Studies on a mycolic acid reductase in *Mycobacterium tuberculosis*

By

Asma Javid

A thesis submitted to The University of Birmingham For the Degree of Doctor of Philosophy

Institute of Infection and Microbiology

School of Biosciences

College of Life and Environment Sciences

The University of Birmingham

September 2017

Abstract

Mycobacterium tuberculosis the causative agent of tuberculosis (TB) infects up to 9 million people per year resulting in approximately 1.5 million deaths due to the disease. With the emergence of multi-drug resistant (MDR) and extremely-drug resistant (XDR) strains arises the need for novel targets for anti-TB therapy. Mycolic acids are essential components of the unique, lipid-rich cell wall of *M. tuberculosis*. However, enzymes involved in the biosynthesis of mycolic acids remain under exploited as drug targets despite one of the early and hallmark anti-TB drug isoniazid which inhibits mycolate biosynthesis. Previous studies from our laboratory identified mycolate processing enzymes and transporters. A mycolic acid reductase was identified to play a role in the final reduction step in mycolic acid biosynthesis in *Mycobacterium smegmatis* and *Corynebacterium glutamicum*. Using gene knockdowns I have now extended these studies to slow-growing mycobacteria-like *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG. The results in this thesis clearly indicated that the mycolic acid reductase Rv2509 involved in the final stages of mycolic acid biosynthesis is essential in *M. tuberculosis* and *M. bovis* BCG. Furthermore using BLAST-P alignments and predictions of a 3D structure we identified unique domains and residues in the mycolate processing enzymes, and present functional studies on the same. This unique domain and other residues identified from *in silico* analysis of the structure of Rv2509 were important for the functionality of the protein following complementation studies. Purification of the protein of Rv2509 gave an insight on the location of the protein and the mechanism of action. The substrate for Rv2509 was also identified through the use of a functional assay. My thesis also looked into the biochemical changes in the structure of the cell wall by extracting lipids

in the outer membrane and hydrophobicity analysis of the mutant due to the change in colony morphology observed.

.

Declaration

The work in this thesis was carried out in the School of Biosciences at the University of Birmingham, UK, B15 2TT during the period of September 2013 to September 2017. The work in this thesis is original except where acknowledged by references.

No portion of the work is being, or has been submitted for a degree, diploma or any other qualification at any other University.

Acknowledgement

Four years since I embarked on this journey to do a PhD which has now finally come to an end. I would like to take this opportunity to thank all those wonderful people who made these four years easy to survive in a different country away from my parents. To begin with I have to thank my father Mr. Javid Hussain because without his support I would not have been able to come this far in life. He has been my main support in encouraging me to achieve my goals right from my undergraduate degree. If it was not for him I would have never been educated. So thank you papa for everything. After my father I would like to thank my mother Mrs. Nilofer Javid who has been with me through my good days and bad days.

I would like to thank my supervisor Dr. Apoorva Bhatt who gave me an opportunity to pursue my PhD in his laboratory under his continuous guidance. His approachable and supportive nature made it easy to work in the laboratory. I would also like to thank my second supervisor Prof. Del Besra for providing his guidance whenever needed.

Thank you to all the past and present lab members, Cristian, Monika, Natacha, Panchali, Padraic, Albel, Veemal, Vijay, Kat, Jon, James, Usha, George, Perla, Nabiela, Rebecca, Patrick, Giacomo, Ramon, Bogdan, Szilvi, Anja, Hayleah, Sid, Luke that made my journey and stay in Birmingham enjoyable.

A special thanks to Kat and Patrick for helping me with my protein work, especially Kat who took out time for me whenever required and patiently took me over every protocol and

procedure. A special thanks to Albel too who guided me through most of the protocols when I was new to the laboratory.

I would like to thank Dr.Rainer and his laboratory for providing the conditional mutant construct without which this thesis wouldn't have taken shape.

Thank you Prof. Milos Ljubisaljevic, Prof. Tibor Pal and Prof. Safa Shehab for encouraging me to pursue my goal of doing a PhD. And finally a big thank you to my brother and sister and the rest of the members of my big fat family for all their support, words of encouragement and love.

This thesis is dedicated to my dad Javid Hussain and

mom Nilofer Javid

Table of Contents

List of Figures

List of Tables

List of Abbreviations

Acp	Acyl carrier protein
acet	acetamide
AcpM	Mycobacterial acyl carrier protein
AG	Arabinogalactan
BCG	Bacille Calmette-Guerin
bp	base pairs
BLAST	basic local alignment search tool
C	Carbon
CESTET	Conditional Expression-Specialised Transduction Essentiality Test
CFU	Colony Forming Units
CPM	Counts per minute
Cg	*Corynebacterium glutamicum*
Cys/C	Cysteine
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate

DOTS	Directly Observed Therapy Shortcourse
FAS	Fatty acid synthase
FAMEs	fatty acid methyl esters
g	gram
H	Hydrogen
His	Histidine
HIV	Human Immunodeficiency Virus
Hyg	Hygromycin
IFN	interferon
IL	interleukin
INH	isoniazid
IMAC	immobilised metal ion affinity chromatography
Kan	Kanamycin
kb	kilobase pairs
L	litre
LAM	Lipoarabinomannan
LOS	lipoologosaccharides

LB	Luria-Bertani
LM	Lipomannan
M	Molar
MA	Mycolic Acids
MAMEs	mycolic acid methyl esters
MDR	multi-drug resistant
mg	milligram
MIC	Minimum inhibitory concentration
min	minute
mL	mililitre
mM	millimolar
Ms	*Mycobacterium smegmatis*
Mtb	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* complex
MmpL	mycobacterial membrane protein large
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate

ng	nanogram
OADC	oleic acid-albumin-dextrose catalase
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PG	peptidoglycan
PDIM	phthiocerol dimycocerosate
pH	'Power of Hydrogen'
RNA	ribonucleic acid
SDM	site-directed mutant/mutagenesis
SDS	Sodium dodecyl sulfate
TB	Tuberculosis
TDM	Trehalose dimycolate
TDR	Totally drug resistant
TLC	Thin Layer Chromatography
TMM	Trehalose monomycolate
TSA	tryptic soy agar
TSB	tryptic soy broth

WHO	World Health Organisation
XDR	extensively drug resistant
%	percentage
Δ	delta = change
[^{14}C]	Radiolabelled Carbon-14
µM	micromolar
µL	microlitre

Chapter 1

General Introduction

1.1 What is Tuberculosis?

Tuberculosis (TB) is one of the leading killers amongst the bacterial pathogens known to date. The bacterium *Mycobacterium tuberculosis* that causes this devastating disease was discovered by Robert Koch in 1882. It is an air borne pathogen that causes this pulmonary infection and is a member of the *Mycobacterium tuberculosis* (*M.tuberculosis*) complex. The complex refers to genetically related mycobacteria that cause infection either in humans or other organisms.

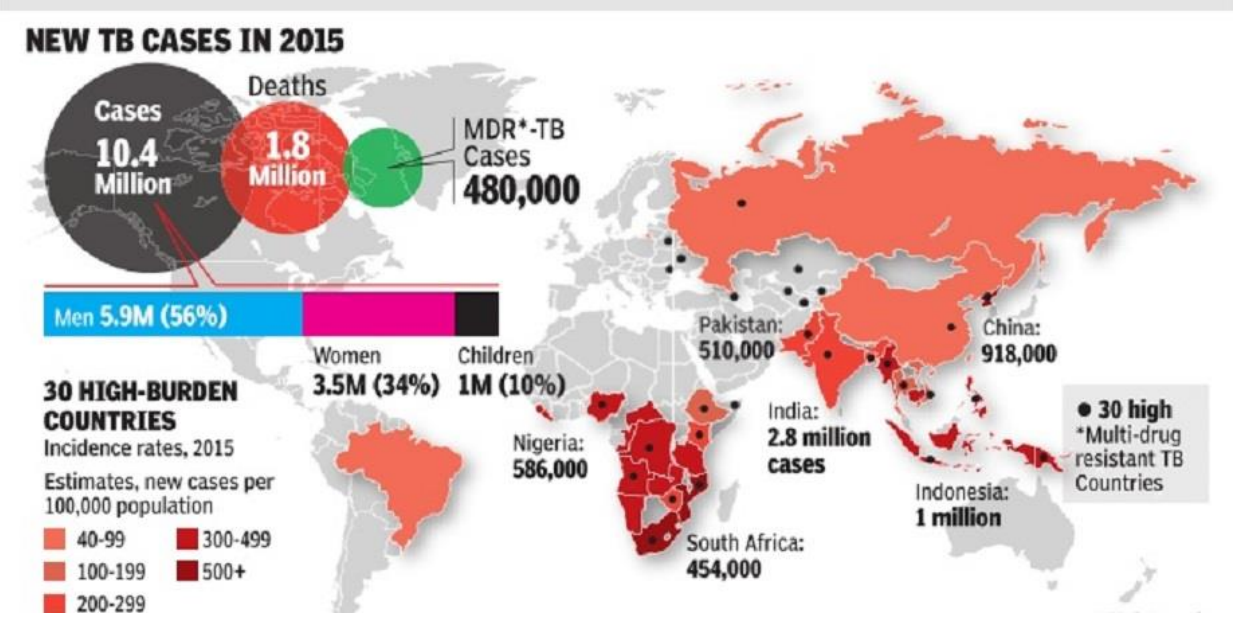

Figure 1.1: **Tuberculosis incidence rates throughout the world in 2013 from the World Health Organization record of 2014.** Adapted from WHO report, 2014.

TB results in two million deaths every year. 6.1 million cases of TB were reported to the WHO in 2013 out of which 5.7 million were of patients that were newly diagnosed. 60% of the cases seen are of multi-drug resistant (MDR) TB and extensively-drug resistant (XDR) TB mainly emerging from India, China, the Russian Federation and South Africa.

Factors that led to the reemergence of tuberculosis (mainly in Eastern Europe, UK and certain parts of USA) include poor drug compliance, absence of completely protective TB vaccine, slow development of new drugs, and emergence of new strains like multi drug resistant, extremely drug resistant and totally drug resistant, and individuals with a weakened immune system as in the case of AIDS patients.

Tuberculosis is mainly acquired by inhaling tiny droplets from coughs or sneezes of an infected person. The most common form of TB is the one that infects the lungs but there are other forms that can attack the bones and cause skeletal deformities. Samples obtained from hard tissue of bones are helpful in evolutionary studies of tuberculosis (Issar Smith, 2003). Individuals that are infected can either have one of the two types of TB either latent TB infection or TB disease. In latent infection the bacterium can survive within the individual without making him/her sick, usually without any symptoms and cannot spread the bacterium to others. In some cases the bacterium becomes active in the body and multiplies making the individual go from latent to being sick with TB diseases. Typical symptoms include persistent cough for up to 3 weeks or longer with blood, pain in the chest, weight loss, sweating at night and weakness. Based on the type of TB there are different diagnostic methods used which involve a Mantoux tuberculin skin test (TST) in which a fluid called tuberculin is injected into the skin and reaction observed. Posterior-anterior chest radiography, sputum examination and drug resistance of the bacterium are few other diagnostic methods. Apart from this, interferon gamma release assay (IGRA) is a newer type of blood test that is widely used. This test is especially helpful to differentiate latent TB from those previously administered with BCG vaccine. The test detects for the release of cytokine IFN-gamma from T cells that react to antigens

not found in BCG vaccines. Treatment of TB varies from six to nine months depending on the type of TB, possible drug resistance, location of infection in the body and overall health of the individual. Recent studies show that shorter treatment times of 4 months is more effective and reduces the risk of side effects. The commonly used drugs for tuberculosis are isoniazid, rifampin, ethambutol and pyrazinamide.

1.2 *Mycobacterium* species

Mycobacteria containing GC rich DNA belong to a group of Gram-positive bacteria termed actinomycetes. (Brennan and Nikaido, 1999). Mycobacteria are rod shaped ranging from 0.2-0.6 μm wide and 1.0 to 10 μm long and can be divided into two main groups based on their growth rate as fast-growing and slow-growing species which are listed in table 1.1 below.

