is mature. Extracellular polymeric substances (EPS) secreted by autotransporters (AT) control biofilm formation (Alav et al., 2018).

One other mechanism of antibiotic resistance is the ability for microorganisms to form biofilms. Biofilms are colonies of bacteria that clump together and secrete extracellular matrices. They typically form when planktonic cells attach to surfaces where they can proliferate and mature

into colonies. A biofilm is considered mature when they begin releasing planktonic cells (Figure 3). They are also not composed of one single bacterial species. They can incorporate many bacterial species and even fungi (Abd El-Baky et al., 2019; Alav et al., 2018; Baugh et al., 2012; Flemming & Wingender, 2010; Heersema & Smyth, 2019; Richards & Ojha, 2014; Zambrano & Kolter, 2005). While they are clumped, they will begin secreting a film composed of enzymes, proteins, lipids, carbohydrates, quorum sensors, and extracellular matrices. This film can account for as much as 90% of the total mass. In this film they can communicate with one another to help regulate their surroundings via quorum sensing (Miller & Bassler, 2001; Subhadra et al., 2018).

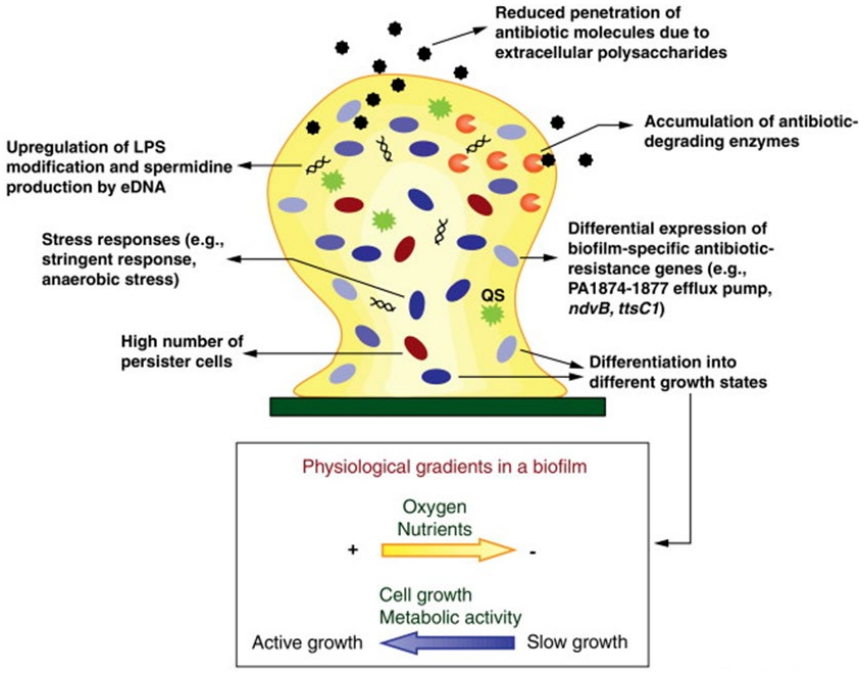

Figure 4. Composition of a Biofilm. A biofilm is composed of many different compounds that are not cellular organisms. These compounds include enzymes, extracellular polysaccharides, eDNA, lipids, and quorum sensing autoinducers. The biofilms also cause bacteria to have unequal access to nutrients, which changes their metabolic state (de la Fuente-Núñez et al., 2013).

Biofilms regulate the local environment and protect bacteria from harmful compounds. This film also presents its own challenges to the bacteria habituating the film. Planktonic cells are freely floating and therefore have equal opportunity and access to any nutrients present. Biofilm cells are clumped and surrounded by a film. This contributes to bacteria having unequal access to nutrients. This prevents uniform metabolism and can act as another measure in limiting the effectiveness of antibiotics, which rely on bacteria to grow to be effective (Figure 4). The film

itself also contains enzymes that help break down harmful compounds such as antimicrobials (Alav et al., 2018; Pang et al., 2012).

Biofilms also contribute to antibiotic resistance in multiple ways. Biofilms are formed when bacteria are subjected to stress and begin to secrete compounds that make up the extracellular matrices mentioned earlier. Stressed bacteria will begin upregulating genes necessary for survival. They will also increase efflux capacity and allows for bacteria to be more resistant to harmful compounds.

1.6 Biofilms in *Mycobacterium abscessus*

Biofilms help contribute to antibiotic resistance in *M. abscessus*. Biofilm formation causes gene upregulation for efflux pumps which help increase levels of biofilm formation. *M. abscessus* also has increased efflux capacity and it has more efflux pumps than other mycobacteria, which help contribute to improved efflux of various potentially harmful compounds (Marini et al., 2019; Richards & Ojha, 2014; Wright et al., 2017). *M. abscessus* can also utilize lipids as a food source and allows it to store energy reserves for future use during infections (Deb et al., 2009; Mavi et al., 2019). The increased presence of lipids and gene upregulation also contributes to increased levels of antibiotic resistance in *M. abscessus*. The presence of accumulated lipids allows it to enter a dormant state and reduce the effectiveness of antibiotics.

There are several studies reported in mycobacteria on biofilm formation. Many of them have focused on the increase in antibiotic tolerance (de la Fuente-Núñez et al., 2013; Greendyke & Byrd, 2008). Some studies demonstrated that culture media can affect the ability of biofilms to increase antibiotic tolerance (Hunt-Serracin et al., 2019). These investigations highlighted the importance of creating experimental conditions that mimic human physiology as accurately as possible. It was shown that a reducing environment created by the addition of dithiothreitol (DTT) to the culture medium induced the formation of biofilms in *Mtb* (Trivedi et al., 2016). Thiol-reductive stress creates a reducing environment that is similar to what mycobacteria experience during an infection. We hypothesized that, *M. abscessus* would also form biofilms under *in vitro* thiol reductive stress and that *M. abscessus* in biofilms would show increased resistance to antibiotics.

1.7 Efflux Pump Inhibitors

Efflux pumps play an important role in developing antibiotic resistance. They are also present in all organism and has been shown that efflux pump genes transplanted from bacterial to mammalian cells will still work (Blanco et al., 2016; Martinez et al., 2009). This demonstrates that efflux pumps play a variety of functions in multiple organisms. They play an important role in maintaining homeostasis by expelling harmful compounds such as heavy metals and other antimicrobials. It has been shown that efflux pumps play a role in maintaining antibiotic resistance (Baugh et al., 2012; Blanco et al., 2016). It has been shown that including medications that are known to inhibit efflux pump have contributed to clearing infections from patients. However, incorporating them into treatment regimens will be difficult. Some efflux pump inhibitors are used in clinical settings such as verapamil but have severe cytotoxic effects. The presence of efflux pumps in all organisms makes it a difficult to develop compounds maintain their effectiveness without eliciting harmful effects to human hosts (Chen et al., 2018; Vianna et al., 2019).

Efflux pumps are also able to regulate cell signaling in bacteria which is caused by quorum sensing. Quorum sensing is used by bacterial populations to help regulate their environment by releasing autoinducers (Pearson et al., 1999). This also helps maintain symbiosis of biofilms and is another important part of developing antibiotic resistance in bacteria (Miller & Bassler, 2001; Subhadra et al., 2018).

Bacterial efflux pumps have been classified into five families: 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. Almost all the efflux pump superfamilies use proton motive force and sodium/hydronium ion electrochemical gradients to function. They can also be referred to as H^+ drug antiporters. The ABC superfamily is an exception, which instead relies on ATP to generate energy (Alav et al., 2018; Baugh et al., 2014; Blanco et al., 2016; Martinez et al., 2009; Matsumura et al., 2011; Pule et al., 2016). After generating energy, all superfamilies can expel harmful compounds.

The MFS transport family is one of the largest and are located in the cytoplasmic membrane. These transport various molecules such as simple sugars, amino acids, oligosaccharides, and other compounds in addition to conferring antibiotic resistance. The MFS transporter family of efflux pumps plays an integral role in preventing fluroquinolones and tetracyclines from working

properly. It is believed that this coincides with their normal function of transporting toxic chemicals outside the cell (Alav et al., 2018; de la Fuente-Núñez et al., 2013).

The SMR family predominantly expel cationic compounds. The RND family allow for the direct export of drugs into external medium. They are unable to pump any drugs that belong to the aminoglycoside family of antibiotics. They are also able to pump out dyes, detergents, and solvents. This generates a large advantage for the bacteria as it requires the effluxed compounds to circumvent the external cell membrane again instead of being able to immediately reenter the cell. One disadvantage is the removal or inhibition of this efflux pump eliminates any resistance for the bacteria (Alav et al., 2018; Nikaido, 2009).

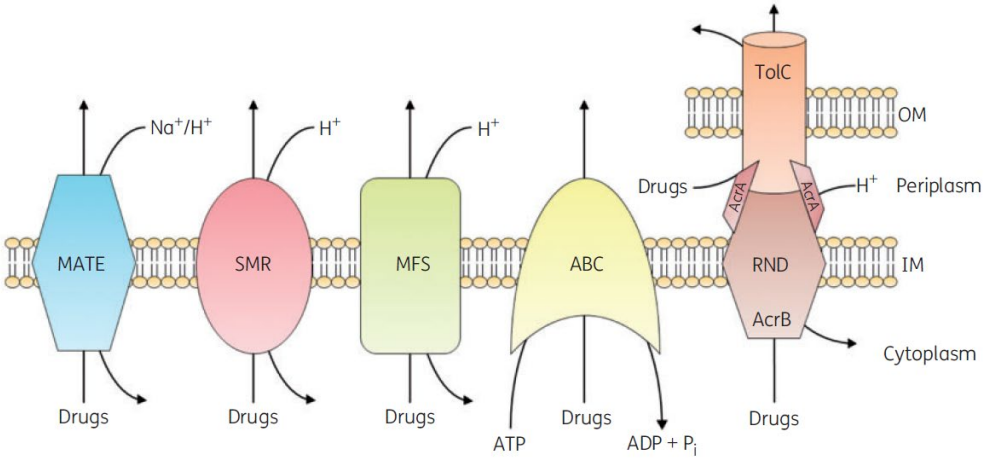

Figure 5. Known major efflux pump superfamilies in *M. abscessus*. Several compounds have been discovered to directly affect the ability of several efflux pump superfamilies in *Mtb* (Alav et al., 2018).

Scientists have managed to catalog various compounds that are able to neutralize the efflux pumps. Reserpine will limit the effectiveness of efflux activity in the RND superfamily. Verapamil and piperine limit ABC transporters. CCCP limits the MFS superfamily as well as the electron transport chain (ETC) (Figure 5). We are currently unaware of any compounds that inhibit the effectiveness of SMR family (Abd El-Baky et al., 2019; Alav et al., 2018; Pule et al., 2016).

1.8 Efflux pumps and biofilm formation in mycobacteria

The roles of efflux pumps in the export of antibiotics has been studied in depth (Alav et al., 2018; Fyfe et al., 2016; Vianna et al., 2019). However, the roles of efflux pumps on biofilm

formation are not well described in bacteria. In *Escherichia coli*, efflux pump genes were upregulated under anaerobic conditions and during biofilm formation (Ito et al., 2009; Stewart & Franklin, 2008). When genes that encoded efflux pumps were knocked out, there was a decrease in the amount of biofilm formation. It was determined that these genes encoded for RND, MFS, and SMR families of efflux pumps (Kvist et al., 2008; Matsumura et al., 2011; Schembri et al., 2003). In *Pseudomonas aeruginosa*, efflux pump genes were upregulated during biofilm formation (Pearson et al., 1999; Waite et al., 2005). In *Acinetobacter baumannii*, it was determined that efflux pumps secrete sugars that contribute to biofilm formation. It was also found that the efflux pump AdeFGH contributes to biofilm formation and that a certain level of efflux pump gene expression was needed to increase biofilm formation (Abd El-Baky et al., 2019; Flemming & Wingender, 2010; He & Ahn, 2011; He et al., 2015).

We found one study on a mycobacterial efflux pump involved in biofilm formation. In this study, the authors overexpressed a mycobacterial efflux pump in *E. coli*, and demonstrated that it increased the levels of biofilm formation (Bansal et al., 2016). We were unable to find any studies on the effects of efflux pumps and biofilm formation in *M. abscessus*.

1.9 Lipids and *Mycobacterium abscessus*

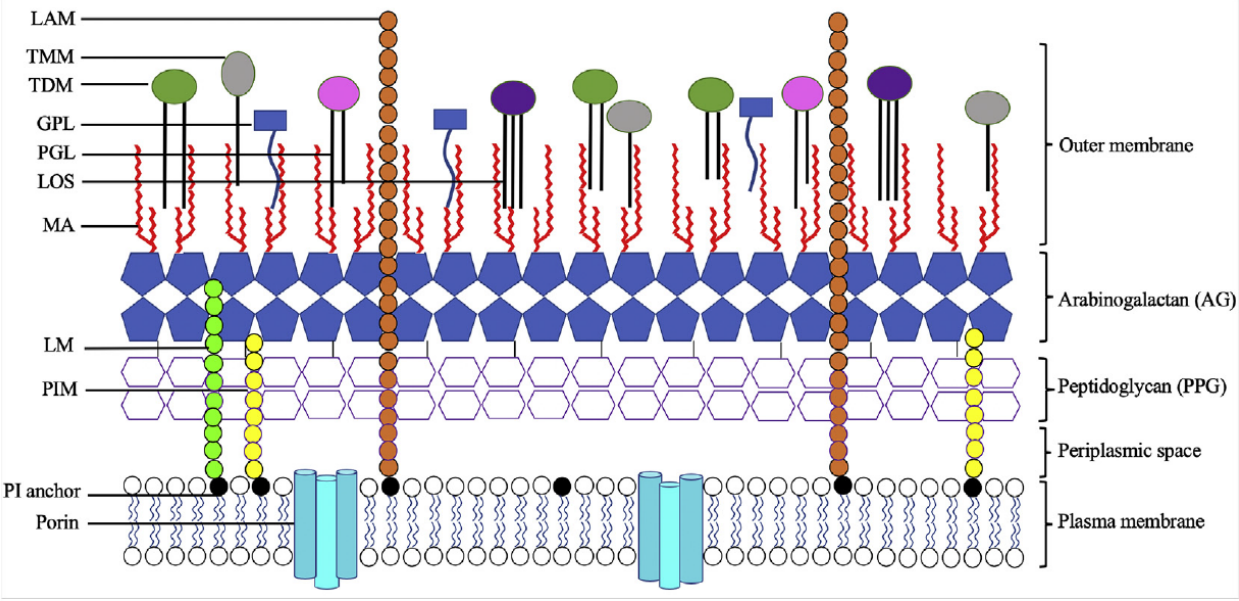

Figure 6. Composition of mycobacterial cell walls. Mycobacteria possess cell walls that possess several different types of lipids which contribute to a waxy cell wall (Tran et al., 2019)

Lipids play an important part in mycobacterium infections. The mycobacterium cell membrane has many lipids present on the both the inner and outer membranes that contribute to host infections (Figure 6). Mycobacteria are also unique in that several species including *Mtb* and *M. abscessus* are able to store host-derived lipids and use them as source of food at a later point in the infection (Daniel et al., 2011; Deb et al., 2009; Viljoen et al., 2016). In fact, lipid-loaded macrophages are considered a symptom of long-term tuberculosis infections from *Mtb*. It was also originally believed that this was limited only to *Mtb*, but recent literature suggests that *M. abscessus* also has the capability to do so. This is because genes that have been determined to be essential for allowing lipid accumulation in *Mtb* have had homologous versions detected in *M. abscessus*. This may allow *M. abscessus* to enter a dormant state and prolong infections (Daniel et al., 2016; Viljoen et al., 2016). It has also been determined that various types of lipids also play a part in mediating and eliciting an immune response for mycobacteria. Membrane lipids such as phosphatidylinositol mannnosides (PIMs) can interfere with phagosome maturation. Lipomannan (LM) lipids promote strong proinflammatory cytokine responses. Trehalose-based lipooligosaccharides (LOS) have been found to act as antigens for *Mycobacterium kansasii* (Tran et al., 2019). This highlights the importance that lipids have on modulating the host immune response for mycobacterium.

While lipids important for mycobacterium have been studied in some capacity, our knowledge is still limited. This is exacerbated because most of the studies have focused primarily on *Mtb* and have neglected other mycobacterium. It has been shown that there are slight differences on what lipids may be used for in specific mycobacterium. There have also been no studies that attempt to focus on lipids that may be present or contribute to biofilm formation in *M. abscessus* or even other mycobacterium. We postulated that efflux pump proteins in the pathogen can transport lipids synthesized within the cell and cause their deposition on the outer cell wall of the bacterium thereby contributing to biofilm formation.

1.10 Metabolic radiolabeling of *M. abscessus* biofilm lipids using ^{14}C-acetic acid.

Lipids play important roles in mycobacterial physiology and have been shown to be critical in mycobacterium infections in several studies. The biosynthesis and accumulation of lipids have normally been studied using radiolabeled biochemical precursors of lipids. Acetic acid is the two-

carbon building block used by fatty acid synthase to make fatty acids which are the building blocks of complex lipids in all organisms (Daniel et al., 2014; Rücker et al., 2015). Metabolic radiolabeling using ^{14}C-labeled acetic acid is routinely used to study lipid metabolism since the radiolabeled lipids can resolved on silica-thin layer chromatography (TLC), imaged using autoradiography and selected lipids can be quantitated by scintillation counting. Since mycobacterial lipids have been extensively characterized, the retention factor (R_f) values for important mycobacterial lipids on several silica-TLC solvent systems are known. Such previously reported R_f values form the basis for identification of lipids in any new study (Daniel et al., 2011; Howard et al., 2006; Hunt-Serracin et al., 2019; Rhoades et al., 2009). However, the lipids critical for *M. abscessus* during biofilm formation under thiol-reductive stress have not been studied using ^{14}C-labeled acetic acid. Therefore, we applied this method to study lipid biosynthesis and metabolism in *M. abscessus* cells in biofilms. We hypothesized that efflux pump inhibitors will prevent the formation of biofilms and that metabolic radiolabeling of cells using a precursor of fatty acids would reveal the lipids that were involved in various stages of biofilm formation.

CHAPTER 2. METHODS

2.1 Culture conditions

Mycobacterium abscessus ATCC 19977 (ATCC, Manassas, VA) was routinely grown as planktonic cultures in Middlebrook 7H9 broth (BD Life Sciences, Sparks, MD) supplemented with ADC (Albumin-Dextrose-Catalase, BD Life Sciences), 0.05% Tween 80, and 0.5% glucose. A saturated starter culture was grown in 7H9 medium from glycerol stock stored in ultracold freezer and used to inoculate 10 mL broth culture. Cultures were grown at 37 °C at 225 RPM until they reached an OD_{600} of 0.3 (early-log phase) or 0.7 (log-phase).

2.2 Biofilm formation under Thiol Reductive Stress

We followed modifications of an earlier protocol that caused *Mtb* to form biofilms when exposed to dithiothreitol (DTT) (Trivedi et al., 2016). Cultures for biofilm formation were grown in Middlebrook 7H9 without Tween 80 (7H9_noTw). They were grown without shaking at 37 °C until they reached an OD_{600} of approximately 0.3 (early log-phase). Cultures were then aliquoted to a 96-well plate before DTT was dispensed into each well to reach the following desired concentrations: 0 mM, 1 mM, 2 mM, 4 mM, and 8 mM. The cells were incubated at 37 °C without shaking over a period of 1, 2, 4 and 8 days. At each respective time-point, the wells containing culture media were removed, leaving biofilm formation cells behind to be assessed (Trivedi et al., 2016).

2.3 Crystal Violet Assay

Biofilm under thiol reductive stress was measured using crystal violet following a modification of previously described procedures (Pang et al., 2012). This assay utilizes 1 % crystal violet in distilled water, which was dispensed into wells that previously contained culture media and allowed to incubate at 37 °C for 10 min. The crystal violet was removed, and the wells were washed three times with water. Then, 95 % ethanol was added and after an incubation for 10 min, an aliquot was collected from each well for a 1:2 dilution. The absorbance of the extracted 1:2 dilution was then read at 600 nm on a microplate spectrophotometer (Thermo Fisher AccuSkan).

2.4 Antibiotic Resistance Determination

Planktonic cells were grown to OD_{600} of 0.3 before being diluted to reach approximately 10^4 CFUs/mL. Cells were then aliquoted into 96-well plates. Cells for biofilm formation were grown to OD_{600} of 0.3 prior to the addition of DTT to 4 mM. Cells were then dispensed into wells in 96-well plate and incubated for 4 days. Then one of the following antibiotics was added to the following final concentration: clarithromycin (0.5 $\mu g*mL^{-1}$; Alfa Aesar, Ward Hill, MA), amikacin (1.25 $\mu g*mL^{-1}$; MP Biomedicals, Solon, OH), and streptomycin (2.5 $\mu g*mL^{-1}$; Sigma-Aldrich, St.Louis, MO). Control wells did not receive any antibiotic. The final well volume was then brought up and equalized to 200 µL with media dependent on the type of culture as described above. The cells were incubated with antibiotic at 37 °C for three days. Free-floating cells were removed and the biofilm was gently washed with 7H9_noTw to remove any remaining excess planktonic cells. Colony forming units (CFU) plating was accomplished by pooling technical replicates from the same 96-well plates into 1.5 ml tubes before being sonicated and vortexed to break up biofilms and ensure proper serial dilution. Once completed, cells were serially diluted in 7H9 media containing Tween 80 and appropriate dilutions were plated to obtain 30-300 CFUs per plate. The CFUs/ml for antibiotic-treated samples were divided by the CFUs/ml for the untreated controls to determine the percent survival values.

2.5 Analysis of effects of efflux pump inhibitors

Biofilm cells were grown to OD_{600} of 0.3 before DTT was added to the final concentration of 4 mM. The cells were then aliquoted into a 96-well plate. Then one of the following efflux pump inhibitors was added to the following final concentrations: verapamil (50 $\mu g*mL^{-1}$ & 10 $\mu g*mL^{-1}$; TCI America, Portland, OR), CCCP (25 $\mu g*mL^{-1}$ & 3 $\mu g*mL^{-1}$; Alfa Aesar, Ward Hill, MA), berberine (30 $\mu g*mL^{-1}$ & 5 $\mu g*mL^{-1}$; TCI America, Portland, OR), piperine (30 $\mu g*mL^{-1}$ & 3.5 $\mu g*mL^{-1}$; Alfa Aesar, Ward Hill, MA), and reserpine (40 $\mu g*mL^{-1}$ & 5 $\mu g*mL^{-1}$; Alfa Aesar, Ward Hill, MA). The plate was then covered and wrapped with parafilm and incubated for four days at 37°C.

2.6 Analyses of lipid accumulation in *M. abscessus* biofilms

Lipid analyses were conducted using the following method. Cultures were grown for both planktonic and biofilm cells as described above. Cultures induced for biofilm formation were centrifuged at 200 X g for 10 min to collect adhered and aggregated cells in biofilms. The supernatant containing floating planktonic cells was then centrifuged again at 16,000 X g for 10 min to collect the cells, when needed, and the supernatant containing cell-free medium was separated. The wet pellets were then weighed and resuspended in 5 mL of isopropanol and shaken at 225 rpm for 10 min to gently extract cell-surface lipids. The cells were collected by centrifugation. The supernatant isopropanol extract was transferred to a new tube and the volumes were equalized. The cells that were extracted for surface lipids using isopropanol were then extracted with chloroform/methanol (2:1, v/v) for 16 h at room temperature to extract total cellular lipids not removed by the previous isopropanol extraction. The lipids were collected after washing the organic phase with acidified water three times. Lipid extracts were normalized across samples by cell wet weight prior to loading (Figure 7). Lipids were resolved on silica-thin layer chromatography (TLC) plates (Silica G Uniplate, Analtech, Newark, DE) using chloroform/methanol/ water (60:30:6, v/v/v) as a solvent system to resolve phosphatidylinositol mannosides (PIMs) or chloroform/methanol/water (100:14:0.8, v/v/v) to resolve glycopeptidolipids as described by others earlier (Rhoades et al., 2009). Lipid extracts were resolved on hexane/diethyl ether/ acetic acid (90:10:1, v/v/v) to resolve neutral lipids (Daniel et al., 2011).

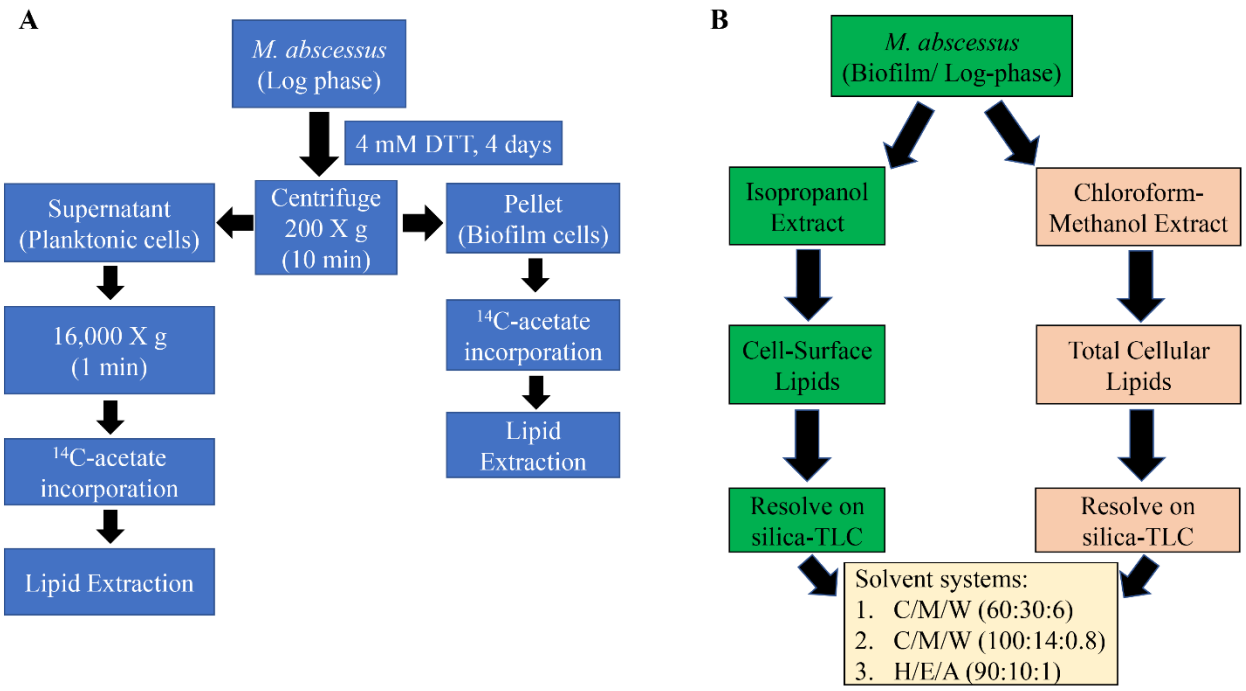

Figure 7. Radiolabeling and lipid extraction protocol. A. Steps for radiolabeling protocol. **B.** Steps for lipid extractions and analyses.

2.7 Metabolic radiolabeling of *M. abscessus* biofilms with ^{14}C-acetate

The biosynthesis of lipids during the early stages of biofilm formation was investigated by radiolabeling the cells with ^{14}C-acetate at 0 h, 12 h, 24 h, and 48 h after the initiation of biofilm formation upon addition of DTT. ^{14}C-acetic acid (Moravek, Inc., CA; 59 mCi/mmol) was added (2 µCi/2.5 mL cells) and cultures were then incubated at 37 °C with shaking at 225 rpm following a modification of procedures described earlier (Daniel et al., 2004). At each of these time-points, the radiolabel was added and cells were incubated with the radiolabel for an additional 12 h with shaking at 225 rpm at 37 °C to allow metabolic incorporation into lipids. At each time point, duplicate aliquots of cells were collected and centrifuged at 200 X g for 10 minutes. The supernatant was then collected and centrifuged at 16,000 X g for 1 min. The extracellular media was removed and lipids in the cell pellets were then extracted and analyzed as described below.