Table 1.1: List of fast and slow growing mycobacteria

Fast Growing mycobacteria	Slow Growing mycobacteria
Mycobacterium smegmatis	*Myocbacterium tuberculosis*
Mycobacterium chelonae	*Myobacterium bovis*
Mycobacterium pulveris	*Mycobacterium leprae*
Mycobacterium vaccae	*Mycobacterium kansasii*
Mycobacterium gadium	*Mycobacterium canettii*
Mycobacterium phlei	*Mycobacterium africanum*
Mycobacterium aurum	*Mycobacterium microtii*
Mycobacterium flavescens	*Mycobacterium marinum*
Mycobacterium fortuitum	*Mycobacterium xenopi*
Mycobacterium duvalii	*Mycobacterium ulcerans*
Mycobacterium simiae	*Mycobacterium haemophilumi*
Mycobacterium farcinogenes	

A characteristic feature of members belonging to this genus is a lipid rich cell envelope which comprises the long chain α alkyl β hydroxyl mycolic acids. The presence of mycolic acids attributes to the low permeability of their cell wall. The *Mycobacterium tuberculosis* complex (MTBC) refers to species that have genetic similarities and comprises of *Myocobacterium tuberculosis, Mycobacterium africanum*, *Mycobacterium canettii, Mycobacterium bovis, Mycobacterium microti, Mycobacterium caprae* and *Mycobacterium pinnipedii* (Ventura et al, 2007, Brosch et al, 2002, Smith et al, 2006). Members of the *Mycobacterium tuberculosis* complex have descended from a common ancestor and have undergone a series of deletions or insertions which now determines

their differences in pathogenicity (Smith et al, 2006). There have been 14 regions of difference (RD1-14) (Forrellad et al, 2013) been identified through genomic studies. *M. tuberculosis*, the most pathogenic member of the *Mycobacterium tuberculosis* complex infects nearly two thirds of the world's population plus infecting animals in contact with infected humans. Other members like *M. pinnipedii* have been studied in seal infections, *M. microti* which is a rodent pathogen isolated from voles has shown to cause infection in immunocompromised individuals, *M. caprae* have been isolated from goats causing infection in them, *M. africanum* and *M. canettii* which have been isolated from African patients, have been shown to be closely related to *M. tuberculosis* causing tuberculosis in humans. *M. bovis* comprises the largest spectrum of host infections right from causing infections in humans to goats and wild or domestic bred cows. BCG (Bacillus Calmette-Guerin) which is an attenuated strain of *M. bovis* has been used as a prophylactic vaccine against tuberculosis for well over 90 years (Singh et al, 2015)

The genus comprises of other species like *M. smegmatis*, *M. leprae*, *M. chelonae*, *M. abscessus*, *M. fortuitum*, *M. kansasii* and *M. marinum* which are not grouped under *Mycobacterium tuberculosis* complex. *M. smegmatis* a non-pathogenic, fast-growing mycobacterium is commonly found in soil and water (Etinne et al, 2005). It is easily grown in laboratory media and as it in non-pathogenic serves as a surrogate for genetic analysis of *M. tuberculosis*. *M. leprae* the microorganism responsible for the chronic disease leprosy is the common cause of nontraumatic peripheral neuropathy worldwide. It is a major human pathogen but has been observed to cause leprosy in some primates and nine banded armadillo (Ramesh and Chitra, 2012). *M. leprae* has never been successfully cultured *in vitro* but can be grown *in vivo* by injecting it into the footpads of

mice. The opportunistic pathogens like *M. kansasii* causes a disseminated disease in immunocompromised individuals and *M. marinum* which is a fish pathogen causes skin infection in immunocompromised patients (Ramesh and Chitra, 2012).

1.3 The causative agent of tuberculosis: *Mycobacterium tuberculosis*

Mycobacterium tuberculosis is an acid fast bacteria belonging to the *M. tuberculosis* complex. The *M. tuberculosis* complex includes *Mycobacterium africanum*, *Mycobacterium bovis, Mycobacterium cannetti* and *Mycobacterium microti* all of which are tuberculosis causing agents in distinct hosts. *Mycobacterium tuberculosis* also belongs to the order Actinomycetales which also includes other bacteria like *Corynebacterium*, *Norcardia* and *Rhodococcus*. Mycobacteria are usually found in soil and water with *M. tuberculosis* being the human pathogen, acid fast bacilli, non-motile, non-sporulating, slow-growing characterized by 12 to 24 hour division rate and under the microscope may appear straight or rods. *Mycobacterium tuberculosis* is stained using the Ziehl-Neelsen technique developed in early 1890s which uses phenol based stains to penetrate the lipid rich hydrophobic cell envelope that is resistant to decolourisation by acid alcohol.

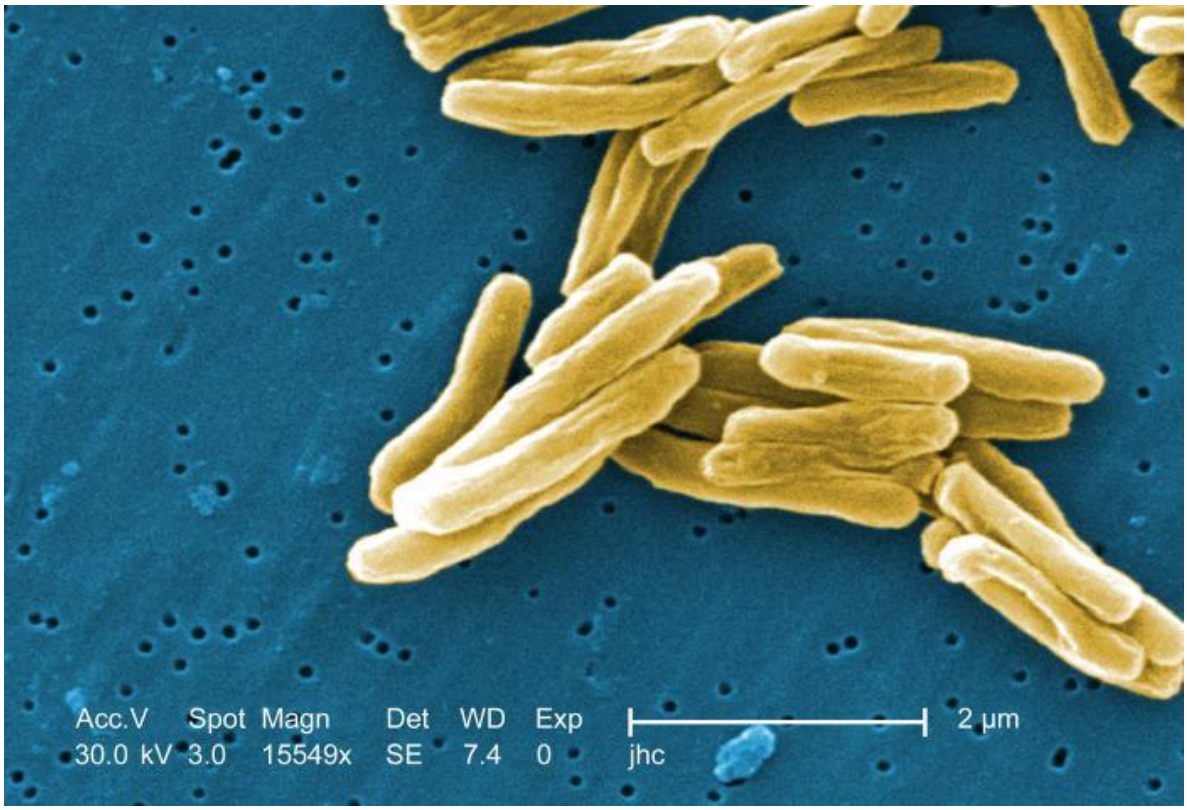

Figure 1.2: Scanning electron microscopic (SEM) of *Mycobacterium tuberculosis*. High magnification of 15549x and the scale used is 2 µm. Adapted from CDC, Dr.Ray Butler.

Acid fastness of this bacterium is attributed to the unique nature of its cell envelope, is a physical property of certain bacteria rendering it impermeable to certain dyes and stains. Mycobacteria belonging to the order Actinomycetales possess mycolic acids in their cell envelope that play a critical role in the structure and function of the cell envelope (Radhakrishnan et al, 2014). The most commonly used strain in the laboratory is *Mycobacterium tuberculosis* H37Rv whose genome consists of around 4000 genes and 4.4×10^6 bp. Studies have shown some unique features of the genome where there are over 200 genes encoding enzymes for metabolism of fatty acids, over 100 genes involved in

β-oxidation of fatty acids and the presence of unrelated Pro-Glu (PE) and Pro-Pro-Glu (PPE) families of acidic, glycine-rich proteins. The PE and PPE genes are thought to play a role in virulence. (Smith, 2003).

1.4 Epidemiology

The number of TB related deaths have increased considerably due to the emergency of multi-drug resistant and now extremely-drug resistant strains. Apart from this, with the increase in HIV incidences so has the TB in HIV related cases increased. In 2015, alone there were an estimated of 480,000 new cases of multi-drug resistant TB (MDR-TB) with an addition of 100,000 new cases of extensively drug resistant TB (XDR-TB). 45% of these cases were seen in India, China and the Russian Federation (WHO, 2016). Worldwide there was an estimated of 10.4 million new incidences of TB in 2015 out of which 5.9 million cases were seen in men, 3.5 million seen in women and 1.0 million seen in children and 1.2 million were seen in people with HIV. India, Indonesia, China, Nigeria, Pakistan and South Africa accounted for 60% of the 10.4 million new TB cases seen in 2015. In 2015 apart from the new TB cases identified there were 1.4 million TB deaths and in addition to this 0.4 million deaths resulting from TB disease among people with HIV. TB treatment did avert 49 million deaths globally between 2013-2015 but majority of the cases seen to date have been due to poor diagnostics and poor treatment regimens. Between 2013-2015 around 6 million new cases were notified to WHO out of which around 580,000 cases were eligible for MDR-TB treatment but only 125,000 cases received them. Availability and accessibility to TB treatments need to be expanded especially in countries with MDR and XDR TB. In September 2015 WHO also

announced the TB target of millennium development goal which includes halting the spread of the disease and ending the TB epidemic by 2030.

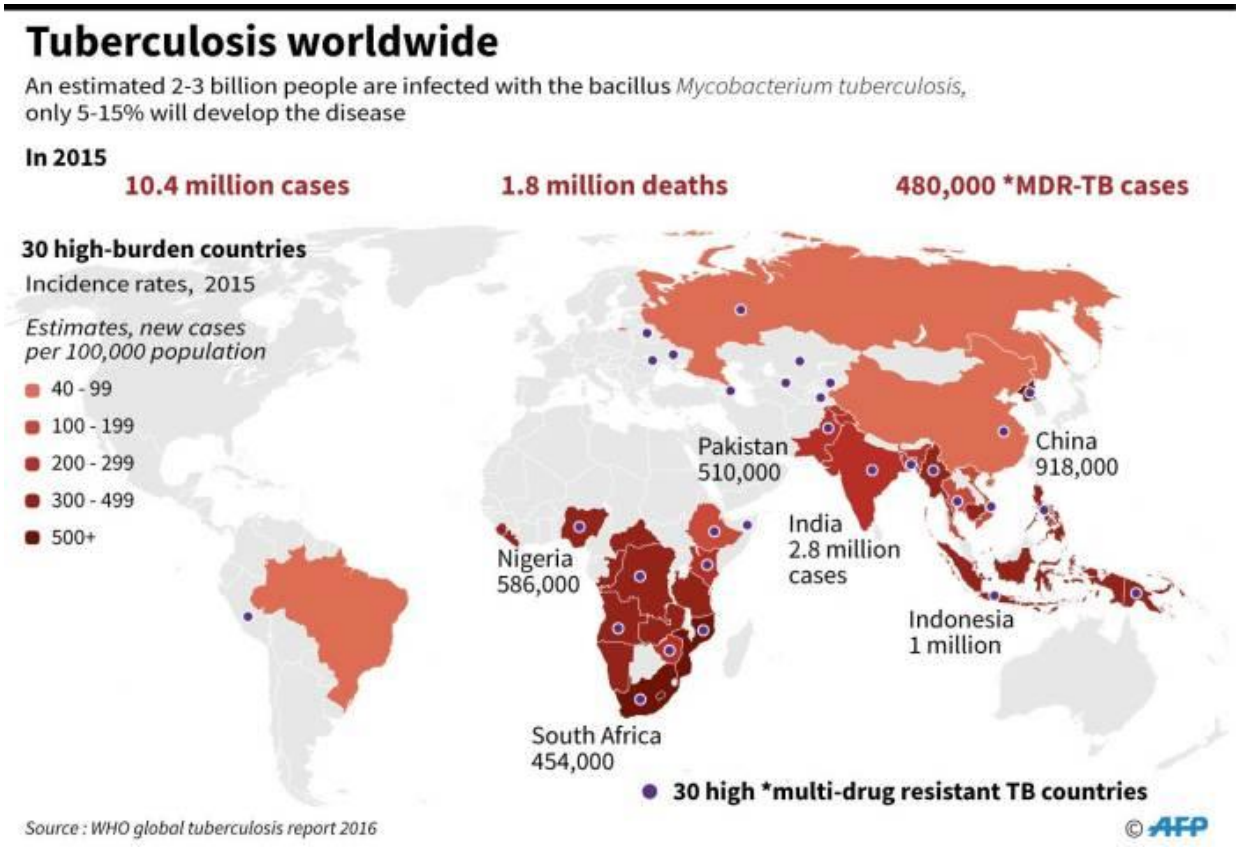

Figure 1.3: Map illustrating the number of tuberculosis related deaths worldwide due to *Mycobacterium tuberculosis*. Adapted from WHO global tuberculosis report 2016.