Cultures were grown in 7H9_noTw at 37 °C until they reached OD_{600} of 0.7 after which DTT was added to a final concentration of 4 mM for inducing biofilm formation. The cells were incubated in standing conditions for 96 h at 37 °C. Aggregated and adhered biofilm cells were centrifuged at 200 X g for 10 min and the supernatant containing floating cells collected for

analysis when needed. Planktonic controls were grown in 7H9 broth at 37 °C with shaking at 225 rpm until they reached an OD_{600} of 0.7. Cells in the planktonic culture were pelleted by centrifugation at 16,000 X g, 1 min and the supernatant was discarded. The biofilm and planktonic cell pellets were then resuspended with double the previous volume of media. ^{14}C-acetic acid was added (2 µCi/2.5 mL cells) and cultures were then incubated at 37 °C with shaking at 225 rpm. The metabolic turnover of radiolabeled lipids was then analyzed by incubating the cells with the radiolabel for 6 h, 24 h, and 72 h. At these time-points, duplicate aliquots of cells were collected for lipid extractions by centrifugation at 16,000 X g for 1 min (Daniel et al., 2014).

In order to assess the effects of exogenously supplied fatty acids, palmitic acid was added to a final concentration of 100 µM along with DTT (final concentration 4 mM). The cells were incubated in standing conditions for 4 days. Planktonic controls also received the fatty acid supplementation immediately when the cells reached OD_{600} of 0.7. The potential inhibitory activity of the efflux pump inhibitor verapamil in the synthesis of lipids was investigated by adding verapamil to a final concentration of 50 µg/mL 15 min prior to the addition of ^{14}C-acetic acid. Verapamil is expected to inhibit the efflux of lipids synthesized inside the cell using the radiolabeled precursor and potentially involved in biofilm formation. The cultures were then incubated at 37 °C with shaking at 225 rpm for 6 h.

2.8 Lipid extraction and analysis by silica-thin layer chromatography

The lipids on the cell surface of radiolabeled cells were extracted using an alcohol (isopropanol) following which the cellular lipids were extracted using organic solvents chloroform-methanol (2:1). The radiolabeled cell pellets were resuspended in isopropanol and shaken at 225 rpm for 10 min to gently extract cell-surface lipids. Then, the cells were collected by centrifugation at 16,000 X g, 1 min. The isopropanol extract supernatant containing cell surface lipids were collected into fresh tubes. The cell pellets were then resuspended and shaken in chloroform/methanol (2:1, v/v) for 16 h at room temperature to extract total cellular lipids. The radioactivity in the isopropanol extracts and in total lipid extracts was determined by liquid scintillation counting.

The extracted lipids were analyzed by silica-thin layer chromatography using three different solvent systems. In order to normalize the levels of total lipids across samples for TLC analysis, the lipid extracts were loaded on silica-TLC plates (Silica G Uniplate, Analtech, DE) by

equal amounts of total radioactivity across samples. This was to account for differences in cell numbers and lipid extraction efficiencies across samples.

The isopropanol extracts and chloroform/methanol extracts were resolved on silica-TLC plates using chloroform/methanol/water (60:30:6, v/v/v) as a solvent system to resolve phosphatidylinositol mannosides (PIMs) and other lipids or chloroform/methanol/water (100:14:0.8, v/v/v) to resolve glycopeptidolipids and other lipids (Howard et al., 2006; Hunt-Serracin et al., 2019; Rhoades et al., 2009). Hexane/diethyl ether/acetic acid (90:10:1, v/v/v) was used to resolve triacyglycerols and other neutral lipids (Daniel et al., 2011). Plates were then exposed to autoradiography film for one week at -70 °C. Each lipid band of interest in the respective solvent systems were identified on the basis of its known retention factor (R_f) from previously published studies on lipids from *M. abscessus* in the same solvent systems. Radioactivity in lipid bands was determined by scraping the silica from the TLC plate followed by liquid scintillation counting. In order to normalize across samples, the radioactivity counts in lipid bands were expressed as a percent of the total radioactivity in the respective lipid extract.

CHAPTER 3. RESULTS

3.1 Thiol reductive stress stabilizes biofilms formed by *Mycobacterium abscessus*

We tested four separate growth conditions using several different concentrations of dithiothreitol (DTT) at various time-points to determine the optimal method for forming biofilms under thiol reductive stress. The crystal violet assay was used to quantify biofilm formation. We found that *M. abscessus* grown to early logarithmic phase without shaking in the absence of Tween 80 was the most effective at developing a biofilm. We observed that biofilm formation by *M. abscessus* occurred without DTT but the levels of biofilm decreased after 48 h. When DTT was added, the biofilms that were formed were more stable and persisted until 96 h in the presence of 4 mM DTT. At 96 h, biofilm levels were nearly 4-fold higher in the presence of 4 mM DTT (OD_{600} 0.31 ± 0.11) compared to 96 h in the absence of DTT (OD_{600} 0.08 ± 0.03). However, this was not statistically significant.

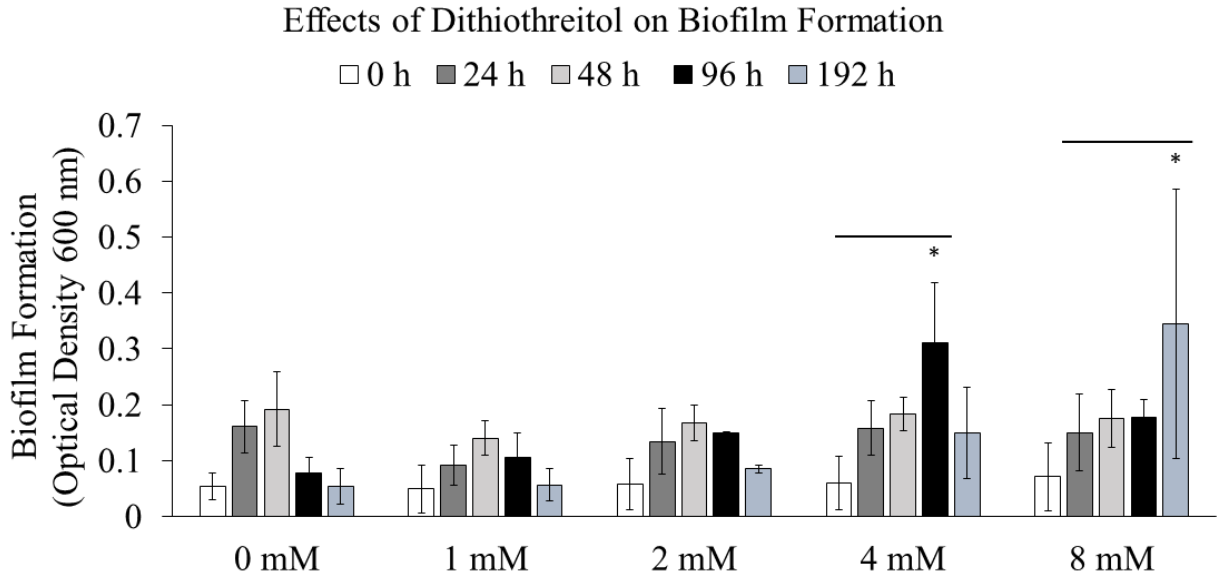

Figure 8. Thiol reductive stabilizes biofilms formed by *M. abscessus*. On days 0, 1, 2, 4, and 8 after DTT addition, crystal violet assay was performed. Representative of two independent repeats. Values are average ± SD of duplicates from two independent experiments. ANOVA was performed followed by Tukey's post-hoc test. *, $p<0.05$.

There was no statistically significant difference between 4 mM DTT at 96 h and 8 mM DTT at 192 h (Figure 8). We also did not observe reliable biofilm formation when *M. abscessus* cells were incubated with shaking in the presence of Tween 80 or when DTT was added to fresh or early logarithmic phase shaking cultures (data not shown).

3.2 Biofilms formed by *Mycobacterium abscessus* do increase antibiotic tolerance

M. abscessus cultures demonstrated increased resistance to clarithromycin, amikacin, and streptomycin when they were induced to form biofilms when compared to their planktonic states. Clarithromycin resistance increased from 3% (± 0%) survival to 27% (± 15%) survival, which was statistically significant. Amikacin resistance did not show any noticeable increase. Streptomycin resistance increased from 21% (± 13%) survival to 73% (± 3%) in biofilm cells and was statistically significant. The antibiotic resistance of *M. abscessus* in biofilms is shown as percent survival respective to the untreated control (Figure 9).

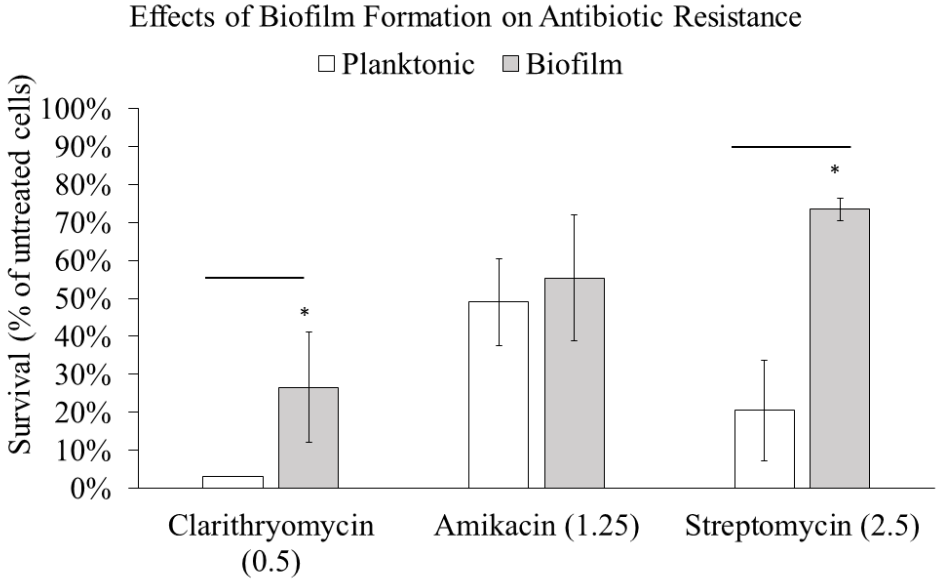

Figure 9. Biofilms formed by *M. abscessus* increase antibiotic tolerance. Percent survival is shown relative to untreated controls. Values are in $\mu g*mL^{-1}$. Values are average ± SD of duplicate measurements from two independent experiments. ANOVA was performed followed by Tukey's post-hoc test. *, $p<0.05$.

3.3 Efflux pump inhibitors prevent biofilm formation in *Mycobacterium abscessus* cultures

We evaluated the effects of efflux pump inhibitors on biofilm formation in *M. abscessus* cultures. We tested the efflux pump inhibitors at two concentrations described in the methods. All efflux pump inhibitors except for reserpine prevented biofilm formation at higher concentrations (Figure 10 A). Verapamil and CCCP were both observed to cause over a three-fold decrease in biofilm formation when compared to biofilms with no inhibitors present at high concentrations. Berberine and piperine at high concentration demonstrated over a two-fold decrease in biofilm formation. All four were demonstrated to have statistically significant decreases in biofilm formation compared to controls. The efflux pump inhibitor reserpine did not inhibit biofilm formation at all.

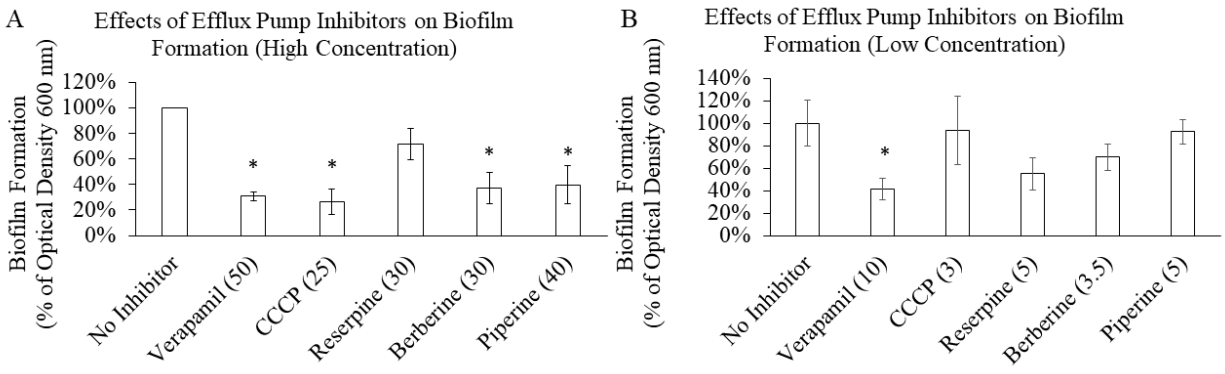

Figure 10. Efflux pump inhibitors disrupt biofilm formation at high concentrations. Values are in $\mu g*mL^{-1}$. Cells were incubated with efflux pump inhibitor and biofilm formation was assessed as described in methods. **A.** Efflux pump inhibitor at high concentrations. Values are average ± SD of triplicate measurements from two independent experiments. **B.** Efflux pump inhibitors at low concentration with one experiment performed. Values are average ± SD of triplicate measurements from a single experiment. ANOVA was performed followed by Tukey's post-hoc test. *, $p<0.05$.

We also evaluated the effectiveness of the same efflux pump inhibitors at low concentrations. Of these, only verapamil was able to decrease biofilm formation at statistically significant levels (Figure 10 B). Verapamil showed almost a two-fold decrease in biofilm formation compared to controls.

3.4 Biosynthesis of trehalose mycolates and phosphatidylinositol mannosides is elevated during early biofilm formation

In order to investigate the biosynthesis of lipids during biofilm formation, we performed metabolic radiolabeling of cells using ^{14}C-acetate which is the precursor molecule of fatty acids which are the building blocks of lipids. Radiolabeled lipids extracted from the cell surface of *M. abscessus* induced to form biofilms under thiol-reductive stress were resolved on silica-TLC using chloroform/methanol/water (60:30:6) as the solvent system. At the indicated times after initiation of thiol-reductive stress, cells were radiolabeled with ^{14}C-acetate for 12 hours and lipids were extracted as described. We observed that radiolabel accumulation in certain cell-surface lipids was increased early during biofilm formation as shown in Figure 11 A.