1.5 Stages of infection

There are different stages of TB infection and the involved organ systems. Tuberculosis that affects the lungs is the only form that is contagious and infectious and is acquired by prolonged exposure to someone with the illness. Majority of the population exposed to

tuberculosis has either latent TB infection which can progress to active infection based on factors mentioned in the earlier section or have active TB disease. The severity of infection depends on the host immune response and the strategies the bacterium develops to evade the host response. The initial stage of infection involves the transmission of the bacterium through air droplets from an infected person to a healthy individual. The bacterium is then engulfed by the alveolar epithelial type II (AE 2) pneumocytes and dendritic cells. AE2 cells termed as the defenders of the alveolus are bacteriostatic, enhance clearance of inhaled particles and form the epithelial component of the thin air blood barrier (Fehrenbach, 2001). The AE2 cells should kill the bacterium but this is not always the case. Factors that could influence the bacterium evade include the intrinsic microbicidal capacity of the host phagocytes and virulence of the bacterium strain. The alveolar epithelial cells and the dendritic cells are thought to play important role in the early stages of infection by activating the T cells in response to specific *Mycobacterium tuberculosis* antigens (Long and Schwartzman, 2014). Surfactant protein A and protein D found on the alveolar surface enhance the uptake of the bacteria by regulating the mannose receptor activity. Some studies have shown that the human toll-like receptor 2 (TLR-2) also play a major role in the uptake of *Mycobacterium tuberculosis* (Russell, 2007). The next stage involves the response of cell mediated immunity and formation of granulomas (Sasindran et al, 2011). Granulomas are the hallmark of tuberculosis infection and are developed by the host to contain the infection and eliminate the bacteria. Bacilli capable of escaping the alveolar macrophages multiply and destroy the alveolar macrophages which attract monocytes and neutrophils to the site of infection (Glikman et al, 2001). These monocytes mature to form antigen presenting dendritic cells and

alveolar macrophages which do not effectively kill the bacilli (Sakamoto, 2012). At this stage when the bacterium grow with minimal tissue damage the antigen presenting dendritic cells travel to the lymph nodes which in turn activates the T lymphocytes (Smith, 2003, Fogel, 2015). These T lymphocytes are recruited to the site of infection where they proliferate to form initial stage granulomas and activate macrophages which kill the intracellular bacilli. To evade the phagocytosis, bacteria have come up with different mechanisms to avoid the hostile vacuolar microenvironment. In the case of *Listeria* they physically escape the phagosomes and replicate in the cytoplasm, *Salmonella enterica* serovar Typhimurium require acidification of the phagosomes to survive (Sasindran et al, 2011). With *M. tuberculosis* phagosome-lysosoma fusion is inhibited by the exclusion of proton ATPases from the mycobacterial phagosome. Not much is known about the later stages of infection and how the bacteria survives in the lungs (Glikman et al, 2001). The bacteria go into latency surviving within the alveolar macrophages and more than half the infected individuals are asymptomatic (Fogel, 2015). Either in the case of reactivation of latent TB or the active disease the later stage results in the disruption of the granulomas in turn resulting in lung cavitation and disease (Sakamoto, 2012). Reason for reactivation can be poor host immunity due to various factors that may include environmental and genetic causes that relate to failure in immune signals. In cases where an individual is infected with HIV the most important cause for susceptibility of TB is because the host is immune compromised for CD4 T cells (Smith, 2003).

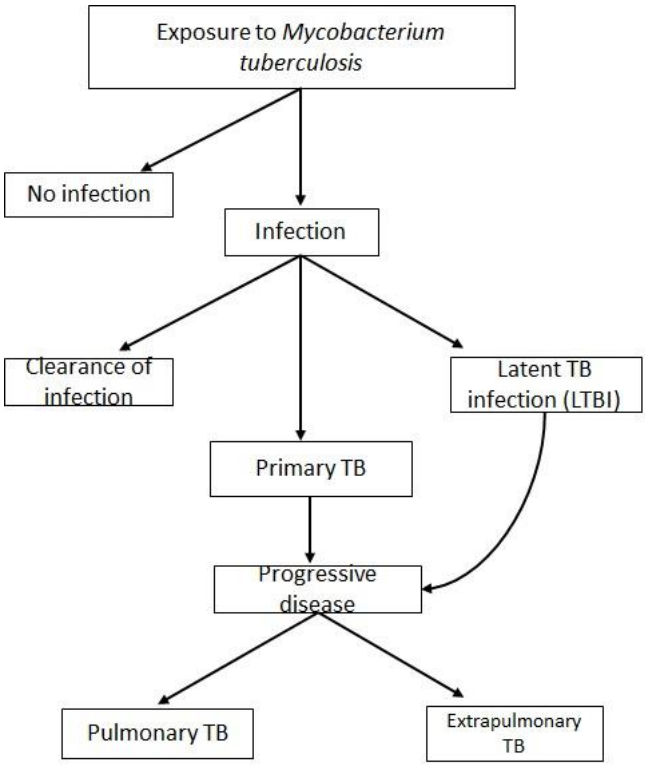

Figure 1.4: **Stages in the infection process of *Mycobacterium tuberculosis*.**

1.6 Anti-TB Drugs

The treatment of tuberculosis in 1946 began with the administration of the antitubercular drug streptomycin (Anon 1946) (Jones et al, 1944) and since then there has been a continuous research on new drugs and therapies to cure tuberculosis. The standard regimen for the clearance of tuberculosis infection is a two month intensive course of a cocktail of four drugs which are rifampicin (RIF) (Sensi 1983), isoniazid (INH) (Steenken and Wolinsky, 1952), pyrazinamide (PZA) (Yeager et al, 1952; Tompsett et al, 1954) and ethambutol (EMB) (Thomas et al, 1954). This is followed by a longer administration of rifampicin and isoniazid to eliminate the remaining bacteria that would have entered the

dormant, slow replicating latent phase. Recently the bacterium has become resistant to most of the available drugs resulting in multi-drug (MDR) resistant and extensively-drug resistant (XDR) strains which has resulted in an urgency to develop new agents to combat MDR and XDR. For a novel agent to work against these resistant strains, it should have a novel mechanism of action to attenuate cross resistance, should have low drug to drug interactions especially when administered to HIV patients, should be optimized in a way to be administered once daily orally, and in order to reduce the duration of course should have rapid bactericidal activity (Hogland et al, 2016). Apart from these they should be extremely safe to be administered to pregnant women and children. Most of the first and the second line TB drugs target mycolic acid biosynthesis. They have been designed to inhibit at least one of the enzymes involved in the biosynthesis pathway of mycolic acids which includes acyl-CoA carboxylase inhibition, β-ketoacyl ACP synthase inhibition, enoyl reductase inhibition and dehydratase inhibition. Thiolactomycin (TLM) a naturally occurring molecule in *Norcardia* is a good inhibitor of the mycobacterial KasA and KasB enzymes. It is composed of thiolactone ring which preferentially binds to the malonate portion of KasA acyl enzyme which inhibits mycolic acid biosynthesis (Wright et al, 2007). Isoniazid (INH) and ethambutol (ETH) both have been demonstrated to target *Mycobacterium tuberculosis* enoyl-ACP reductase InhA (Dover et al, 2004). Both are prodrugs which are activated by KatG and EthA respectively (Rawat et al, 2003). In the case of isoniazid mycolic acid biosynthesis is inhibited when INH activated by the catalase peroxidase KatG forms an adduct with NAD which inhibits *inhA* encoded NADH dependent enoyl ACP reductase of the fatty acid synthase (FAS) II (Zhang et al, 1992). In the case of ethambutol it gets activated by a flavin monooxygenase EthA to

form a covalent ETH-NAD adduct which inhibits InhA which in turn inhibits mycolic acid biosynthesis leading to the accumulation of long chain fatty acids and ultimately cell death (Kremer et al, 2003). Drugs like Isoxyl (ISO) and thiacetazone (TAC) are also prodrugs which are activated by mycobacterial monoxygenase EthA and act on the dehydratase step catalyzed by β-hydroxyacyl ACP dehydratases (Quemard et al. 1995). These drugs are effective in inhibiting the initial elongation of meromycolate chain in fatty acid synthase (FAS) II (Banerjee et al, 1998).

Bendaquiline which is a diarylquinolones has been the first novel antitubercular drug to be approved in over a decade (Sloan and Lewis, 2015). This drug acts by specifically inhibiting ATP synthase activity in actively replicating mycobacteria plus mycobacteria in dormant phase (Andries et al, 2005). Bedaquiline has a distint mechanism of action when compared to rifampicin and isoniazid making it effective against multi-drug resistant strains. This drug is especially useful for latent *M. tuberculosis* infection as the bacteria in this latent phase maintains an energized membrane using a pool of ATP produced by ATP synthases and bedaquiline inhibits this ATP synthase. Another attractive drug target is the MmpL3, the only essential member of the mycobacterial membrane protein family large (MmpL) family (W.Li et al, 2014; Biava et al, 2007). As MmpL3 is involved in the export of mycolic acids to the outer membrane in the form of trehalose monomycolates, inhibition of MmpL3 could be the mechanism of action of novel TB drugs. SQ109 which is a structural derivative of ethambutol diamine moiety has been identified as the latest MmpL3 inhibitor. It has been shown that treatment with SQ109 led to the accumulation of trehalose monomycolate in *M. tuberculosis* (Tahlan et al, 2012) and also inhibition of menaquinone synthesis and cellular respiration.

Faropenem which is structurally similar to carbepenems, has been approved for respiratory infections in humans as it has demonstrated bactericidal activity metabolic active *M. tuberculosis* cells (M.Lee et al, 2012; Gracia et al, 2014; Anger et al, 2010). Two of the oxazolidinones, linezolid and tedizolid have been clinically approved based on their antibacterial activity. As these are protein synthesis inhibitors they work by binding the 50s ribosomal subunit of the 23s rRNA making the bacteria highly susceptible. All these new drugs have shown potential against drug resistant tuberculosis and apart from synthesises of new drugs major research has been directed towards new vaccines (WHO,2013).

Table 1.2: Clinically relevant antitubercular drugs and their mechanism of action.

Drug Name	Mechanism(s) of Action	Common Mechanism(s) of Resistance
Rifampicin	RNA synthesis inhibition	Mutation of *rpo*B induces a conformational change at β-subunit of RNA polymerase causing a decrease in binding affinity (Heep et al, 2001, Somoskovi et al, 2001, Philey and Grifith, 2015).
Delamanid	Mycolic acid biosynthesis	Mutation of reductive activating Rv3547 gene (Stover et al, 2000, Matsumoto et al, 2006)

Clarithromycin	Protein synthesis inhibition (50S subunit)	Low cell wall permeability and the expression of *emr*37, confers 23S rRNA site modulation. (Nasiri et al, 2017).
Thiacetazone	Inhibits methyltransferases in mycolic acid biosynthesis	*eth*A mutation minimizes prodrug activation and mutations to hadABC operon affecting dehydratase activity. (Nasiri et al, 2017).
Streptomycin	Protein synthesis inhibition	Mutations in rpsL and rrs confer binding site modulation. (Finken et al, 1993, Da Silva and Palomino, 2011, Verma et al, 2014, Philey and Griffith, 2015).
Isoniazid	Mycolic acid biosynthesis inhibitor and effects on DNA, lipid, carbohydrate and NAD metabolism	*Kat*G suppression causing decreased prodrug activation and a mutation in the promoter region of *Inh*A causing an over expression of InhA. (Somoskovi et al, 2001, Ramaswamy et al, 2003,

		Zhang et al, 2005, Guo et al, 2006).
Pyrazinamide	Not fully resolved, may include membrane potential disruption	Mutations in *pnc*A reducing conversion to active acid form. (Somoskovi et al, 2001, Shi et al, 2011, Feuerriegel et al, 2013, Zhang et al, 2013, 2014).
Ethambutol	Arabinogalactan biosynthesis inhibition	Mutations in *emb*B at codon embB306 (Brown-Elliott et al, 2012, Palomino and Martin, 2014, Philey and Griffith, 2016).
Kanamycin	Protein synthesis inhibition	16s rRNA target site modulation. Increased drug inactivation via over expression of *eis* aminoglycoside acetyltransferase. (Zaunbrecher et al, 2009, Da Silva and Palomino, 2011, Georghiou et al, 2012).

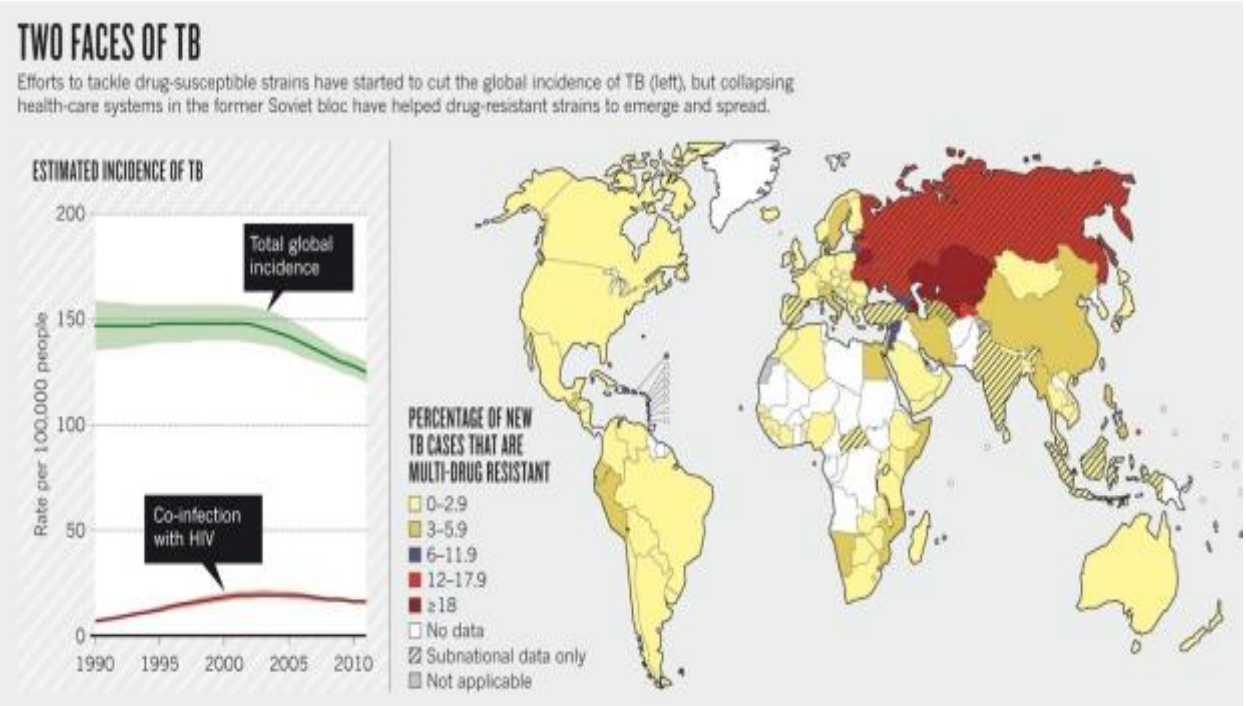

Figure 1.5: Map illustrating percentage of new cases due to multi drug resistant *Mycobacterium tuberculosis* strains. Adapted from WHO global report on tuberculosis, 2016.

1.6.1 DOTS

Directed observed treatment therapy, short course known as DOTS was a strategy recommended by WHO in 1994 to cure tuberculosis. There are five principles to this strategy which can be summarized as follows. The first principle involves the commitment of the government and health authorities towards cure of tuberculosis. The next step is to diagnose new cases primarily by sputum smear microscopy after which the standard course of treatment of tuberculosis with the first line drugs be taken under direct supervision. The next step is to ensure there is adequate supply of drug and the final is monitoring and recording the progress of every diagnosed patient. The short course treatment regimen of DOTS involves a six month treatment span with the administration

of rifampicin, isoniazid, ethambutol and pyrazinamide for the first months and the prolonged administration of rifampicin and isoniazid for the remaining four months (Cox et al, 2006). In 2006, WHO started a DOTS-plus program to be used along with DOTS for multi-drug (MDR) resistant and extensively (XDR) drug resistant strains.

1.7 The Mycobacterial cell wall

The mycobacterial cell envelope is unique, impermeable to and resistant to most antibacterial agents and is associated with pathogenicity. The mycobacterial cell envelope architecture was first proposed by Minnikin in 1992 and modified in 2002 by Minnikin et al. The thick cell wall acts as a protective barrier providing intrinsic protection against antibiotics and bactericidal components of the host immune response (Radhakrishnan et al, 2014). The cell wall consists of an inner and outer layer that surrounds the plasma membrane. The outer layer consists of lipids and proteins and the inner layer consists of three covalently attached polymers: peptidoglycan, arabinogalactan and the mycolic acids forming the mycolyl arabinogalactan-peptidoglycan (mAGP) complex (Brennan and Besra, 1997). This forms the insoluble core and is essential for the viability of the cell and has been addressed in context to new drug development. Between the outer and inner layers are present the lipomannan (LM), lipoarabinomannan (LAM), phosphatidylinositol mannosides (PIMs) and the phthiocerol. Understanding of the biosynthesis of the mAGP complex is especially important for identifying new drug targets.

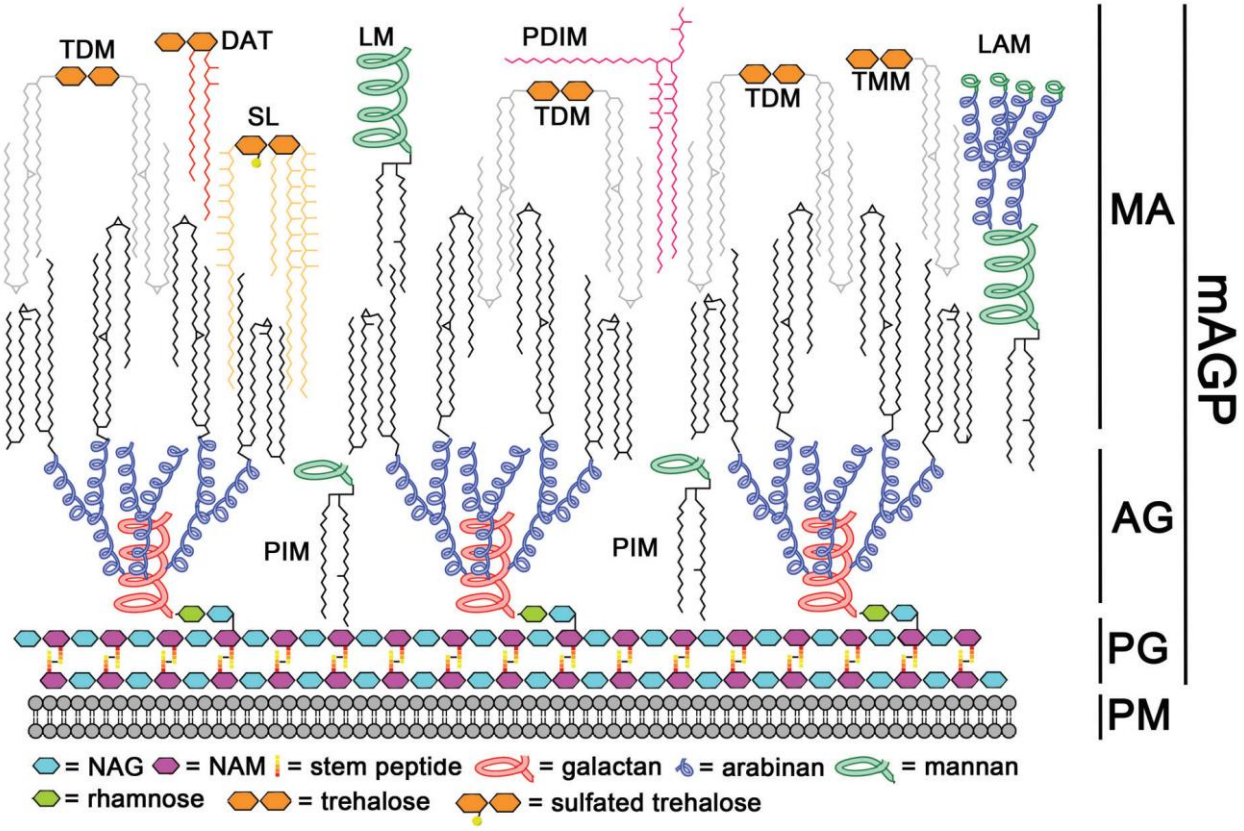

Figure 1.6: **Schematic representation of the cell wall of *Mycobacterium tuberculosis*.** Adapted from Alderwick et al, 2015.

1.7.1 Plasma membrane

The plasma membrane in mycobacteria is a less complex structure and appear as a thick outer layer and this thickness has been attributed to the presence of a carbohydrate, phosphatidylinositol mannosides (PIMs) (Silva and Macedo, 1983) The presence of PIMs has been restricted to acitnomycetes, forming the major plasma membrane component. Apart from this it also forms the lipid base of lipoarabinomannan and lipomannan. The most common phospholipids found in the mycobacterial cell envelop are phosphatidylglycerol, diphosphatidylglycerol and phosphatidylinositol mannosides

(Brennan and Nakaido, 1995). The typical structure of PIMs constitutes of a single α-D-mannopyranosyl group at position 2 and a mannose oligosaccharide. Apart from the PIMs plasma membrane consists of a large number of polyterpene based products such as carotenoids and menaquinones and glycosylphosphopolyprenols. Carotenoids protect the bacterium from photolytic damage and give *M. kansasii* and *M. gordonae* their characteristic yellow color. Menaquinones are associated with electron transport and glycosylphosphopolyprenols involved in cell wall biosynthesis (Brennan and Nakaido, 1995).

1.7.2 Peptidoglycan

Peptidoglycan is a rigid versatile substance (Alderwick et al, 2007) present in all Gram-positive as well as Gram-negative bacteria, giving it shape, rigidity and protecting the bacteria from osmotic stress. Gram-negative bacteria contain limited peptidoglycan and are mainly composed of lipopolysaccharides and lipoprotein. The majority of the cell wall of Gram-positive bacteria are composed of peptidoglycan and polysaccharides or teichoic acid (Schfleifer and Kandler, 1972). Peptidoglycan is a heteropolymer and all bacteria share the same structure of peptidoglycan consisting of glycan strands crosslinked through short peptides. The glycan strands are typically composed of β (1-4) linkages of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid residues (MurNAc) (Jankute et al, 2015). The short peptides bind to carboxyl group of muramic acids via their N terminus and contain alternating L and N amino acids. Mycobacterial peptidoglycan differ from other peptidoglycan in a way that MurNAc is oxidized and present as N-glycolylmuramic acid (MurNGlyc) and they use L-alanyl-D-iso-glutaminyl-

meso-diaminopimelic acid (DAP) as residues to cross link peptidoglycan. So mycobacteria have a chemotype IV cell wall which contain peptidoglycan with meso-DAP and arabinogalactan. The modifications in the mycobacterial peptidoglycan attribute to their decreased susceptibility to lysozyme and even increase their overall strength by providing sites for hydrogen bonding. Apart from linkages between two meso-DAP, linkages between one meso-DAP and D-alanine (Zhang et al, 2003) have been identified, which leads to a high percentage of cross linking in mycobacteria making the peptidoglycan the principal structural framework of the cell wall (Brennan and Nikaido, 1995).

1.7.3 Arabinogalactan

The arabinogalactan is the major polysaccharide of the cell envelope and is important for cell envelope intergrity and anchoring the mycolic acids to the peptidoglycan. It is mainly composed of the arabinan (Ara*f*) and galactan (Gla*f*) and the arabinan is attached to the galactan at the 5 position of the 1-5 linkages. The arabinogalactan is linked to the peptidoglycan by a phosphoryl-N-acetlyglucosaminosyl rhamnosyl linkage. The arabinogalactan is attached to the mycolic acids via ester links (Jankute et al, 2014). The mycolic acids are present in clusters on the terminal pentarabinofuranosyl units and out of these mycolic acids present only two thirds are mycolylated under physiological conditions (Daffe, 2015). From gas chromatography mass spectrometry (GS-MS) and fast atom bombardment mass spectrometry (FAB-MS) (Brennan and Nikaido, 1995) studies on mycobacteria certain characteristics within arabinogalactan were identified. The first feature was that all arabinan and galactan residues were in the furanose ring

form. The arabinan domain is highly branched and is essential to mycobacteria when compared to the linear galactan component. The arabinan and galactan units are linked through the C5 and in some cases C6 Gal*f* units. Some studies identified a nonacylated galactosamine ($_D$-GalN) in the arabinogalactan of slow growing mycobacteria like *M. tuberculosis* which could contribute to the rigidity and tightness of the cell wall in slow-growing mycobacteria (Jankute et al, 2012).