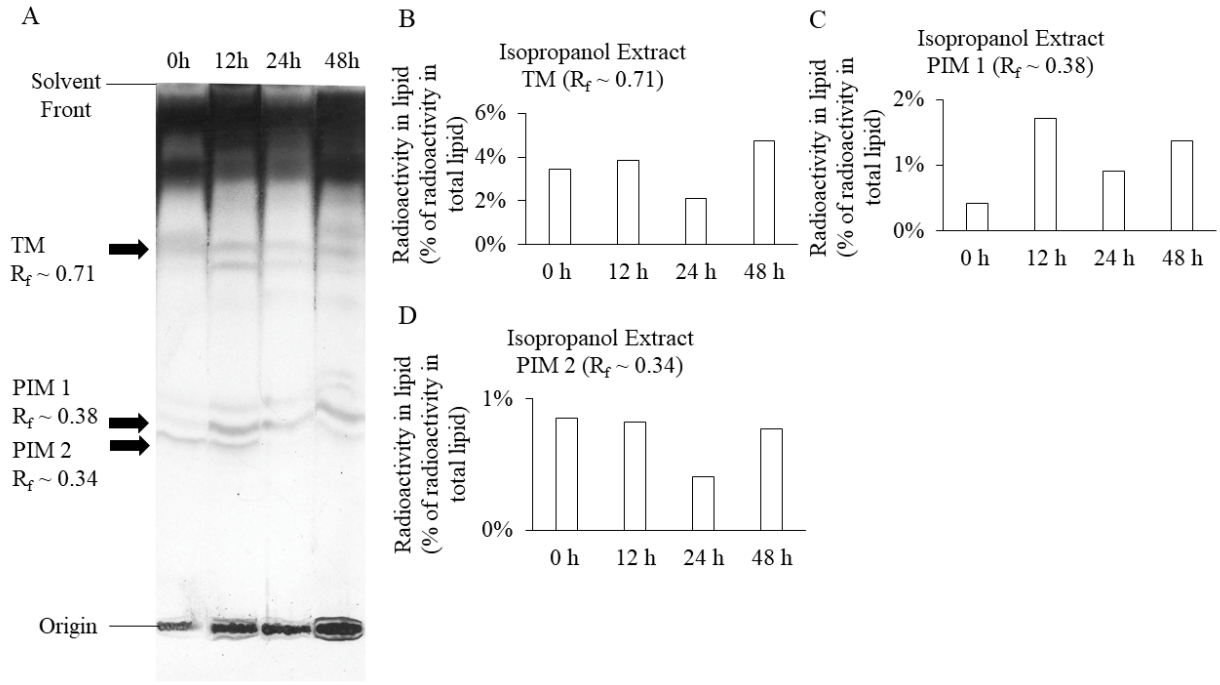

Figure 11. The biosynthesis of the cell-surface lipid phosphatidylinositol mannoside is elevated during biofilm formation. Cell-surface lipids were extracted with isopropanol and resolved on silica-TLC using chloroform/methanol/water (60:30:6, v/v/v) as solvent system. **A.** Autoradiogram of TLC plate showing TM (R_f ~0.71), PIM_1 (R ~ 0.38), and PIM_2 (R ~ 0.34). **B, C, D.** Radioactivity in lipids TM, PIM_1, and PIM_2 respectively, shown as percent of radioactivity in total lipids. Data from one experiment shown.

We tentatively identified these lipids as trehalose mycolates (TM; R_f ~0.71) and phosphatidylinositol mannosides (PIM_1; R_f~0.38 and PIM_2; R_f ~ 0.34) from previous reports

(Howard et al., 2006; Hunt-Serracin et al., 2019; Rhoades et al., 2009). Radioactivity in trehalose mycolates increased from 3.5% to 4.8% at 48 hours (Figure 11 B). PIM_1 increased more than 3-fold from 0.4% to 1.4% of total lipid in 12 hours and maintained a similar level at the 48-hour period (Figure 11 C). PIM_2 species showed a decrease at 24 hours but no noticeable changes over the 48-hour period (Figure 11 D).

3.5 Glycopeptidolipid biosynthesis is elevated during early biofilm formation

M. abscessus biofilm cells were extracted with isopropanol to isolate the cell-surface lipids which were resolved on silica-TLC using chloroform/methanol/water (100:14:0.8) as the solvent system. As shown in Figure 12 A, we identified the prominently-labeled lipids with $R_f \sim 0.3$ as glycopeptidolipids (GPL) based on previous reports (Howard et al., 2006; Hunt-Serracin et al., 2019; Rhoades et al., 2009). The lipid resolving with an $R_f \sim 0.6$ was not identifiable based on previous reports. This unknown lipid was also elevated in the total lipid concentration from 6.8% to 9.9% (Figure 12 B). We observed that radioactivity in glycopeptidolipids increased slightly during biofilm formation. The radioactivity in glycopeptidolipids originally only constituted 6.3% of radioactivity in total lipids in the isopropanol extract at 0 hour but increased to 8.6% of total lipid radioactivity by the 48-hour period (Figure 12 C).

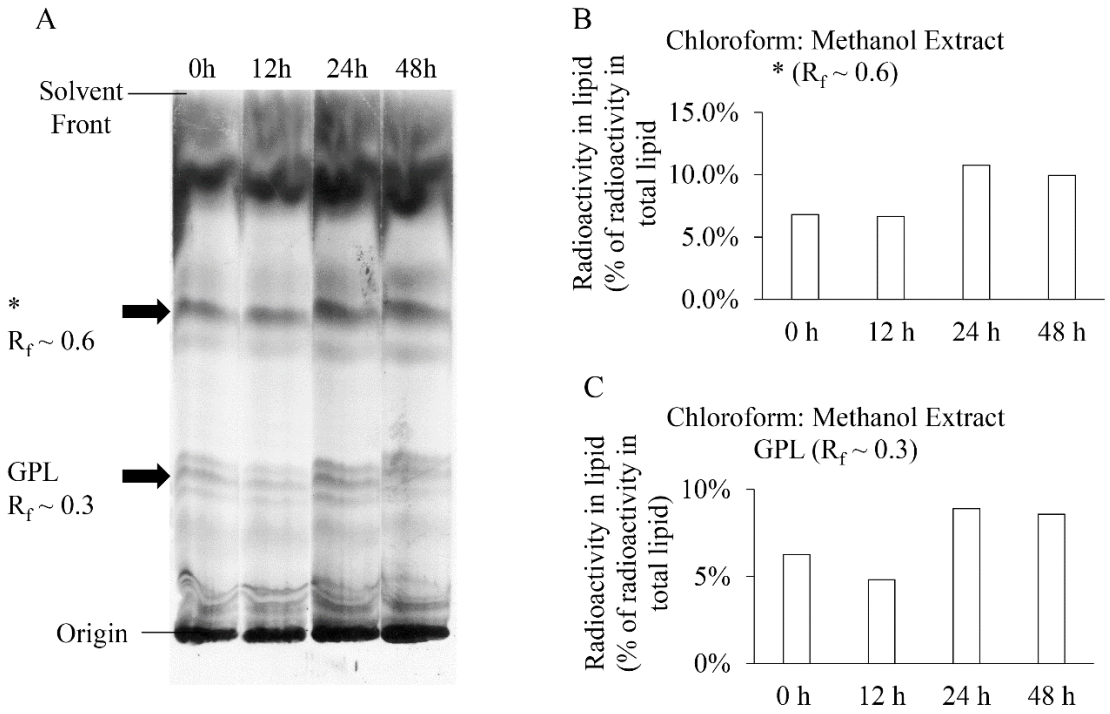

Figure 12. *M. abscessus* synthesizes increasing amounts of glycopeptidolipids during early biofilm formation. Cell-surface lipids were extracted with isopropanol and resolved on silica-TLC using chloroform/methanol/water (100:14:0.8, v/v/v) as solvent system. **A.** Autoradiogram of TLC plate showing unidentified lipid (*; R_f ~ 0.6) and glycopeptidolipids (GPL; R_f ~ 0.3). **B., C.** Radioactivity in unidentified lipid and in GPL shown as percent of radioactivity in total lipids. Data from one experiment shown.

3.6 *M. abscessus* cells in biofilms synthesize increasing levels of triacylglycerol and fatty acids

Total cellular lipids radiolabeled by ^{14}C-acetate were extracted from *M. abscessus* cells in biofilms and resolved on silica-TLC using hexane/diethyl ether/acetic acid (90:10:1) to identify neutral lipids. As shown in Figure 13 A, we identified the prominently-radiolabeled lipids as triacylglycerol (TAG; Rf ~ 0.58) and fatty acids (FA; R_f ~ 0.4) based on a previously published study (Daniel et al., 2011). The radioactivity in triacylglycerol was observed to be 4.9% of total lipid radioactivity at 0 hour and had increased to 8.5% by 48-hours of biofilm formation (Figure 13 B). Fatty acids demonstrated a slight increase in the amount of total radioactivity present in the total lipid concentration over the 48-hour period from 4.3% to 5.3% (Figure 13 C).

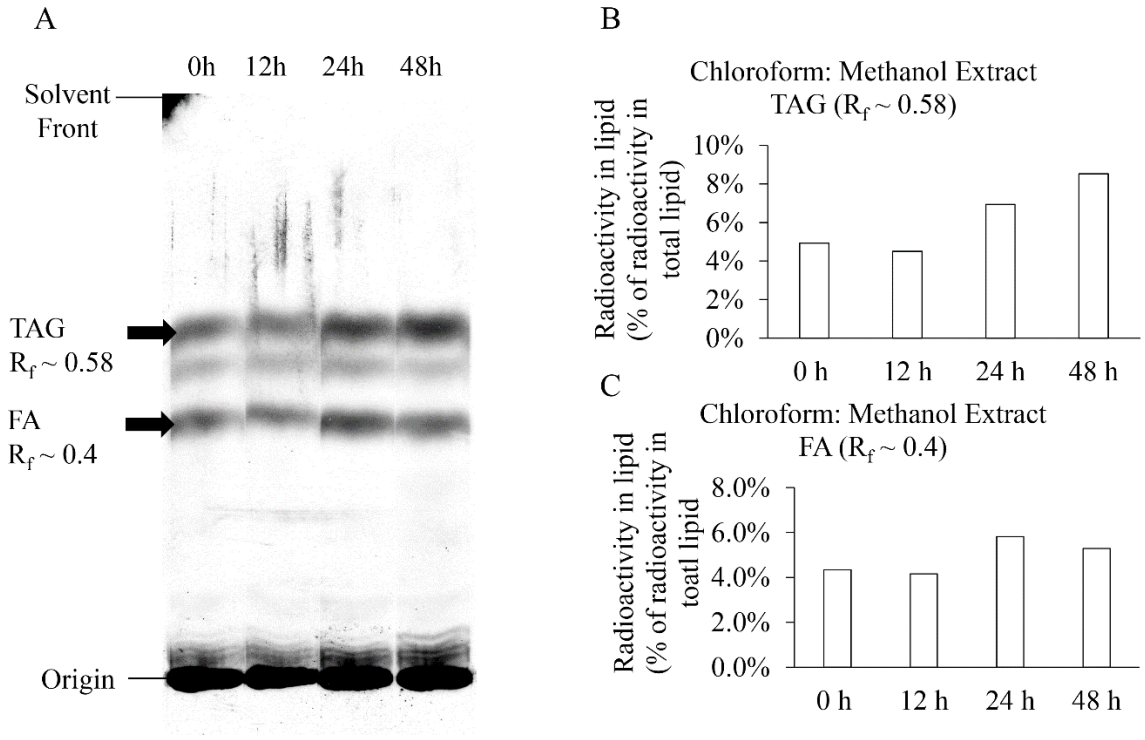

Figure 13. *M. abscessus* synthesizes increasing amounts of triacylglycerol during early biofilm. Cellular lipids were extracted with chloroform/methanol (2:1, v/v) and neutral lipids were resolved on silica-TLC using hexane/diethyl ether/acetic acid (90:10:1, v/v/v) as solvent system. **A.** Autoradiogram of TLC plate showing triacylglycerol (TAG; R_f ~ 0.58) and fatty acids (FA; R_f ~ 0.4). **B, C.** Radioactivity in TAG and FA shown respectively as percent of radioactivity in total lipids. Data from one experiment shown.

3.7 Metabolism of lipids is higher in planktonic cells than in biofilm cells

We investigated the metabolic turnover of lipids in *M. abscessus* in biofilms and in planktonic log-phase growth. Biofilm cells and planktonic log-phase cells were generated as described in Methods and incubated with ^{14}C-acetate for 6 h, 24 h and 72 h. During this incubation with the radiolabel, it was expected that the cells in a rapid growth state or lipids that were being metabolized at a high rate would show a shorter time of retention of the radioactivity while the cells in a slower growth phase or lipids that were being metabolized at a low rate would retain the radioactivity longer. We observed that radioactivity in the lipid extracts from cell-surface (isopropanol extracts) and from the total cell (chloroform/methanol extracts) followed a similar pattern. Radioactivity from planktonic log-phase cell lipids decreased with time but radioactivity from biofilm cell lipids remained stable overall (Figure 14). Radioactivity in cell-surface lipids from planktonic cells decreased from 143,902 (± 6700) DPM at 6 hours to 82,199 (± 2562) DPM

at 72 hours of incubation with the radiolabel suggesting a high rate of turnover. However, in biofilm cells, radioactivity remained stable at each time-point tested (Figure 14 A). A similar observation was made with radioactivity from total cellular lipids as the radioactivity in lipids from planktonic log-phase cells decreased from 459,000 (± SD 95,609) DPM at 6 hours to 277,200 (± SD 37,412) DPM at 72 hours (Figure 14 B).