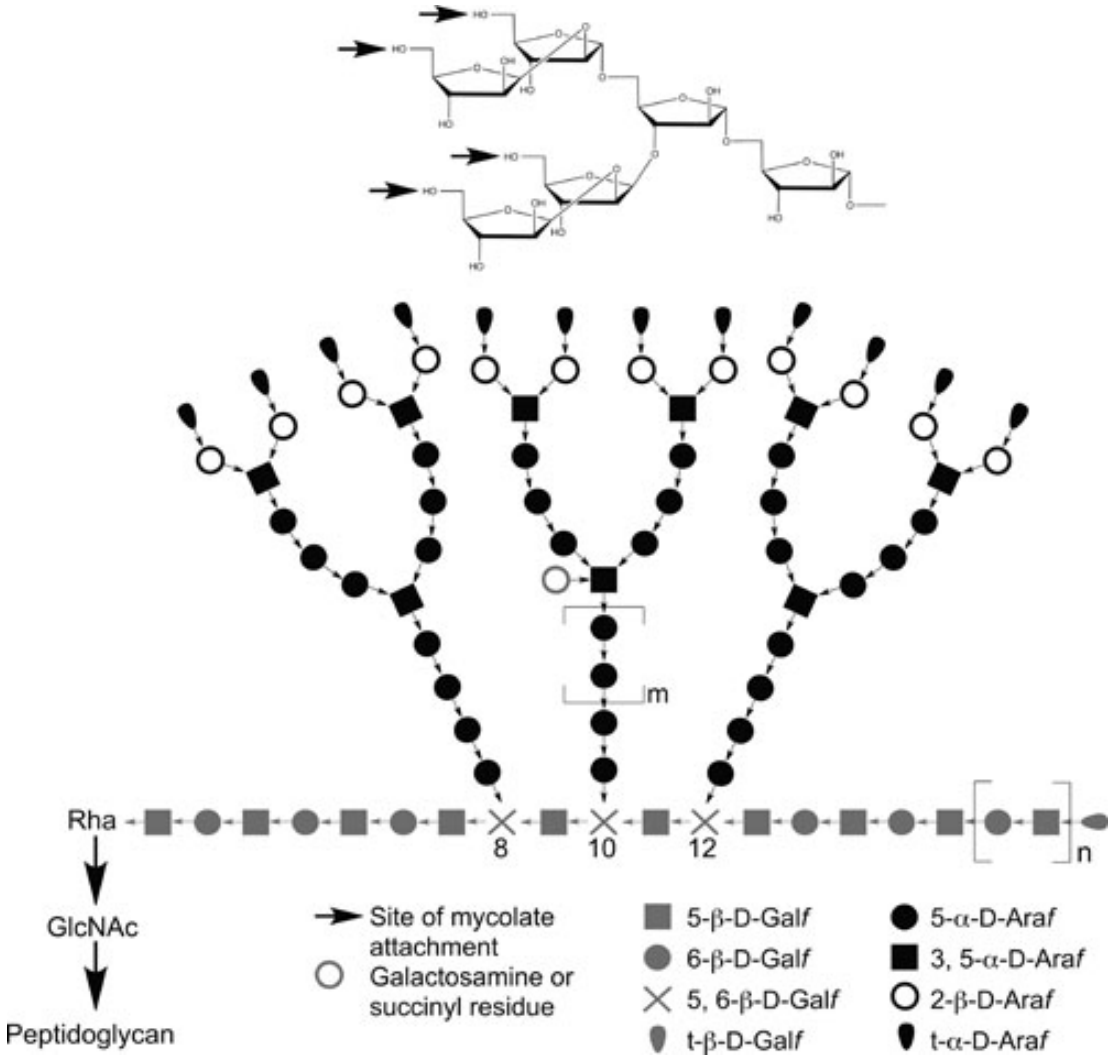

Figure 1.7: Schematic representation of mycobacterial arabinogalactan. A linkage unit, composed of rhamnose and *N*-acetyl-glucosamine residues, anchors the whole AG structure to peptidoglycan. The galactan domain is composed of alternating β(1→5) and

β(1→6) galactofuranose residues with three chains of arabinan attached to each linear galactan chain at positions 8, 10, and 12. The highly branched nonreducing end of AG terminates with a hexa-arabinofuranoside motif, two-thirds of which is substituted with mycolic acids. Adapted from Jankute et al, 2014.

1.7.4 Mycolic Acids

Mycolic acids are short α-alkyl long β-hydroxyl fatty acids ranging from 60 to 80 carbons per chain. Mycolic acids occur in all mycobacterial species and are used as taxonomic markers. Chain lengths vary from the shortest found in *Corynebacterium* 28-38 carbon atoms, 46-69 in *Norcardia* to the longest being upto 100 in *Segniliparus* (Marrakchi et al, 2014). They are mainly found as esters of the non-reducing arabinan terminus of the arabinogalactan and also present as free lipids within the cell wall associated to trehalose to form trehalose dimycolates (TDM). Trehalose dimycolate (TDM), known as 'cord factor' has been shown to be a major virulence factor in *in vivo* models. Presence of TDM alone exerts strong immunological effects and in turn mediates humoral and cell mediated immunity (Verschoor et al, 2012). Mycolic acids are important as they exhibit important characteristics such as biofilm formation (Ojha et al, 2005), low permeability to hydrophobic antibiotics, virulence (Glickman et al, 2001), foamy macrophage formation in TB granulomas (Marrakchi et al, 2014) and in addition to this enzymes involved in mycolate biosynthesis are important for mycobacterial survival hence representing good drug targets (Bhatt et al, 2007). Mycolic acids include various functional groups such as α-methyl ketones, α-methyl methyl ethers, *cis*- or trans- cyclopropanes (Yaun et al, 1998). It has been shown in animal models that deletion of any one of the proximal cyclopropane rings of the α or ketomycolates has led to restricted growth.

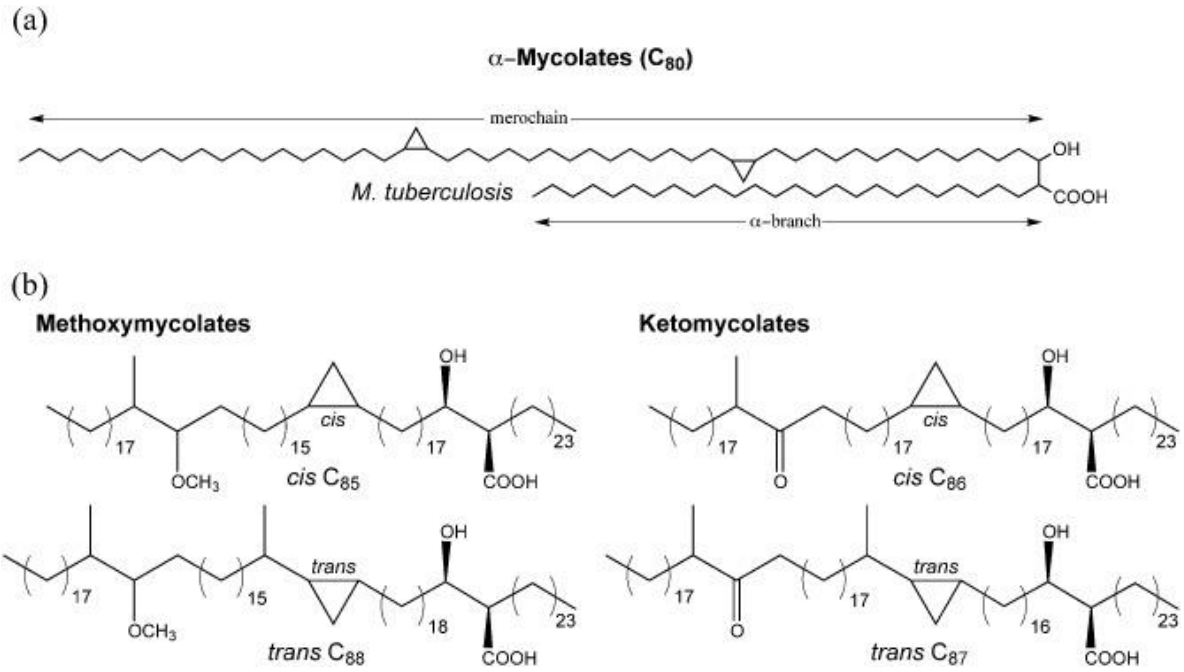

Figure 1.8: Structures of mycolic acid subclasses from *Mycobacterium tuberculosis*.

The synthesis pathway of mycolates involves two fatty acid synthases synthesised by the repetitive addition of two carbon units to the growing end of a hydrocarbon chain. The two types involve fatty acid synthase (FAS) type I and type II.

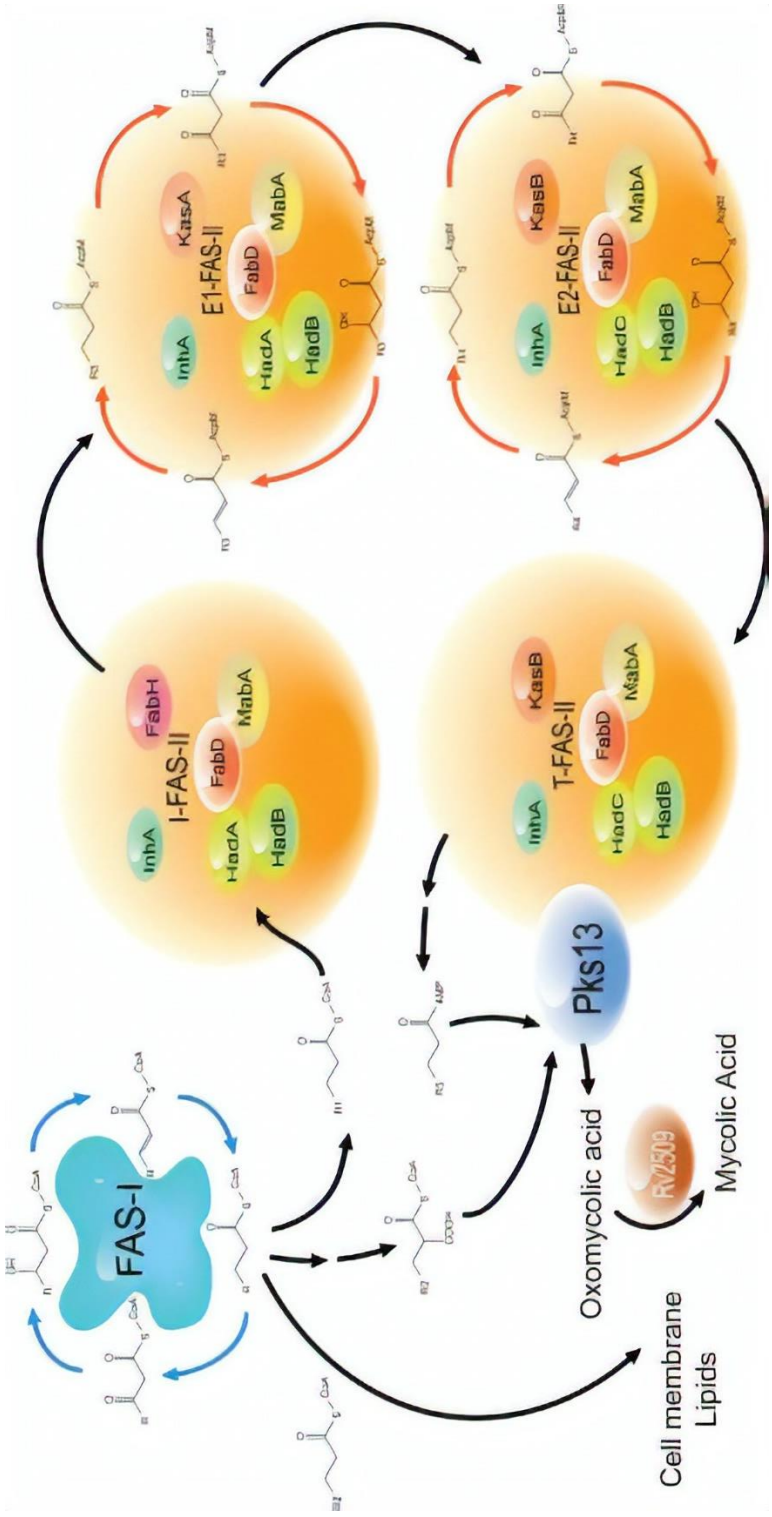

Figure 1.9: Biosynthetic pathway of mycobacterial mycolic acids. Adapted from Nataraj et al, 2015.

FAS I is a large multifunctional enzyme common in animals and fungi that catalyzes the de novo synthesis of fatty acids from acetyl Co enzyme A in a bimodial distribution, C_{16}-C_{18} and C_{24}-C_{26} acids with the latter corresponding to the α-branch found in mycolic acids. FAS II also known as bacterial like mono functional enzyme is responsible for the elongation of fatty acids at the origin of the very long meromycolic chains. The final step involves the condensation of the α-branch and the meromycolate chain, catalyzed by Pks 13. Antigen 85 complex assists in the transfer of these mature mycolates to the arabinogalactan and other acceptors like trehalose. Antigen 85 complex consists of mycolyl transferases that catalyse the transfer of mycolic acid from one trhalose monomycloate (TMM) to another to form trehalose dimycloate (TDM) or the AG termini. A detailed illustration of all genes, enzymes and inhibitors involved in mycolic acid biosynthesis has been summarised in a recent review (Marrakchi et al, 2014) and involves the following stages.

The synthesis of the α-alkyl branch is catalyzed by a single mycobacterium FAS I encoding gene (*fas*, Rv2524c) which is essential in *Mycobacterium smegmatis* and suggested to be essential in *Mycobacterium tuberculosis* (Zimhony et al, 2004 and Griffin et al, 2011). The fatty acids synthesised are in a bimodial distribution, C_{16}-C_{18} and C_{24}-C_{26} long chain acyl CoAs with the latter participating in mycolic acid biosynthesis after carboxylation by acyl CoA carboxylases. The mycobacterial FAS II elongates the C_{12}-C_{16} fatty acids to yield C_{18}-C_{30} acyl-acyl carrier proteins catalysed by acyl carrier protein (AcpM, Rv2244) (Bloch et al, 1977) through a series of cycles resulting in the formation of the required length of meromycolate which participates in the final stages of mycolic acid biosynthesis. The essential proteins that participate in the biosynthesis of the long

chain meromycolic chain involve InhA, MabA, HadB and KasA (Bhatt et al, 2005; Brown et al, 2007; Parish et al, 2007; Sacco et al, 2007).