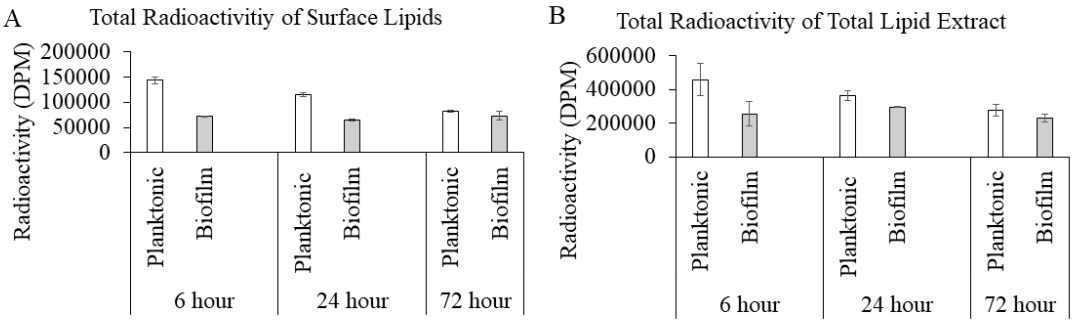

Figure 14. Uptake and incorporation of ^{14}C-acetate into surface lipids and total lipids by planktonic and biofilm cells of *M. abscessus*. The mycobacterial cells were incubated with the radiolabel as described in methods for the indicated time periods. **A.** Radioactivity incorporated into surface lipids extracted with isopropanol. **B.** Radioactivity incorporated into total cellular lipids extracted with chloroform/methanol. Data shown is average ± SD from replicates in the initial exploratory experiment. ANOVA was performed by Tukey's post-hoc test.

3.8 Trehalose mycolate lipids are synthesized and metabolized at high levels in *M. abscessus* biofilms

Cell-surface lipids were extracted from radiolabeled *M. abscessus* cells as described above and resolved on silica-TLC using chloroform/methanol/water (60:30:6) as the solvent system. As shown in Figure 15 A, we tentatively identified a prominently-radiolabeled lipid as trehalose mycolates ($R_f \sim 0.25$) based on previous literature (Howard et al., 2006; Hunt-Serracin et al., 2019; Rhoades et al., 2009). The radioactivity incorporated into TM decreased from 0.7 % at 6 hours to 0.1% of total lipid radioactivity at 72 hours. Radioactivity in TM in planktonic log-phase cells was much lower than biofilm cells suggesting that TM was not a major lipid synthesized in log-phase growth (Figure 15 C). We also observed the radioactivity in several lipids that were resolved (Figure 15 B, D, E). However, we could not identify these lipids since there are no reports of a lipid with such an R_f in this solvent system.

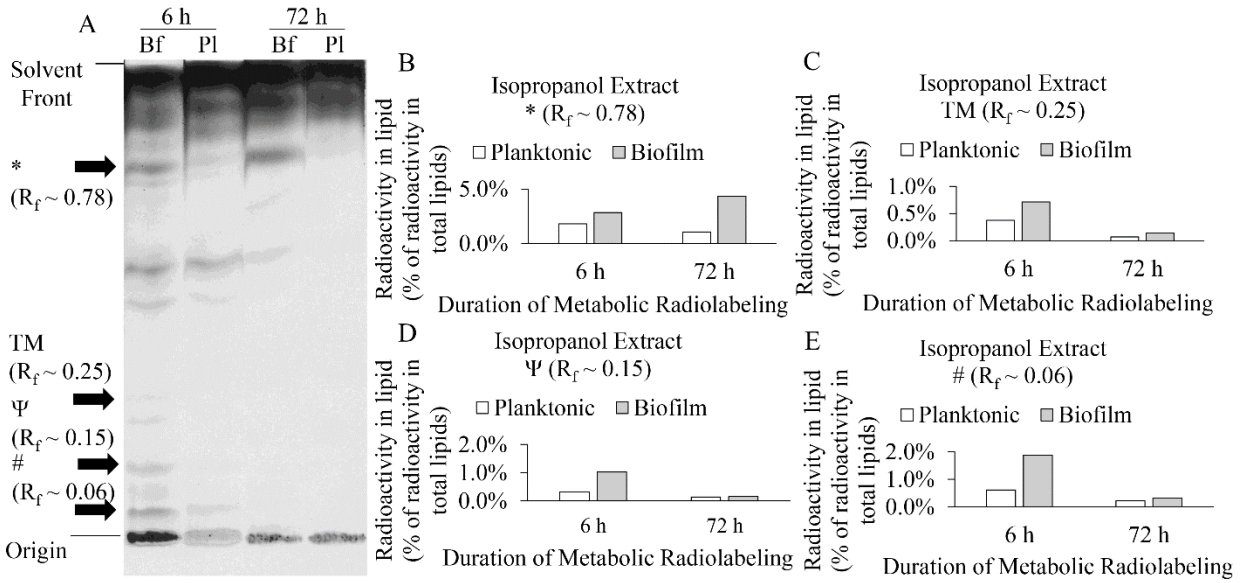

Figure 15. Trehalose mycolates decrease overtime after biofilm formation in *M. abscessus* cells. Cell-surface lipids were extracted with isopropanol and analyzed by silica-TLC using chloroform/methanol/water (60:30:6) as solvent system. **A.** Autoradiogram of TLC plate showing unidentified lipid * (R_f ~ 0.78), trehalose mycolates (R_f ~ 0.25), unidentified lipids Ψ (R_f ~ 0.15), and lipid # (R_f ~ 0.06). **B, C, D, E** Radioactivity in unidentified lipid * (R_f ~ 0.78), trehalose mycolates (R_f ~ 0.25), unidentified lipids Ψ (R_f ~ 0.15), and lipid # (R_f ~ 0.06), respectively, shown as percent of radioactivity in total lipids. Data from one experiment shown.

3.9 *M. abscessus* cells in biofilms synthesize high levels of glycopeptidolipids and trehalose mycolates

Cell-surface lipids radiolabeled with ^{14}C-acetate for 6 hours, 24 hours and 72 hours were resolved on chloroform/methanol/water (100:14:0.8) as the solvent system. As shown in Figure 16 A, we tentatively identified three prominently-radiolabeled lipids as glycopeptidolipids (GPL; R_f ~0.31) and trehalose mycolates (TM; R_f ~ 0.26, 0.19) based on previous reports (Howard et al., 2006; Hunt-Serracin et al., 2019; Rhoades et al., 2009). Radiolabel in glycopeptidolipids in biofilms increased with time from 6.8% of total lipid extract to 12.3% over a 72-hour period (Figure 16 B). TM_1 (Rf ~ 0.26) showed a two-fold increase in total lipid extract over the 72-hour period (Figure 16 C). There was no significant change for TM_2 (Rf ~ 0.19) over the 72-hour period (Figure 16 D). Our observations suggest that GPL and TM were synthesized at higher levels in biofilms compared to planktonic log-phase cells. However, these lipids were not being turned over rapidly in biofilms since the radiolabel persisted in this lipid for longer periods of time.

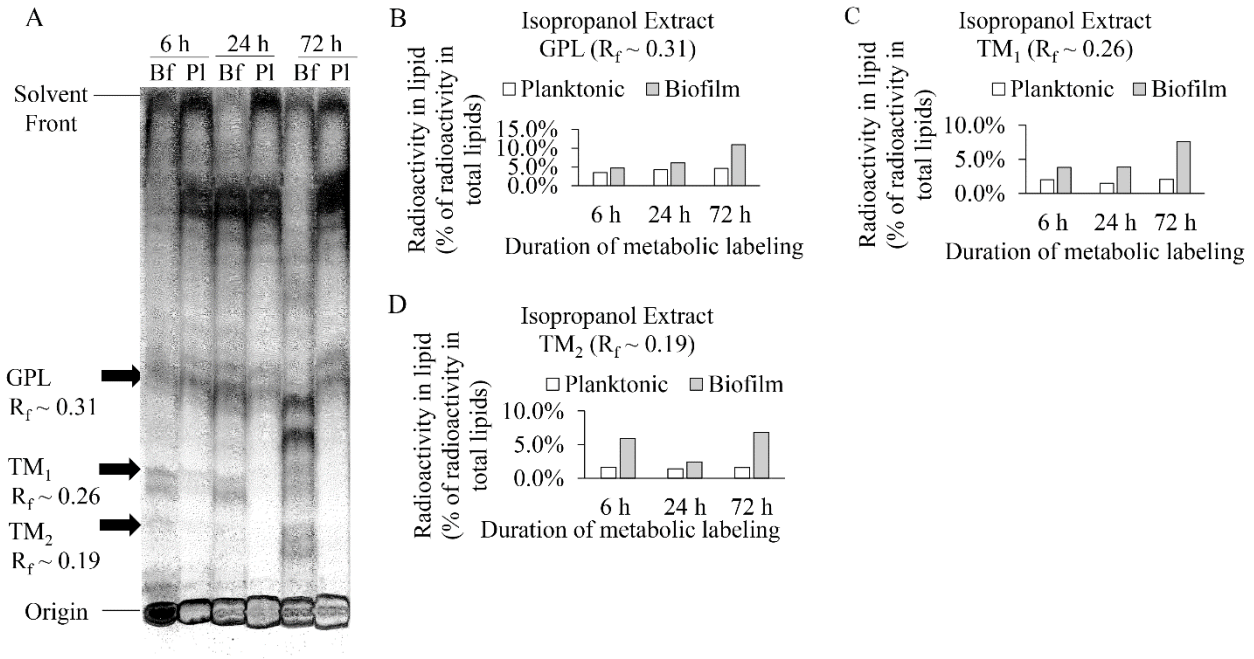

Figure 16. Glycopeptidolipids and trehalose mycolates in the cell surface are synthesized at high levels in *M. abscessus* biofilms. Cell-surface lipids were extracted with isopropanol and analyzed by silica-thin layer chromatography using chloroform/methanol/water (100:14:0.8, v/v/v) as solvent system. **A.** Autoradiogram of TLC plate showing glycopeptidolipids (GPL; Rf ~0.31), trehalose mycolates (TM_1; R_f ~ 0.26,), and TM_2 (R_f ~ 0.19). **B, C, D.** Radioactivity in lipids GPL (R_f ~ 0.31), TM_1 (R_f ~ 0.26) and TM_2 (R_f ~ 0.19) respectively, shown as percent of radioactivity in total lipids. Data from one experiment shown.

In total cellular lipids extracted from *M. abscessus* biofilms. Radiolabeled lipids from cells incubated with ^{14}C-acetate for 6 hours and 72 hours extracted using chloroform/methanol (2:1) were resolved on silica-TLC using chloroform/methanol/water (100:14:0.8) as the solvent system. We located a lipid band with R_f ~ 0.8 (Figure 17 A) that showed elevated levels of radioactivity in biofilm cells. We also tentatively identified the radiolabeled lipid band with R_f ~ 0.47 (Figure 17 A) as glycopeptidolipids (GPL) based on previous reports (Howard et al., 2006; Hunt-Serracin et al., 2019; Rhoades et al., 2009). The radioactivity in GPL in biofilm cells was about half the levels of GPL in planktonic cells at 6 hours but reached similar levels by 24 hours of metabolic labeling (Figure 17 C).

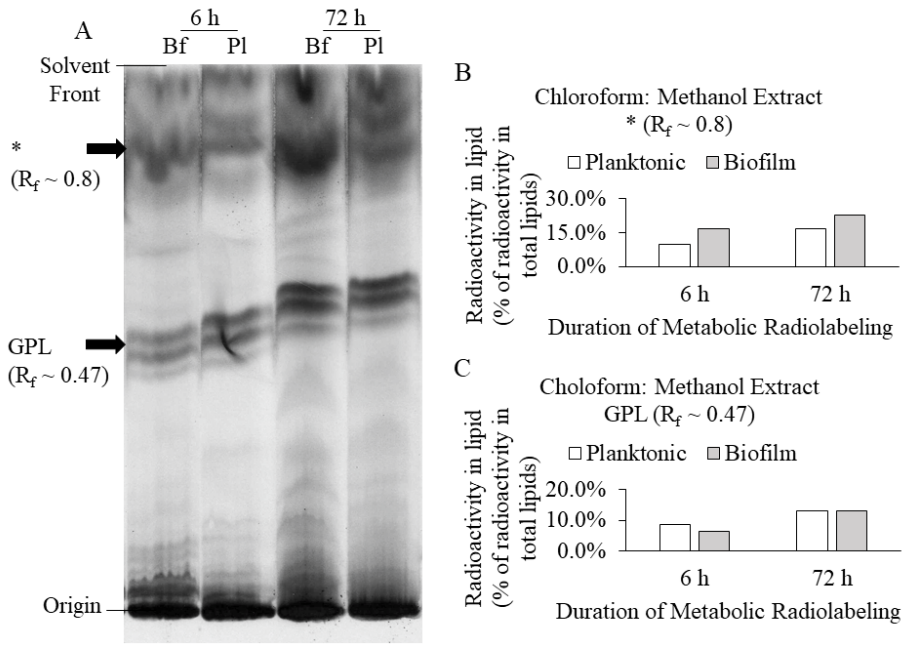

Figure 17. **Metabolic radiolabeling of glycopeptidolipids increases with incorporation time of ^{14}C-acetate into total lipid in *M. abscessus* biofilm cells.** Total lipids were extracted with chloroform: methanol and analyzed with by silica-thin layer chromatography using chloroform/methanol/ water (100:14:0.8, v/v/v) as solvent system. **A**. Autoradiogram of TLC plate unknown lipid (*; R_f ~ 0.47) and Glycopeptidolipids (GPL; R_f ~ 0.47). **B**, **C.** Radioactivity in unknown lipid (*; R_f~ 0.47) and Glycopeptidolipids (GPL; R_f~ 0.47), respectively was determined using a scintillation counter. Data from initial exploratory experiment shown.

3.10 Effects of the efflux pump inhibitor verapamil and exogenous fatty acids on the biosynthesis of lipids in *M. abscessus* biofilms

We postulated that efflux pumps in *M. abscessus* might be involved in the export of lipids to the cell surface of the bacterium and that exogenously provided fatty acids might influence the synthesis of those lipids synthesized during biofilm formation. In order to investigate this hypothesis, we induced biofilm formation in the presence of palmitic acid and treated the cells with verapamil, a known efflux pump inhibitor, immediately before radiolabel addition. The radioactivity in the total extracts of cell-surface (isopropanol extracted) lipids and total cellular (chloroform/methanol extracted) lipids from biofilm cells did not show major changes due to addition of verapamil or palmitic acid (Figure 18 A, B). However, we observed a marked decrease in the radioactivity levels in total cellular (chloroform/methanol extracted) lipids from planktonic log-phase cells due to a combined treatment of verapamil and palmitic acid (Figure 18 B).