Considerable amount of research is gone into identifying the key enzymes involved in FAS type II system when compared to penultimate and key step leading to the formation of mycolic acid motif. The final step involves the formation of a new carbon-carbon bond by a Claisen condensation of both fatty acids and polyketide synthase (Heath et al, 2002) by condensing enzymes. Pks 13, polyketide synthase 13 which is a member of the type I Pks family was identified as the enzyme responsible for the final step of mycolic acids formation in *Mycobacterium smegmatis* and *Corynebacterium glutamicum* (Portevin et al, 2004). The final mycolic acid condensation not only involves Pks13 but also another gene *fad*32. Fad32 acts in concert with Pks13 and activates the very long meromycolic acid before its condensation with C_{24}-C_{26} fatty acid (Marrkchi et al, 2014). The condensation product by Pks13 is the mycolic β-ketoester that needs to be reduced to produce the mature mycolate. Work done by Lea Smith et al (2007) and Bhatt et al (2008) suggested that *NgCl*2385 and *MSMEG*_4722 in *C. glutamicum* and *M. smegmatis* respectively as the candidate gene involved in the reduction of the β-keto ester motif. Not a lot was known about the transportation of the mature mycolic acid to the outer membrane but it has been suggested by Takayama et al in 2005 that mycolic acids could be transported in the form of trehalose monomycolates (TMM). It was hypothesized that a member of the mycobacterial membrane protein large (MmpL) part of the resistant nodulation division family (RND) of efflux pumps was involved in the transportation of TMM. MmpL3 belonging to this membrane protein large family was identified as a likely candidate which was involved in the transportation. Studies by Varela et al, 2012

demonstrated that MmpL3 was an essential gene in mycobacteria and that the conditional depletion of MmpL3 in *M. smegmatis* led to the accumulation of trehalose monomycolate intracellular (Varela et al, 2012).

1.7.4.1 Mycolic acid synthesis in *Corynebacterium*

Corynebacterium glutamicum a member of *Corynebacterianeae* share similar cell wall architecture and biosynthetic machinery as *Mycobacterium tuberculosis*. Previous studies have shown that it can tolerate mutations to essential genes in homologues of *M. tuberculosis* and for this reason it has been a good model of the study of essential genes involved in mycolic acid biosynthesis of *M. tuberculosis* (Dover et al, 2004, Portevin et al, 2004). While *Mycobacterium* species require two fatty acid synthases (FAS) for the biosynthesis of mycolic acids, corynebacteria just use one which is the multifunctional type 1 fatty acid synthase (FAS 1) which is incorporated as part of the cell membrane phospholipids. The presence of fatty acid synthase type 11 (FAS 11) is missing in corynebacteria, hence the corynomycolic acids are synthesized via the condensation of fatty acid synthase type 1 end products (Kalinowski et al, 2003). Corynebacteria contain two FAS 1 enzymes which are FAS A and FAS-1A essential for the cell survival and FAS B and FAS-1B which is supplementary but is required for producing the lipid environment (Subtile et al, 1996). There are exceptions which contain only one FAS 1 enzyme, like the pathogenic *Corynebacterium diphtheriae* and *Coyrnebacterium urealyticum*, which depend on exogenous fatty acids for their growth (Tauch et al, 2005). In corynebacteria FAS A synthesizes palmitic ($C_{16:0}$) and oleic ($C_{18:0}$) acids and FAS B synthesizes saturated $C_{16:0}$ and $C_{18:0}$ fatty acids. Studies have shown that the FAS A and

FAS-1A contain a FAS A like domain which is essentiality the type 11 FAS in *Mycobacterium* species which is involved in the synthesis of unsaturated fatty acid (Radmacher et al, 2005)

1.7.5 Lipoarabinomannan

The outer and inner membranes of mycobacteria are composed of abundant amounts of glycophospholipids (phosphatidyl-*myo*-inositol mannosides (PIMs)) and lipomannan (LM) and Lipoarabinomannan (LAM). These glycolipids play an important role in modulating the host immune response during infection. The basic structure of PIMs is composed of a phosphatidyl-myo-inositol (PI) unit, up to four acyl chains and one to six α-$_D$-mannopyranosyl (Man*p*) residues (Jankute et al, 2015). The most commonly found PIMs in mycobacteria are the tri and tetra-acylated phosphor-*myo*-inositol dimannosides ($A_{c1}PIM_2$ and $A_{c2}PIM_2$) and tri and tetra-acylated phospho-*myo*-inositol hexamannosides ($A_{c1}PIM_6$ and $A_{c2}PIM_6$). Out of these PIMs the $A_{c1}PIM_2$ and $A_{c1}PIM_6$ contain two acyl groups at 3-OH of *myo*-inositol and $A_{c2}PIM_2$ and $A_{c2}PIM_6$ possess four acyl groups. The lipoarabinomannan of *M. tuberculosis* consists of an α(1-6) linked $_D$-Man*p* backbone to which are attached shorter side chains of α(1-5) linked $_D$-Ara*f* residues. Lipomannan (LM) are similar to LAM, they are essentially LAM without an arabinose containing side chain. In LM the mannan chain length and degree of branching is species specific (Besra et al, 1997). Further structural studies on LAM resulted in two distinct arrangements of the arabinan at the non reducing termini. These branching were either a branched hexa arabinofuranosides as [β-$_D$-Ara*f* (1-2)-α-$_D$-Ara*f*]$_2$-3,5-α-$_D$-Ara*f*-(1-5)-α-$_D$-Ara*f* and a linear β-$_D$-Ara*f*-(1-2)-α-$_D$-Ara*f*-(1-5)-α-$_D$-Ara*f* (Brennan and Nakiado, 1995). Apart from

this there were three types of LAM isolated from *M. tuberculosis*. The first one termed ManLAM was the one in which the arabinan termini was capped with Manp residues. The second one termed AraLAM were isolated from fast growing *M. smegmatis* and were devoid of Man caps. And the third type were partially capped with inositol-P residues (Chatterjee et al, 1993).

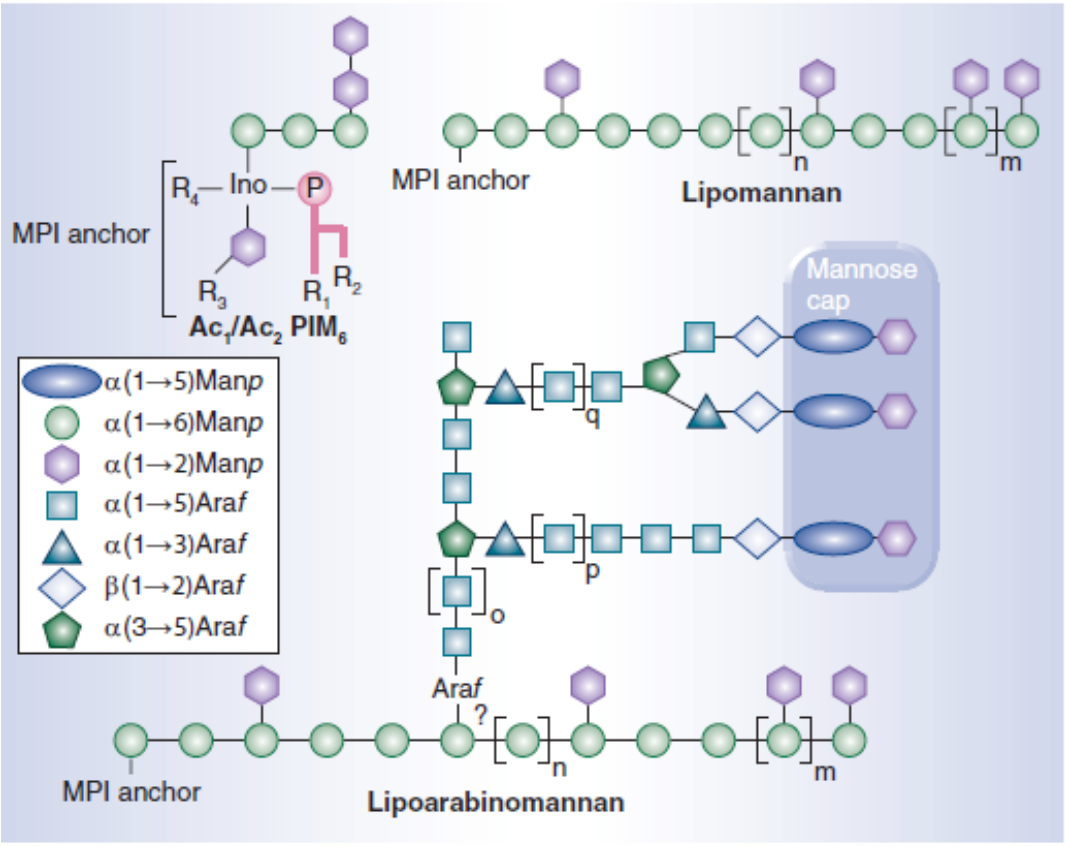

Figure 1.10: Structural features of lipomannan (LM) and lipoarabinomannan (LAM). Adapted from Jankute et al, 2012.

1.7.6 Extractable lipids in the mycobacterial cell wall

Apart from the above discussed component of the cell wall skeleton there are a number of covalently bound lipids within the hydrophobic mycolic acids. Few of these lipids are

species specific like sulfolipids (SL), lipooligosaccharides (LOSs) and phenolic glycolipids (PGLs). Common to all mycobacteria is the presence of trehalose dimycolates (TDM). These components present in the outer membrane have been associated with pathogenicity (Minnikin et al, 2015).

1.7.6.1 Lipooligosaccharides (LOSs)

Lipooligosaccharides have been mainly found in opportunistic and non-pathogenic strains of mycobacteria and are highly polar and trehalose associated glycolipids. LOSs were first identified in *M. kansasii* (Hunter et al, 1985) and then later in *M. gordonae* (Besra et al, 1993), *M. butyricum*, *M. marinum* (Minnikin et al, 1989) and *M. szulgai*. In *M. kansasii* eight LOSs are present and their structure is composed of residues of xylose, 3-*O*-methylrhamnose, fucose and N-acylkansosamine linked to core which contains α,α' trehalose moiety (Hunter et al, 1983). LOSs have also been discovered in *M. smegmatis* in which the oligosaccharide unit of LOS contains only D-glucose residues. LOSs are not produced in the pathogenic *M. tuberculosis* and *M. leprae* and have thought to play a role in virulence and sliding motility.

1.7.6.2 Phenolic glycolipids (PGLs)

Glycosylphenolphthiocerol dimycocerosates also known as PGLs form the second class of glycolipids and are found in *M. kansasii*, *M. marinum* and *M. bovis*. PGLs are hydrophobic molecules that form a large part of the outer membrane of the cell wall. This glycolipids have different classes like myoside A in *M. kansasii*, myoside G in *M. marinum* and myoside B in *M. bovis*. The typical structure of PGLs consists of a large

hydrophobic moiety, the length of which differs according to species, consisting mainly of deoxy sugars that are *O*-methylated and C36 diol substituted by two molecules of mycocerosate (Daffe and Laneelle 1988). Apart from PGLs there are also GPLs which form major cell surface antigens in *M. avium* and *M. intracellular* (Minnikin et al, 2002).

1.7.6.3 Sulfolipids

Chemically defined by Goren and Brennan in 1979, sulfolipids also known as sulfatides belong to acylated trehalose family. They were first isolated in 1959 from *M. tuberculosis* by Middlebrook et al. Sulfolipids are either found as tri or tetra acylated trehalose-2-sulfate and the acyl function can either be palmitate, sterate or hydroxyphthiocerate (Brennan, 1988). Some of the studies have shown that sulfolipids are involved in phagosome inactivation and few more studies have also shown that the presence of sulfolipids in *M.tuberculosis* is correlated to its persistent pathogenicity in guniea pig infection model (Pabst et al, 1988).

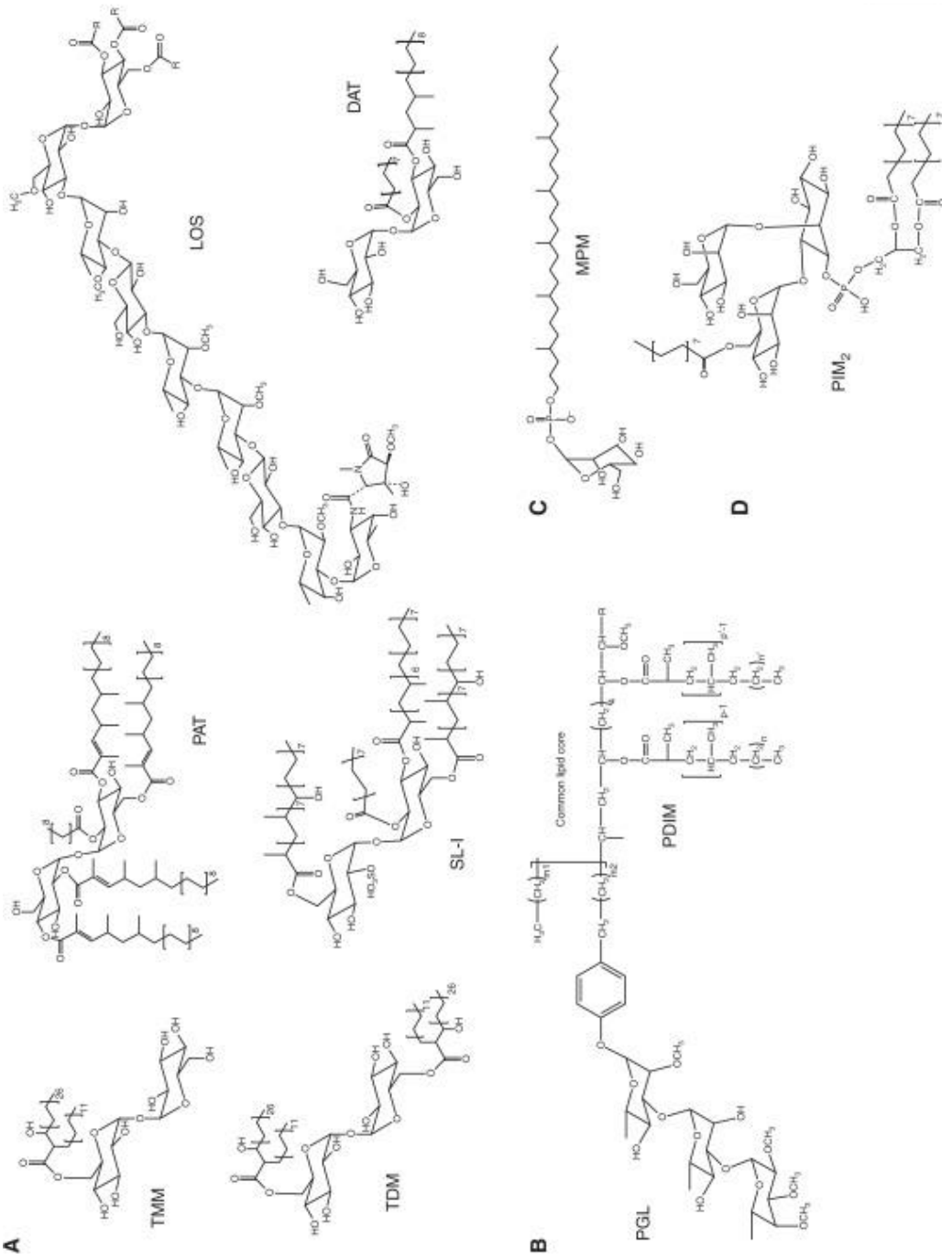

Figure 1.11: Structure of lipids of *Mycobacterium tuberculosis*. Adapted from Mary Jackson, 2014.

1.7.6.4 Trehalose containing glycolipids

The largest and most common glycolipid found in both virulent and avirulent strains of mycobacteria are the trehalose monomycolates (TMM) and trehalose dimycolates (TDM). In 1884 the growth of *M. tuberculosis* in culture was described as rope like structures by Robert Koch. It was not until 1950 that this substance which was responsible for the cord formation in *M. tuberculosis* was identified to play a role in virulence and was termed a toxic substance (Bloch, 1950). It was also shown that the substance involved in the cord formation could be removed using petroleum ether and did not affect the growth of the bacterium suggesting that this substance was present on the outer surface. In 1956 Noll identified this substance as 6.6'-di mycolate (TDM) (Noll, 1956). Trehalose di mycolates consists essential of trehalose which is found in abundance in mycobacteria and is esterified to two mycolic acid residues whose length is variable amongst *Mycobacterium* species (Elbein and Mitchell, 1973 and Elbein et al, 2003). Within the host TDM has shown to inhibit the phagosome lysosome fusion hence enabling the bacterium to survive within the host's phagosome. The biosynthesis of trehalose involved three pathways and trehalose can be synthesized from:

1. Glucose-6-phosphate catalyzed by trehalose-6-phosphate synthase (Ots A) and trehalose-6-phosphate phosphatase (Ots B) (Pan et al, 2002). This pathway that utilizes Ots A and B is essential in *M. tuberculosis*.
2. Maltooligosyltrehalose synthase (TreY) and maltooligosyltrehalose trehalohydrolase (TreZ) generate trehalose grom glycogen.
3. Trehalose synthase catalysis the conversion of maltose to trehalose.

Lpq-SugA-SugB-SugCATP-binding cassette is the transporter system used for the transport of trehalose which recycles the release of trehalose. Antigen 85 is involved in the release of trehalose which occurs as a byproduct of the biosynthesis of mycolic acids. The Antigen 85 mycolyltransferases transfer trehalose monomycolate (TMM) to either another TMM forming trehalose di mycolate (TDM) or to the arabinogalactan.

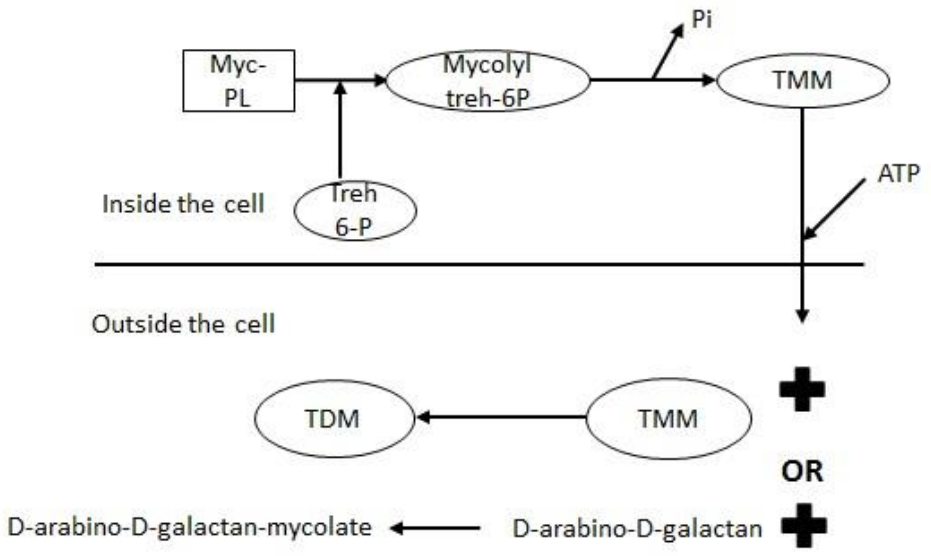

Figure 1.12: Mechanism of incorporation of newly synthesized mycolic acids into major cell wall components. Drawn by myself for the use in my thesis**.**

Figure 1.12 depicts the mechanism that is involved in the incorporation of new synthesised mycolic acids into the cell wall components. The diagram shows that TDM is synthesised with the outside the cell wall and TMM exported from the cytoplasm in order to prevent its degradation by Antigen 85 (Ag85) which is ubiquitously present (Sathyamoorthy and Takayama, 1987). The biosynthesis of trehalose di mycolate (TDM) involves the following steps : first a cytoplasmic mycolyltransferase 1 is involved in the

transfer of a mycolyl group from mycolyl-S-Pks13 (mycolyl-S-PPB) to D-mannopyranosyl-1-phosphoheptaprenol to yield Myc-PL 6-*O*-mycolyl-β-D-mannopyranosyl-1-phosphoheptaprenol (Myc-PL) (Besra et al, 1994). Next the migration of Myc-PL to the inner surface of the cell membrane involves docking itself to an ABC transporter with the help of it hydrophobic heptaprenol tail. The third step involves a mycolyltransferase 11 which transfers the mycolyl group to trehalose-6-phosphate forming TMMphosphate. Later trehalose-6-phosphate phosphatases removes the phosphate group from TMMphosphate resulting in TMM. This TMM along with the mycolyltransferases of Antigen 85 complex transfers the glycolipid TMM to another TMM forming trehalose di mycolate or to the cell wall arabinogalactan forming arabinogalactan-mycolate (Figure 1.11) (Takayama et al, 2005 and Sanki et al, 2009). In *M. tuberculosis*, the antigen 85 complex belonging to the α/β hydrolase superfamily is composed of Ag85A, Ag58B and Ag85C as the predominant secreted proteins. They all three catalyze hydrolysis of ester and amide bonds and all three contain two carbohydrate binding sites to which the TMM binds (Ronning et al, 2004).

1.8 The reductase that catalyses mycolic acid synthesis

The α-branch and the meromycolic acid synthesised by FAS-I and FAS-II respectively are condensed by the polyketide synthase Pks13. This forms a precursor 2-alkyl-3-keto fatty acid which is then reduced by a mycolic acid reductase (Lea-Smith et al, 2007) (Bhatt et al,2008) to form the mature mycolic acids. The mature mycolic acid that is formed is linked to 6-hydroxyl of disaccharide trehalose which forms trehalose

monomycolate (TMM) (mycobacteria) or trehalose monocorynomycolate (TMCM) (corynebacteria).

The mycolic acid reductase involved in final step of the formation of mature mycolic acids is a reductase gene identified from studies by Lea-Smith et al, 2007 and Bhatt et al, 2008. Lea Smith et al, 2007 generated a slow-growing mutant in *Corynebacterium glutamicum* whereas Bhatt et al, 2008 did the same in *Mycobacterium smegmatis*. The results from Lea Smith et al, 2007 showed that the reductase gene was not essential in *C. glutamicum* and Bhatt et al, 2008 showed that the reductase gene was non-essential in *M. smegmatis*. Also Bhatt et al, 2008 attempted to generate a knock-out of the reductase in *M. tuberculosis* and *M. bovis* BCG but were unable to do so probably because the gene is essential in the slow-growing mycobacterial species.

Rv2509 from *Mycobacterium tuberculosis* is the proposed ortholog of NCgl2385 (Lea-Smith et al, 2007) and MSMEG4722 (Bhatt et al, 2008) based on strong amino acid similarity between the predicted proteins and synteny between the regions in which the genes are found. This reductase gene is highly conserved in all sequenced genomes of mycobacterial species including *Mycobacterium leprae*. It was also found to be highly conserved in all *Corynebacteriacea*, *Segnilliparius* and *Norcardia* presumably encoding the same activity. The formation of mycolic acids is by the condensation of two fatty acids by Pks13 which is followed by the reduction to form β-hydroxy acid. Studies by Lea Smith focuses on the enzyme that catalyzes the final reduction step in the formation of mature mycolic acids.

1.8.1 Studies in *Corynebacterium glutamicum* NCgl2385

With the help of bioinformatics studies Lea Smith et al looked into the potential genes that encoded reductase activities in the genomes of *Corynebacterium glutamicum* and *Mycobacterium tuberculosis* and identified NCgl2385 in *C. glutamicum* the orthologue of Rv2509 in *M. tuberculosis* as a potential reductase. Deletion analysis resulted in a mutant ΔNCgl2385 with a slow-growth phenotype. The lipid analysis of the mutant ΔNCgl2385 showed the presence of two novel glycolipids which corresponded to trehalose monocorynomycolate (TMCM) and trehalose dicorynomycolate (TDCM). MALDI-TOF analysis of TMCM and TDCM of the wild type cells revealed the presence of a series of sodiated molecular ions which are consistent with TMCM and TDCM whereas the MALDI-TOF analysis of the mutant ΔNCgl2385 also revealed the presence of a similar cluster of solidated molecular ions but with 2 to 4 atomic mass difference when compared to the authentic TMCM and TDCM. The presence of this atomic mass difference could be due to the presence of an unreduced β-keto ester motif in the mutant ΔNCgl2385. The delipidated pellets which contain only the wall bound mycolates from the wild type and mutant ΔNCgl2385 were subjected to further extractions to determine if the defect in the mutant reflected on any defects in the other cell wall components. The results showed that ΔNCgl2385 showed a reduced level of arabinogalactan bound corynomycolates though the levels of LAM/LM and arabinogalactan were normal in the mutant. This conferred that the defect in the mutant ΔNCgl2385 affected the transport and attached of the newly synthesized corynomycolates to arabinogalactan. The presence of the novel glycolipids was confirmed by sodium borodeuteride treatment and mass

spectrometry (MS) analysis of ΔNCgl2385. Analysis of the data obtained from the treatment and MS demonstrated that the mutant ΔNCgl2385 corynomycolic acids contain a free keto group and therefore deficient in reducatase acitivity in corynomycolic acids. To conclude the findings of Lea Smith et al, NCgl2385 in *C. glutamicum* the orthologue of Rv2509 in *M. tuberculosis* was identified as the reductase involved in the final steps of synthesis of corynomycolic acids. HPTLC analysis of the mutant ΔNCgl2385 showed elevated expression of TMCM and TDCM when compared to the wild type cells and also showed a decrease in the transfer of mycolates to the arabinolgalactan in the cell wall. The decrease was evident with the transfer of mycolates to the arabinogalactan but not for the mycolyl reactions that lead to the transfer of mycolates in the formation of TDMC and TMCM. Complementation of NCgl2385 with the mutant ΔNCgl2385 restored the formation of mature corynomycolic acids demonstrating that the loss of NCgl2385 was the sole source reduction in the corynomycolic acids.