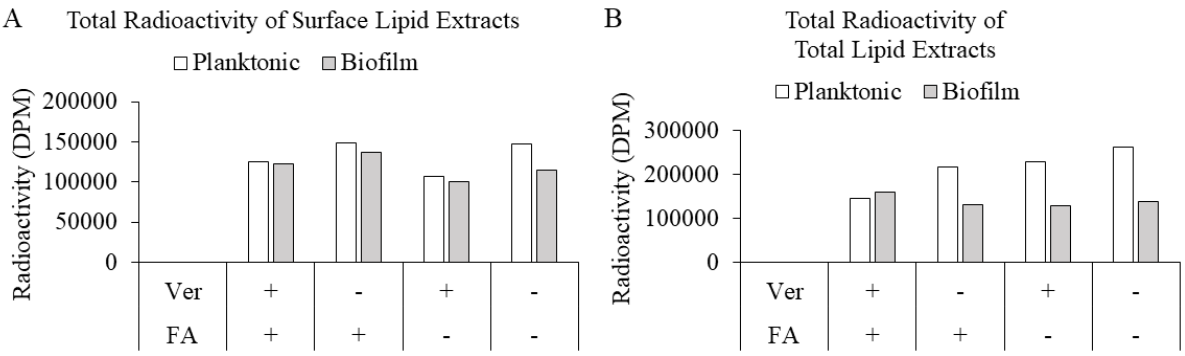

Figure 18. The biosynthesis of lipids by planktonic cells of *M. abscessus* is decreased by exogenous fatty acids and the efflux pump inhibitor verapamil. Surface lipids were extracted with isopropanol and total cellular lipids were extracted with chloroform/methanol (2:1, v/v). Radioactivity in lipid extracts was determined by scintillation counting. Data from one experiment shown.

3.11 Phosphatidylinositol mannoside synthesis in *M. abscessus* biofilm cells could utilize exogenous fatty acids when available

We investigated whether exogenously provided fatty acid would affect the synthesis of the phosphatidylinositol mannoside (PIM; $R_f \sim 0.3$; chloroform/methanol/water, 60:30:6 as solvent system) lipid in *M. abscessus* biofilms. We found that the incorporation of the ^{14}C-acetate radiolabel into PIM in total cellular lipids was diminished when exogenous fatty acids were provided (Figure 19 A, B). Radioactivity in PIM showed a two-fold decrease with the addition of exogenous fatty acid when compared to the biofilm control. Verapamil caused a decrease from 2.1% to 1.6% in the radioactivity incorporated into PIM.

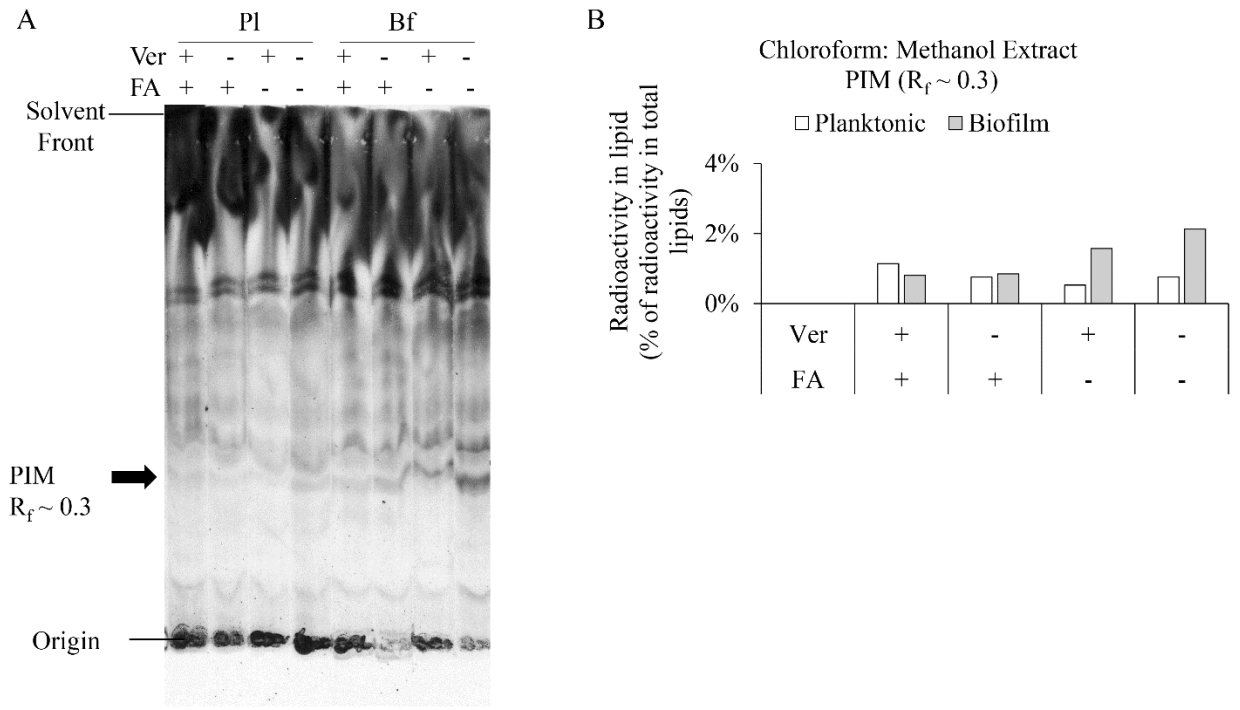

Figure 19. Synthesis of phosphatidylinositol mannosides by *M. abscessus* biofilm cells is diminished in the presence of exogenous fatty acids. Cellular lipids were extracted with chloroform/methanol (2:1, v/v) and were resolved on silica-TLC using chloroform/methanol/water (60:30:6, v/v/v) as solvent system. **A.** Autoradiogram of TLC plate showing phosphatidylinositol mannoside (PIM; Rf ~ 0.3). **B.** Radioactivity in PIM, shown as percent of radioactivity in total lipids. Data from one experiment shown.

3.12 Trehalose mycolates are inhibited by the addition of exogenous fatty acid and verapamil in 96-hour biofilm cells

Cellular lipids extracted with chloroform/methanol and resolved on silica-TLC using chloroform/methanol/water (100:14:0.8) as solvent system showed several lipid bands that were prominently radiolabeled. Among these, one lipid showed a marked decrease when cells were treated with the efflux pump inhibitor verapamil (Figure 20 A). We tentatively identified this lipid as trehalose mycolate (TM; R_f ~ 0.14) based on previous reports (Howard et al., 2006; Hunt-Serracin et al., 2019; Rhoades et al., 2009). We observed a two-fold decrease in biofilm cells in the radiolabeling of trehalose mycolate with the addition of exogenous fatty acid and verapamil when compared to the control. The presence of exogenous fatty acid caused trehalose mycolate to decrease from 11% to 7% of the total lipid content. Verapamil alone caused trehalose mycolate to decrease from 11% to 8% in total lipid content (Figure 20 B).

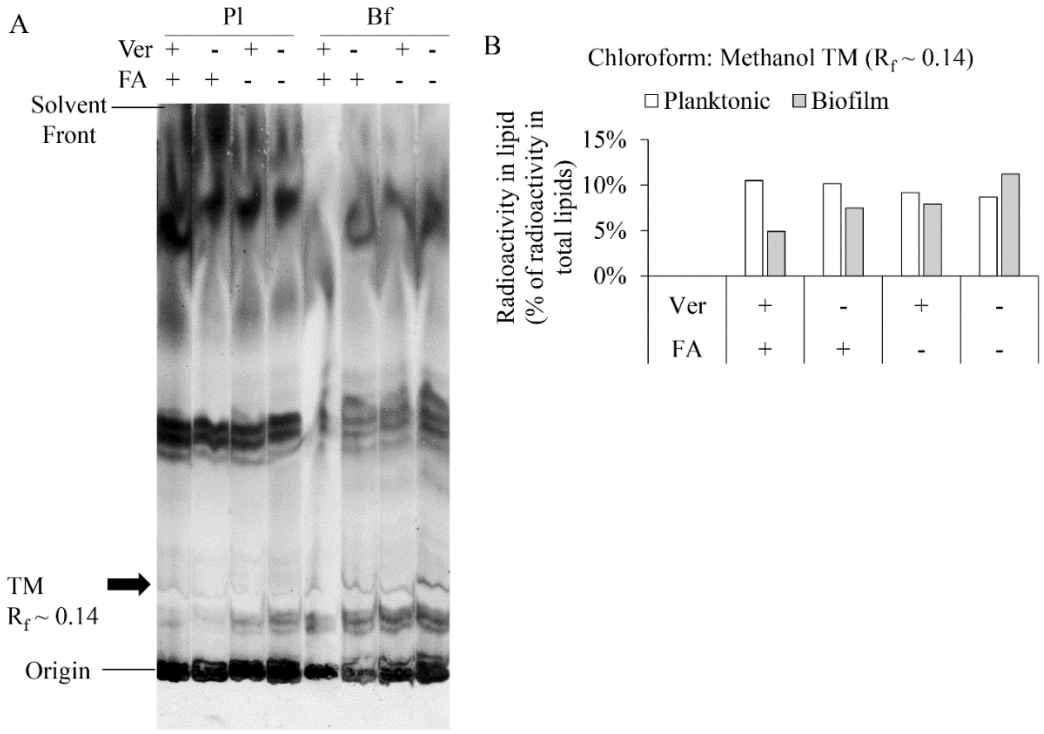

Figure 20. Biosynthesis of trehalose mycolate in biofilm cells is inhibited by exogenous fatty acids and the efflux pump inhibitor verapamil. Cellular lipids were extracted with chloroform/methanol (2:1, v/v) and analyzed by silica-TLC using chloroform/methanol/water (100:14:0.8, v/v/v) as solvent system. **A.** Autoradiogram of TLC plate showing trehalose mycolates (TM; Rf ~ 0.14) that was inhibited by fatty acid addition and by verapamil. **B.** Radioactivity in TM shown as a percent of radioactivity in respective total lipid extract. Data from one experiment shown.

CHAPTER 4. DISCUSSION

M. abscessus is an understudied organism in comparison to its relative, *Mtb*. This is extremely problematic as *M. abscessus* is rapidly becoming a more prevalent issue in developed nations than *Mtb* (Johansen et al., 2020; Tran et al., 2019). New insights are necessary to develop a more accurate understanding of *M. abscessus*. In this study, we co-opted an *Mtb* protocol that uses thiol-reductive stress to form biofilms. This method has never been utilized in *M. abscessus* and is notable because it creates a hypoxic environment that is like what *M. abscessus* would experience physiologically when infecting a host (Trivedi et al., 2016). This had the potential to produce more reliable model for *in vitro* studies when preparing for *in vivo* studies. This method was also not reliant on limiting nutrients and possesses less variables for easier scalability. It has also been shown that the type of media and access to nutrients do contribute to the biofilms ability to increase tolerance (Hunt-Serracin et al., 2019). This highlights the importance of developing *in vitro* models that replicate the physiological state of the environment that *M. abscessus* would experience during an infection. We discovered that *M. abscessus* biofilms were formed even in the absence of DTT but were stabilized when thiol reductive stress was present (Figure 8). This is different from *Mtb*, which did increase show an increase biofilm formation when subjected to thiol-reductive stress (Trivedi et al., 2016). We hypothesized that the biofilms that were formed would increase antibiotic tolerance. It was determined by CFU-plating and comparing the percent survivals of planktonic and biofilm cells induced by DTT did demonstrate an increase in antibiotic tolerance for biofilm cells (Figure 9).

The increase in antibiotic tolerance for bacteria has made it necessary for scientists to develop new methods that can be used for treatment. Efflux pump inhibitors directly affect one of the methods that bacteria utilize to limit the effectiveness of antibiotics. They have been able to use these compounds to lower the effective concentrations necessary to inhibit bacteria (Vianna et al., 2019). However, clinical knowledge on these compounds is not sufficient and there is no universal treatment plan available. Efflux pumps also play an important part in regulating the environment of the bacteria and play a part in homeostasis. They are heavily conserved across multiple organisms and it is possible to successfully insert bacterial genes for efflux pumps into human eukaryotic cell lines and still function properly (Briffotaux et al., 2017). This makes it difficult to incorporate them into potential treatment regimens because they could disrupt

important and necessary functions in human cells. Verapamil has shown synergies with several antibiotics used to treat *Mtb* infections and is currently used to treat other medical conditions.

However, many other compounds being utilized as efflux pump inhibitors are not as well studied in *M. abscessus*. Efflux pumps secrete many of the extracellular matrices that make up a biofilm such as various enzymes, lipids, proteins, and carbohydrates (de la Fuente-Núñez et al., 2013). These efflux pumps also influence biofilm formation by influencing aggregation. It has been shown that efflux pump inhibitors can be utilized to disrupt biofilm formation in other bacteria (Alav et al., 2018; Banerjee et al., 2000; Chen et al., 2018; Reza et al., 2019). However, no such studies have been reported in *M. abscessus*. We initially utilized high concentrations of efflux pump inhibitors to see if they could limit biofilm formation. The data we obtained showed that biofilm formation was disrupted at a statistically significant level by verapamil, CCCP, berberine, and piperine (Figure 10 A). Going forward, we wanted to see if concentrations could be reduced further and still influence disrupting biofilm formation. Of the ones tested, verapamil was the only one that still showed any statistically significant disruption of biofilm formation (Figure 10 B). However, this was only tested a single time and would need to be verified before suggesting using the lower concentration in further studies.

M. abscessus biofilms have been studied before, however most of this research has been focused on the presence of glycopeptidolipids (GPL). This lipid is not found in *Mtb* but can be found in fast-growing nontuberculous mycobacteria such as *M. abscessus*. GPL is present in the smooth morphotype and must be present in large quantities for *M. abscessus* to be able to develop into a biofilm (Greendyke & Byrd, 2008; Howard et al., 2006; Tran et al., 2019). Studies attempting to identify lipids present in *M. abscessus* are also limited beyond their focus on GPL. There have been no other studies focusing on the lipid composition of biofilms induced using thiol-reductive stress in *M. abscessus*. We used ^{14}C-acetate metabolic radiolabeling, which is a technique regularly used to learn about lipids in mycobacteria (Bansal-Mutalik & Nikaido, 2014; Daniel et al., 2014; Deb et al., 2009; Etienne et al., 2002; Howard et al., 2006; Hunt-Serracin et al., 2019). Using acetate, a building block for fatty acid synthesis and lipid formation, we radiolabeled lipids that could be separated by thin layer chromatography and then quantified by scintillation counting. Using this technique were able to tentatively identify lipids that are present and elevated during biofilm formation by comparing the retention factor (R_f) values from the same solvent systems.

The lipids that were upregulated during biofilm formation and were tentatively identified are trehalose mycolates (TM), phosphatidylinositol mannosides (PIMs), glycopeptidolipids (GPL), triacylglycerols (TAG), fatty acids (FA) and diacylglycerols. During our experiment on early biofilm formation, we were able to quantify that TM and several PIMs showed elevated levels of biosynthesis over the 48-hour period of early biofilm formation (Figure 11). GPL also demonstrated elevated levels of lipid biosynthesis during early biofilm formation (Figure 12). For neutral lipids, we determined that TAG showed elevated levels of lipid biosynthesis during biofilm formation while FA only showed a slight increase (Figure 13).

We also investigated lipids metabolized at a high rate in biofilms after 96-hours of biofilm formation. We determined that TM initially shows elevated levels of lipid biosynthesis, which it obtained from early biofilm formation before decreasing to reach similar levels in planktonic cells (Figure 15). This was the opposite of GPL and the TMs, which continued to increase in lipid concentration after 96-hours of biofilm formation in outer surface lipids (Figure 16). However, in total cell lipids it was determined that GPL levels will increase overtime but will match turnover rate that is present in planktonic cells (Figure 17). The stable accumulation of radiolabel in GPL and TM suggests that these lipids are not metabolized at a high rate in biofilms on surface lipids.

Our investigation into the effects of exogenous fatty acid and efflux pump inhibitor verapamil on biofilm formation showed decreased metabolic labeling detected for PIM when in the presence of exogenous fatty acid and verapamil. Metabolic labeling was still decreased when either exogenous fatty acid or efflux pump inhibitor was added (Figure 18). This was also discovered to be the case for TM as well (Figure 20). Since the ^{14}C-acetate radiolabel is a precursor for endogenous fatty acid synthesis, the reduction in the radiolabel in PIM when exogenous fatty acid was provided suggests that *M. abscessus* could utilize fatty acids from an exogenous source, when available, to synthesize PIM during biofilm formation. Our observations suggest that exogenous fatty acids could substitute endogenous fatty acids in trehalose mycolate synthesis in biofilms. Furthermore, the ability of verapamil to decrease radiolabeling of trehalose mycolates suggests that efflux pumps appear to be involved in the biosynthesis of trehalose mycolates in biofilms of *M. abscessus*. We were unable to perform any statistical analysis on our lipid experiments, which is a result of these studies being exploratory.

These lipids have many functions ranging from structural integrity to modulating immune responses. We can infer the function of some lipids based on studies from *Mtb*. However, our

comprehensive knowledge is lacking. Glycopeptidolipids (GPL) are not present in *Mtb* and can only be found present in nontuberculous mycobacteria. Phenolic glycolipids (PGL) are found in *Mtb* but not in *M. abscessus*. Many of these lipids are also limited only to bacteria, which make them ideal candidates to be targeted by new antibiotics in the future. It has been shown that these lipids play an important part in modulating an immune response (Daffé et al., 2014; Tran et al., 2019). Other lipids include: triacylglycerols, fatty acids, and diacylglycerols. Triacylglycerols play an important part in lipid-accumulation for mycobacteria and can be utilized as an energy source for the bacteria when they enter dormancy. We expected and found elevated levels of triacylglycerols to be present in the biofilm cells because the mycobacteria do not have equal access to nutrients which would change their metabolic state and potentially induce dormancy on the bacteria (Daniel et al., 2011). TMs are not synthesized by any mammals, which has made them a potential target to be used to disrupt mycobacteria (Daffé et al., 2014). They can be incorporated in various glycolipids that help modulate the immune system. They also have been found to contribute to structural integrity of the cell wall in mycobacterium (Tran et al., 2019). PIMs can be found on the inner and outer membrane of *M. abscessus* and usually work to interfere with phagosome maturation (Tran et al., 2019). Further investigation is needed for understanding the roles of these lipids in biofilm formation in *M. abscessus*.

CHAPTER 5. FUTURE STUDIES

The role of efflux pump inhibitors such as verapamil in the lipid biosynthesis and accumulation during early stages of biofilm formation will be investigated using studies involving radiolabeling with lipid precursors and non-radiolabeled lipid accumulation. The effect of exogenous fatty acids will also be investigated in these future studies. Our previous research has shown that the presence of exogenous fatty acid lowers the concentration of several lipid that were tentatively identified. Many of these lipids are hydrophobic and have properties that modulate the immune system. The inhibition of these lipids could decrease the effectiveness of the biofilm and contribute to lowered antibiotic tolerance. It would also be beneficial to separate lipids more using two-dimensional TLC utilizing several different solvent systems at different times for the same experiment. Many of the lipids we tentatively identified were an entire class of lipid and did not go into specifics. A two-dimensional TLC analysis would enable us to tentatively identify these lipids more specifically. It would also be beneficial to properly identify the lipids that were present. Currently we have only been able to tentatively identify them by comparing R_f values from other papers that use the same organism and solvent system, which is not the most accurate method. To properly identify these lipids, Gas-chromatography mass spectrometry (GC-MS) is needed. Another future experiment would be the reverse micellar extraction of the cellular membranes of *M. abscessus* when in a biofilm state (Bansal-Mutalik & Nikaido, 2014). This method would separate the inner membrane, outer membrane, and total lipids from one another and allow for accurate classifications and percentages of lipids that would be present in the cells at that time.

REFERENCES

Abd El-Baky, R. M., Sandle, T., John, J., Abuo-Rahma, G. E.-D. A. A., & Hetta, H. F. (2019, 2019/06/14/). A novel mechanism of action of ketoconazole: inhibition of the NorA efflux pump system and biofilm formation in multidrug-resistant Staphylococcus aureus. *Infection and Drug Resistance, 12*, 1703-1718. https://doi.org/10.2147/IDR.S201124

Alav, I., Sutton, J. M., & Rahman, K. M. (2018, 2018//08/01). Role of bacterial efflux pumps in biofilm formation. *The Journal of Antimicrobial Chemotherapy, 73*(8), 2003-2020. https://doi.org/10.1093/jac/dky042

Banerjee, S. K., Bhatt, K., Misra, P., & Chakraborti, P. K. (2000, 2000/01/01/). Involvement of a natural transport system in the process of efflux-mediated drug resistance in Mycobacterium smegmatis. *Molecular and General Genetics MGG, 262*(6), 949-956. https://doi.org/10.1007/PL00008663

Bansal-Mutalik, R., & Nikaido, H. (2014, 2014/04/01/). Mycobacterial outer membrane is a lipid bilayer and the inner membrane is unusually rich in diacyl phosphatidylinositol dimannosides. *Proceedings of the National Academy of Sciences, 111*(13), 4958-4963. https://doi.org/10.1073/pnas.1403078111

Bansal, A., Mallik, D., Kar, D., & Ghosh, A. S. (2016, Jul). Identification of a multidrug efflux pump in Mycobacterium smegmatis. *FEMS Microbiol Lett, 363*(13). https://doi.org/10.1093/femsle/fnw128

Baugh, S., Ekanayaka, A. S., Piddock, L. J. V., & Webber, M. A. (2012, 2012/10//). Loss of or inhibition of all multidrug resistance efflux pumps of Salmonella enterica serovar Typhimurium results in impaired ability to form a biofilm. *The Journal of Antimicrobial Chemotherapy, 67*(10), 2409-2417. https://doi.org/10.1093/jac/dks228

Baugh, S., Phillips, C. R., Ekanayaka, A. S., Piddock, L. J. V., & Webber, M. A. (2014, 2014/03//). Inhibition of multidrug efflux as a strategy to prevent biofilm formation. *The Journal of Antimicrobial Chemotherapy, 69*(3), 673-681. https://doi.org/10.1093/jac/dkt420

Becker, B., & Cooper, M. A. (2013, 2013/01/18/). Aminoglycoside antibiotics in the 21st century. *ACS Chemical Biology, 8*(1), 105-115. https://doi.org/10.1021/cb3005116

Bento, C. M., Gomes, M. S., & Silva, T. (2020, 2020/01/03/). Looking beyond Typical Treatments for Atypical Mycobacteria. *Antibiotics, 9*(1). https://doi.org/10.3390/antibiotics9010018

Bernard, C., Veziris, N., Brossier, F., Sougakoff, W., Jarlier, V., Robert, J., & Aubry, A. (2015, 2015/03//). Molecular diagnosis of fluoroquinolone resistance in Mycobacterium tuberculosis. *Antimicrobial Agents and Chemotherapy, 59*(3), 1519-1524. https://doi.org/10.1128/AAC.04058-14

Blanco, P., Hernando-Amado, S., Reales-Calderon, J. A., Corona, F., Lira, F., Alcalde-Rico, M., Bernardini, A., Sanchez, M. B., & Martinez, J. L. (2016, 2016/02/16/). Bacterial Multidrug Efflux Pumps: Much More Than Antibiotic Resistance Determinants. *Microorganisms, 4*(1). https://doi.org/10.3390/microorganisms4010014

Briffotaux, J., Huang, W., Wang, X., & Gicquel, B. (2017, 2017//12/). MmpS5/MmpL5 as an efflux pump in Mycobacterium species. *Tuberculosis, 107*, 13-19. https://doi.org/10.1016/j.tube.2017.08.001

Chen, C., Gardete, S., Jansen, R. S., Shetty, A., Dick, T., Rhee, K. Y., & Dartois, V. (2018, 2018//05/). Verapamil Targets Membrane Energetics in Mycobacterium tuberculosis. *Antimicrobial Agents and Chemotherapy, 62*(5). https://doi.org/10.1128/AAC.02107-17

Clary, G., Sasindran, S. J., Nesbitt, N., Mason, L., Cole, S., Azad, A., McCoy, K., Schlesinger, L. S., & Hall-Stoodley, L. (2018, 2018//03/). Mycobacterium abscessus Smooth and Rough Morphotypes Form Antimicrobial-Tolerant Biofilm Phenotypes but Are Killed by Acetic Acid. *Antimicrobial Agents and Chemotherapy, 62*(3). https://doi.org/10.1128/AAC.01782-17

Daffé, M., Crick, D. C., & Jackson, M. (2014, 2014/08//). Genetics of Capsular Polysaccharides and Cell Envelope (Glyco)lipids. *Microbiology Spectrum, 2*(4), MGM2-0021-2013. https://doi.org/10.1128/microbiolspec.MGM2-0021-2013

Dal Molin, M., Gut, M., Rominski, A., Haldimann, K., Becker, K., & Sander, P. (2017, 2017/12/21/). Molecular Mechanisms of Intrinsic Streptomycin Resistance in Mycobacterium abscessus. *Antimicrobial Agents and Chemotherapy, 62*(1). https://doi.org/10.1128/AAC.01427-17

Daniel, J., Deb, C., Dubey, V. S., Sirakova, T. D., Abomoelak, B., Morbidoni, H. R., & Kolattukudy, P. E. (2004, Aug). Induction of a novel class of diacylglycerol acyltransferases and triacylglycerol accumulation in Mycobacterium tuberculosis as it goes into a dormancy-like state in culture. *Journal of Bacteriology, 186*(15), 5017-5030. https://doi.org/10.1128/jb.186.15.5017-5030.2004

Daniel, J., Kapoor, N., Sirakova, T., Sinha, R., & Kolattukudy, P. (2016, 2016//09/). The perilipin-like PPE15 protein in Mycobacterium tuberculosis is required for triacylglycerol accumulation under dormancy-inducing conditions. *Molecular Microbiology, 101*(5), 784-794. https://doi.org/10.1111/mmi.13422

Daniel, J., Maamar, H., Deb, C., Sirakova, T. D., & Kolattukudy, P. E. (2011, 2011/06//). Mycobacterium tuberculosis uses host triacylglycerol to accumulate lipid droplets and acquires a dormancy-like phenotype in lipid-loaded macrophages. *PLoS Pathogens, 7*(6), e1002093. https://doi.org/10.1371/journal.ppat.1002093

Daniel, J., Sirakova, T., & Kolattukudy, P. (2014, 2014). An acyl-CoA synthetase in Mycobacterium tuberculosis involved in triacylglycerol accumulation during dormancy. *PLoS ONE, 9*(12), e114877. https://doi.org/10.1371/journal.pone.0114877

de la Fuente-Núñez, C., Reffuveille, F., Fernández, L., & Hancock, R. E. W. (2013, 2013/10/01/). Bacterial biofilm development as a multicellular adaptation: antibiotic resistance and new therapeutic strategies. *Current Opinion in Microbiology, 16*(5), 580-589. https://doi.org/10.1016/j.mib.2013.06.013 (Antimicrobials • Genomics)

Deb, C., Lee, C.-M., Dubey, V. S., Daniel, J., Abomoelak, B., Sirakova, T. D., Pawar, S., Rogers, L., & Kolattukudy, P. E. (2009, 2009/06/29/). A novel in vitro multiple-stress dormancy model for Mycobacterium tuberculosis generates a lipid-loaded, drug-tolerant, dormant pathogen. *PLoS ONE, 4*(6), e6077. https://doi.org/10.1371/journal.pone.0006077

Dinos, G. P. (2017, 2017/09//). The macrolide antibiotic renaissance. *British Journal of Pharmacology, 174*(18), 2967-2983. https://doi.org/10.1111/bph.13936

Etienne, G., Villeneuve, C., Billman-Jacobe, H., Astarie-Dequeker, C., Dupont, M.-A., & Daffé, M. (2002, 2002/10/01/). The impact of the absence of glycopeptidolipids on the ultrastructure, cell surface and cell wall properties, and phagocytosis of Mycobacterium smegmatis. *Microbiology, 148*(10), 3089-3100. https://doi.org/10.1099/00221287-148-10-3089

Flemming, H.-C., & Wingender, J. (2010, 2010/09/01/). The biofilm matrix. *Nature Reviews Microbiology, 8*(9), 623-633. https://doi.org/10.1038/nrmicro2415

Fyfe, C., Grossman, T. H., Kerstein, K., & Sutcliffe, J. (2016, 2016/10/03/). Resistance to Macrolide Antibiotics in Public Health Pathogens. *Cold Spring Harbor Perspectives in Medicine, 6*(10). https://doi.org/10.1101/cshperspect.a025395

Greendyke, R., & Byrd, T. F. (2008, 2008/06//). Differential Antibiotic Susceptibility of Mycobacterium abscessus Variants in Biofilms and Macrophages Compared to That of Planktonic Bacteria. *Antimicrobial Agents and Chemotherapy, 52*(6), 2019-2026. https://doi.org/10.1128/AAC.00986-07

He, X., & Ahn, J. (2011, Dec). Differential gene expression in planktonic and biofilm cells of multiple antibiotic-resistant Salmonella Typhimurium and Staphylococcus aureus. *FEMS Microbiology Letters, 325*(2), 180-188. https://doi.org/10.1111/j.1574-6968.2011.02429.x

He, X., Lu, F., Yuan, F., Jiang, D., Zhao, P., Zhu, J., Cheng, H., Cao, J., & Lu, G. (2015, Aug). Biofilm Formation Caused by Clinical Acinetobacter baumannii Isolates Is Associated with Overexpression of the AdeFGH Efflux Pump. *Antimicrobial Agents and Chemotherapy, 59*(8), 4817-4825. https://doi.org/10.1128/aac.00877-15

Heersema, L. A., & Smyth, H. D. C. (2019, 2019/05/30/). A Multispecies Biofilm In Vitro Screening Model of Dental Caries for High-Throughput Susceptibility Testing. *High-Throughput, 8*(2). https://doi.org/10.3390/ht8020014

Howard, S. T., Rhoades, E., Recht, J., Pang, X., Alsup, A., Kolter, R., Lyons, C. R., & Byrd, T. F. (2006, 2006/06//). Spontaneous reversion of Mycobacterium abscessus from a smooth to a rough morphotype is associated with reduced expression of glycopeptidolipid and reacquisition of an invasive phenotype. *Microbiology, 152*(Pt 6), 1581-1590. https://doi.org/10.1099/mic.0.28625-0

Hunt-Serracin, A. C., Parks, B. J., Boll, J., & Boutte, C. C. (2019, 2019//07/). Mycobacterium abscessus Cells Have Altered Antibiotic Tolerance and Surface Glycolipids in Artificial Cystic Fibrosis Sputum Medium. *Antimicrobial Agents and Chemotherapy, 63*(7). https://doi.org/10.1128/AAC.02488-18

Ito, A., Taniuchi, A., May, T., Kawata, K., & Okabe, S. (2009, Jun). Increased antibiotic resistance of Escherichia coli in mature biofilms. *Applied and Environmental Microbiology, 75*(12), 4093-4100. https://doi.org/10.1128/aem.02949-08

Johansen, M. D., Herrmann, J.-L., & Kremer, L. (2020, 2020/07//). Non-tuberculous mycobacteria and the rise of Mycobacterium abscessus. *Nature Reviews Microbiology, 18*(7), 392-407. https://doi.org/10.1038/s41579-020-0331-1

Kvist, M., Hancock, V., & Klemm, P. (2008, Dec). Inactivation of efflux pumps abolishes bacterial biofilm formation. *Applied and Environmental Microbiology, 74*(23), 7376-7382. https://doi.org/10.1128/aem.01310-08

Lee, M.-R., Sheng, W.-H., Hung, C.-C., Yu, C.-J., Lee, L.-N., & Hsueh, P.-R. (2015, 2015/09//). Mycobacterium abscessus Complex Infections in Humans. *Emerging Infectious Diseases, 21*(9), 1638-1646. https://doi.org/10.3201/2109.141634

Lopeman, R. C., Harrison, J., Desai, M., & Cox, J. A. G. (2019, 2019/03/22/). Mycobacterium abscessus: Environmental Bacterium Turned Clinical Nightmare. *Microorganisms, 7*(3). https://doi.org/10.3390/microorganisms7030090

Marini, E., Di Giulio, M., Ginestra, G., Magi, G., Di Lodovico, S., Marino, A., Facinelli, B., Cellini, L., & Nostro, A. (2019, 2019/07/01/). Efficacy of carvacrol against resistant rapidly growing mycobacteria in the planktonic and biofilm growth mode. *PLoS ONE, 14*(7). https://doi.org/10.1371/journal.pone.0219038

Martinez, J. L., Sánchez, M. B., Martínez-Solano, L., Hernandez, A., Garmendia, L., Fajardo, A., & Alvarez-Ortega, C. (2009, 2009/03//). Functional role of bacterial multidrug efflux pumps in microbial natural ecosystems. *FEMS Microbiology Reviews, 33*(2), 430-449. https://doi.org/10.1111/j.1574-6976.2008.00157.x

Matsumura, K., Furukawa, S., Ogihara, H., & Morinaga, Y. (2011, 2011/06//). Roles of multidrug efflux pumps on the biofilm formation of Escherichia coli K-12. *Biocontrol Science, 16*(2), 69-72. https://doi.org/10.4265/bio.16.69

Mavi, P. S., Singh, S., & Kumar, A. (2019, 2019/11/19/). Reductive Stress: New Insights in Physiology and Drug Tolerance of Mycobacterium. *Antioxidants & Redox Signaling*. https://doi.org/10.1089/ars.2019.7867

Miller, M. B., & Bassler, B. L. (2001, 2001). Quorum sensing in bacteria. *Annual Review of Microbiology, 55*, 165-199. https://doi.org/10.1146/annurev.micro.55.1.165

Moore, M., & Frerichs, J. B. (1953, 1953/02//). An Unusual Acid-Fast Infection of the Knee with Subcutaneous, Abscess-Like Lesions of the Gluteal Region. *Journal of Investigative Dermatology, 20*(2), 133-169. https://doi.org/10.1038/jid.1953.18

Nikaido, H. (2009, 2009). Multidrug resistance in bacteria. *Annual Review of Biochemistry, 78*, 119-146. https://doi.org/10.1146/annurev.biochem.78.082907.145923

Pang, J. M., Layre, E., Sweet, L., Sherrid, A., Moody, D. B., Ojha, A., & Sherman, D. R. (2012, 2012/02//). The polyketide Pks1 contributes to biofilm formation in Mycobacterium tuberculosis. *Journal of Bacteriology, 194*(3), 715-721. https://doi.org/10.1128/JB.06304-11

Pearson, J. P., Van Delden, C., & Iglewski, B. H. (1999, Feb). Active efflux and diffusion are involved in transport of Pseudomonas aeruginosa cell-to-cell signals. *Journal of Bacteriology, 181*(4), 1203-1210. https://doi.org/10.1128/jb.181.4.1203-1210.1999

Pule, C. M., Sampson, S. L., Warren, R. M., Black, P. A., van Helden, P. D., Victor, T. C., & Louw, G. E. (2016, 2016/01//). Efflux pump inhibitors: targeting mycobacterial efflux systems to enhance TB therapy. *The Journal of Antimicrobial Chemotherapy, 71*(1), 17-26. https://doi.org/10.1093/jac/dkv316

Reza, A., Sutton, J. M., & Rahman, K. M. (2019, 2019/11/19/). Effectiveness of Efflux Pump Inhibitors as Biofilm Disruptors and Resistance Breakers in Gram-Negative (ESKAPEE) Bacteria. *Antibiotics, 8*(4). https://doi.org/10.3390/antibiotics8040229

Rhoades, E. R., Archambault, A. S., Greendyke, R., Hsu, F.-F., Streeter, C., & Byrd, T. F. (2009, 2009/08/01/). Mycobacterium abscessus Glycopeptidolipids Mask Underlying Cell Wall Phosphatidyl-myo-Inositol Mannosides Blocking Induction of Human Macrophage TNF-α by Preventing Interaction with TLR2. *The Journal of Immunology, 183*(3), 1997-2007. https://doi.org/10.4049/jimmunol.0802181

Richards, J. P., & Ojha, A. K. (2014, 2014/10//). Mycobacterial Biofilms. *Microbiology Spectrum, 2*(5). https://doi.org/10.1128/microbiolspec.MGM2-0004-2013

Rücker, N., Billig, S., Bücker, R., Jahn, D., Wittmann, C., & Bange, F.-C. (2015, 2015/10//). Acetate Dissimilation and Assimilation in Mycobacterium tuberculosis Depend on Carbon Availability. *Journal of Bacteriology, 197*(19), 3182-3190. https://doi.org/10.1128/JB.00259-15

Rüger, K., Hampel, A., Billig, S., Rücker, N., Suerbaum, S., & Bange, F.-C. (2014, 2014/01/01/). Characterization of Rough and Smooth Morphotypes of Mycobacterium abscessus Isolates from Clinical Specimens. *Journal of Clinical Microbiology, 52*(1), 244-250. https://doi.org/10.1128/JCM.01249-13

Ryan, K., & Byrd, T. F. (2018, 2018). Mycobacterium abscessus: Shapeshifter of the Mycobacterial World. *Frontiers in Microbiology, 9*, 2642. https://doi.org/10.3389/fmicb.2018.02642

Schembri, M. A., Kjaergaard, K., & Klemm, P. (2003, Apr). Global gene expression in Escherichia coli biofilms. *Molecular Microbiology, 48*(1), 253-267. https://doi.org/10.1046/j.1365-2958.2003.03432.x

Shaw, L. P., Doyle, R. M., Kavaliunaite, E., Spencer, H., Balloux, F., Dixon, G., & Harris, K. A. (2019, 2019/10/30/). Children With Cystic Fibrosis Are Infected With Multiple Subpopulations of Mycobacterium abscessus With Different Antimicrobial Resistance Profiles. *Clinical Infectious Diseases, 69*(10), 1678-1686. https://doi.org/10.1093/cid/ciz069

Stewart, P. S., & Franklin, M. J. (2008, Mar). Physiological heterogeneity in biofilms. *Nature Reviews Microbiology, 6*(3), 199-210. https://doi.org/10.1038/nrmicro1838

Strollo, S. E., Adjemian, J., Adjemian, M. K., & Prevots, D. R. (2015, 2015/10//). The Burden of Pulmonary Nontuberculous Mycobacterial Disease in the United States. *Annals of the American Thoracic Society, 12*(10), 1458-1464. https://doi.org/10.1513/AnnalsATS.201503-173OC

Subhadra, B., Kim, D. H., Woo, K., Surendran, S., & Choi, C. H. (2018, 2018/09/10/). Control of Biofilm Formation in Healthcare: Recent Advances Exploiting Quorum-Sensing Interference Strategies and Multidrug Efflux Pump Inhibitors. *Materials, 11*(9). https://doi.org/10.3390/ma11091676

Towse, A., Hoyle, C. K., Goodall, J., Hirsch, M., Mestre-Ferrandiz, J., & Rex, J. H. (2017, 2017/10/01/). Time for a change in how new antibiotics are reimbursed: Development of an insurance framework for funding new antibiotics based on a policy of risk mitigation. *Health Policy, 121*(10), 1025-1030. https://doi.org/10.1016/j.healthpol.2017.07.011

Tran, T., Bonham, A. J., Chan, E. D., & Honda, J. R. (2019, 2019/03//). A paucity of knowledge regarding nontuberculous mycobacterial lipids compared to the tubercle bacillus. *Tuberculosis, 115*, 96-107. https://doi.org/10.1016/j.tube.2019.02.008

Trivedi, A., Mavi, P. S., Bhatt, D., & Kumar, A. (2016, 2016/04/25/). Thiol reductive stress induces cellulose-anchored biofilm formation in Mycobacterium tuberculosis. *Nature Communications, 7*. https://doi.org/10.1038/ncomms11392

Vianna, J. S., Machado, D., Ramis, I. B., Silva, F. P., Bierhals, D. V., Abril, M. A., von Groll, A., Ramos, D. F., Lourenço, M. C. S., Viveiros, M., & da Silva, P. E. A. (2019, 2019/09/18/). The Contribution of Efflux Pumps in Mycobacterium abscessus Complex Resistance to Clarithromycin. *Antibiotics, 8*(3). https://doi.org/10.3390/antibiotics8030153

Viljoen, A., Blaise, M., de Chastellier, C., & Kremer, L. (2016, 2016//11/). MAB_3551c encodes the primary triacylglycerol synthase involved in lipid accumulation in Mycobacterium abscessus. *Molecular Microbiology, 102*(4), 611-627. https://doi.org/10.1111/mmi.13482

Waite, R. D., Papakonstantinopoulou, A., Littler, E., & Curtis, M. A. (2005, Sep). Transcriptome analysis of Pseudomonas aeruginosa growth: comparison of gene expression in planktonic cultures and developing and mature biofilms. *Journal of Bacteriology, 187*(18), 6571-6576. https://doi.org/10.1128/jb.187.18.6571-6576.2005

Wright, C. C., Hsu, F. F., Arnett, E., Dunaj, J. L., Davidson, P. M., Pacheco, S. A., Harriff, M. J., Lewinsohn, D. M., Schlesinger, L. S., & Purdy, G. E. (2017, 2017//08/). The Mycobacterium tuberculosis MmpL11 Cell Wall Lipid Transporter Is Important for Biofilm Formation, Intracellular Growth, and Nonreplicating Persistence. *Infection and Immunity, 85*(8). https://doi.org/10.1128/IAI.00131-17

Zambrano, M. M., & Kolter, R. (2005, 2005/12/02/). Mycobacterial biofilms: a greasy way to hold it together. *Cell, 123*(5), 762-764. https://doi.org/10.1016/j.cell.2005.11.011