1.8.2 Studies in *Mycobacterium smegmatis* MSMEG4722

Mycobacterium smegmatis is non pathogenic, grows fast in laboratory media and can accommodate the deletion of some genes essential in *Mycobacterium tuberculosis* and for these reasons serves as a surrogate for studying genes involved in mycolic acid biosynthesis in *M. tuberculosis*. Using bioinformatics, MSMEG4722 in *M. smegmatis* was identified as the closest match to Rv2509 in *M. tuberculosis* belonging to shortchain dehydrogenase/reductase family containing conserved active site residues and NAD/NADP binding residues. Deletion of ΔMSMEG4722 resulted in a slow-growth

phenotype which was similar to that obtained with the mutant in *Corynebacterium glutamicum.* Growth of ΔMSMEG4722 on agar plates resulted in irregular and rough colony morphology when compared to the smooth colonies got with the wild type which is shown in the figure below adapted from the paper by Bhatt et al, 2008 with his permission to use this image.

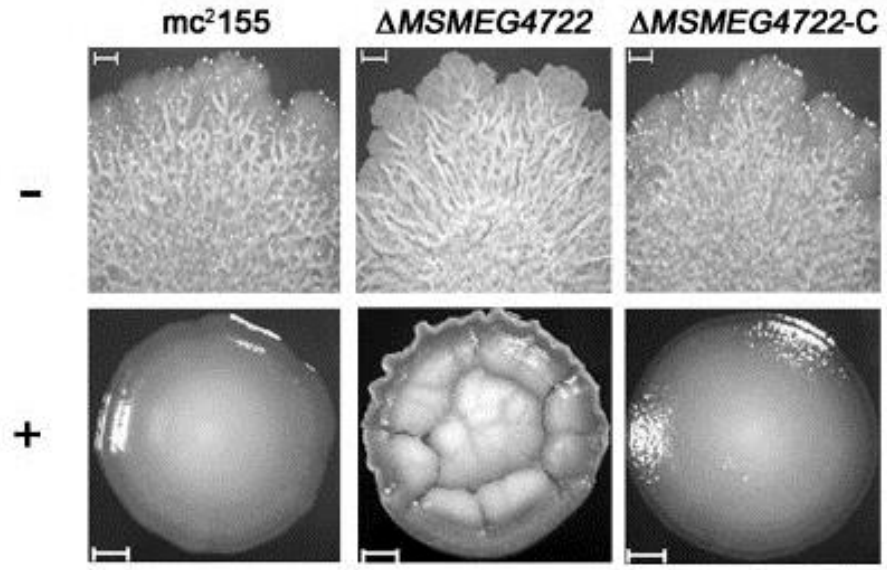

Figure 1.13 Colony morphology of *M. smegmatis* mc²155 WT, ΔMSMEG4722 and complemented strain ΔMSMEG4722-C grown on trytic soy agar without tween (-) and with 0.05% of Tween-80 (+). Adapted from Bhatt et al, 2008.

Lipid analysis showed the presence of α, α' and epoxy mycolic acids in the wild type mc²155 and the complemented strain but were missing in ΔMSMEG4722. Alkaline hydrolysis of ΔMSMEG4722 α-alkyl β-oxo precursors produced unstable β-oxo acids which loose carbon

dioxide to produce long-chain ketones. The same results were got from whole cells which contain both the cell wall bound mycolates and trehalose bound mycolates and from delipidated cells which contain the cell wall bound mycolates. The TLC image below has been adapted from the paper by Bhatt et al,2008 and used in this thesis with his approval.

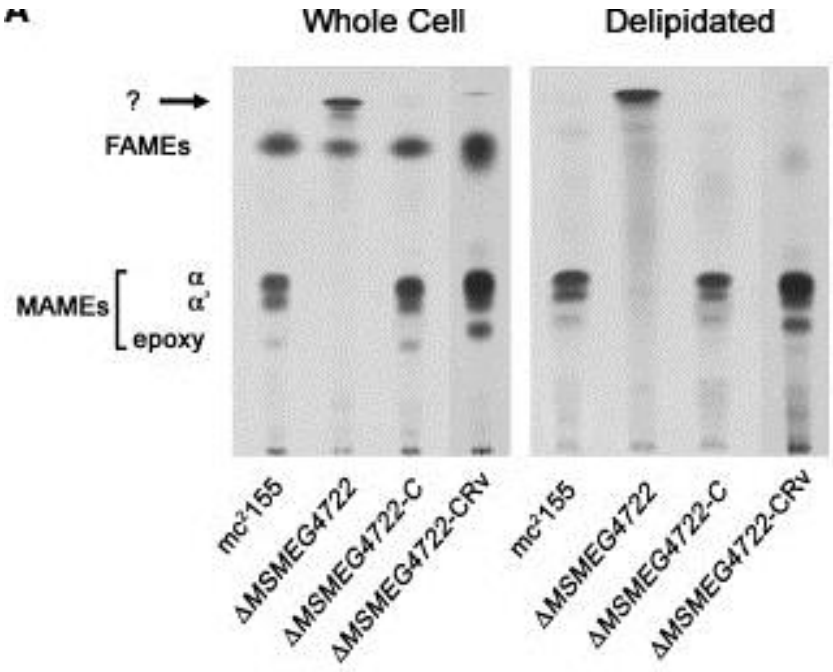

Figure 1.14: Thin layer chromatography (TLC) analysis of MAMEs extracted from mc^{2}155, ΔMSMEG4722, ΔMSMEG4722-C and ΔMSMEG4722-CRv. Adapted from Bhatt et al,2008.

The mutant cells were treated with sodium borohydride to confirm if the unreduced precursors of α, α' and epoxy mycolic acids did exist in the mutant and TLC analysis confirmed the presence of α, α' and epoxy mycolic acid methyl esters (MAMEs) due to the reduction of the β-oxo group after the treatment.The combined data from MALDI-TOF and

MS analysis demonstrated that deletion of MSMEG4722 resulted in loss of mycolic acid reductase function producing the α-alkyl β-oxo intermediates which were transported and subsequently attached to the arabinogalactan in the cell wall. Apart from this TLC analysis of mutant strain showed altered mobility of TDM and TMM when compared to the wild type suggestion that α-alkyl β-oxo precursors were esterified to the trehalose. Similar results were obtained from extractions of whole cell and delipidated cells suggesting that the processing , transport and transfer of mycolic acids to the cell wall could also be achieved using the α-alkyl β-oxo intermediates. This contradicted the result obtained from Lea Smith et al where there was reduced attachment of mycolates to the arabinogalactan in the cell wall. Complementation of MSMEG4722 with the mutant ΔMSMEG4722 restored the formation of mature mycolic acids indicating that the reduction in mycolic acids was due the loss of MSMEG4722.

1.6 Aims and objectives

To date all most genes involved in mycolic acid biosynthesis are essential and serve as potential drug targets. With the increasing cases of tuberculosis related deaths it is essential to find new drugs and reduced treatment options. The aims and objectives of this thesis are listed below.

1. The main aim was to test the essentiality of Rv2509 in *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG using a conditional mutant that silenced gene expression by the addition of anhydrotetracycline (ATc).

2. Next was the *in silico* analysis of the structure of Rv2509 which would give an insight into any unique domains and residues which played a role in the functionality of the protein through complementation studies.
3. Purification of Rv2509 protein and using it in a potential functional assay.
4. Assessing the biochemical changes to the changes in the structure of the cell wall by analyzing lipids extracted from the outer layer and looking into hydrophobicity due the difference in the colony morphology.

Chapter 2

Characterisation of a conditional mutant of the *Mycobacterium bovis* BCG Rv2509 homologue

2.1 Introduction

Mycolic acids form an essential part of the cell wall of *Mycobacterium tuberculosis* and other related mycobacteria. While most of the earlier research focused on studying genes encoding Type I and II fatty acid synthases, very little was known about the latter stages of mature mycolic acid formation. It was recent that two groups, Lea-Smith et al, 2007 and Bhatt et al, 2008 identified a slow-growing mutant in *Corynebacterium glutamicum* (Lea-Smith et al, 2007) and *Mycobacterium smegmatis* (Bhatt et al, 2008) missing a gene encoding a reductase involved in the final step of mature mycolic acid motif synthesis. Bhatt et al, 2008 showed that the alkaline hydrolysis of mycolic acids the parental strain *M. smegmatis* mc^2155 released α, α' and epoxy mycolates whereas the mutant strain ΔMSMEG4722 did not release any of the mycolates but instead showed an accumulation of the degradation products of the premycolates as they are unstable (Bhatt et al, 2008).

Prior to starting my project several attempts were made by Bhatt et al, 2008 to generate knockouts in *M. tuberculosis* and *M. bovis* BCG. Despite several attempts no knockouts were generated prompting that the reductase gene might be essential in *M. tuberculosis* and *M. bovis* BCG. According to transposon mutagenesis studies by Sassetti et al, insertions in Rv2509, the *M. tuberculosis* mycolic acid reductase results in a slow-growing mutant. Rv2509 in *M. tuberculosis* and its homologue Mb2537 in *M. bovis* BCG were identified as the probable reductase gene involved in the formation of mature mycolic acid based on amino

acid similarities with other reductases. *M. tuberculosis* Rv2509 was highly conserved in all related mycobacteria including *M. leprae* which has a highly decayed genome.

To study the essentiality of *M. tuberculosis* Rv2509 and *M. bovis* BCG Mb2537 a conditional mutant was constructed with the help of Dr.Rainer Kalscheurer, Institute for Medical Microbiology and Hospital Hygiene, Heinrich-Heine University, Dusseldorf, Germany. They constructed a conditional mutant in both *M. tuberculosis* and *M. bovis* BCG where the native promoter of the gene is replaced by a Tet-off promoter. The system used was a modification to the original Tet-off system where the addition of anhyrotetracycline (ATc) led to the silencing of the gene and the subsequent depletion of it in the bacterial cell (Ehrt et al, 2009). This chapter focuses on essentiality of reductase gene in *M. tuberculosis* and *M. bovis* BCG through a series of biochemical characterisations of the conditional mutant of Rv2509 and Mb2537.

2.1.1 Regulated gene expression systems in mycobacteria

Regulated gene expression systems were not well characterised in mycobacteria until recently, due to limited knowledge of transcriptional machinery mechanisms. The first successful gene regulated system based on dose responsive gene expression was the acetamidase system used in *Mycobacterium smegmatis*. Conditional mutants in mycobacteria have been generated by using regulated expression systems which silence or induce target gene expression by transcriptional regulation (Schnappinger et al, 2014). The most commonly used expression systems for mycobacteria so far are: acetamidase system based on regulatory elements of the *M.smegmatis* acetamidase gene (Parish et al, 1997 Triccas et

al,1998) and the Tet system based on genes encoding tetracycline exporters in *C. glutamicum*/*E. coli* (Bloch and Segal, 1956, Schnappinger et al,2003). Acetamide and tetracycline serve as inducers of gene expression and hence have to be removed to initiate gene silencing experiments. Bacterial expression systems have mainly two applications which include it being used in protein production and analysis of genes important in bacterial growth (Xinzheng.V.Guo et al, 2007). The most commonly used expression systems for silencing of genes is the inducible expression systems which involves the addition of an inducer. Since inducible expression systems involve the removal of inducer for silencing of gene it can have certain disadvantages. For example, removal of the inducer may affect the biological process under investigation or in some cases the removal can be slow because of its intracellular accumulation or its binding to the cell envelope. For these reasons the best suited expression system for studying silencing of genes are the systems which can be turned off by the addition of a corepressor.

2.1.2 The acetamidase system

In 1967, the presence of an acetamidase enzyme in *Mycobacterium smegmatis* was demonstrated by Draper. It is a highly inducible enzyme and upon induction by acetamide which acts as a substrate can grow on aliphatic amides as the sole carbon source. This was the first regulated gene expression system used in mycobacteria, and enabled first silencing of essential genes like *whm*D and *dna*A in *M. smegmatis* (Gomez and Bishai, 2000, Greendyke R et al, 2002). Apart from gene silencing this system has been used for the overexpression of mycobacterial antigens in *M. smegmatis*. The most commonly used gene essentiality testing method for mycobacteria in my laboratory is CESTET i.e conditional

expression-specialised transduction essentiality test (Bhatt et al, 2005). This gene essentiality method is based on the use of specialised transduction in conjunction with an inducible acetamidase promoter. The first step for this conditional expression essentiality test is the construction of a merodiploid strain. This strain essentially consists of a second copy of the essential gene fused transcriptionally to the acetamidase promoter. The second step involves the generation of a specialised transduction phage. This phage is designed to contain an allelic exchange substrate that replaces the target gene with hygromycin cassette. The final step is the transduction of the merodiploid strain. This is done prior to the growth of it in the presence or absence of acetamide. As with any system the drawbacks with the acetamidase system is low level induction even after the removal of inducer, cannot be used for *in vivo* studies, requires a special growth medium for optimal activity and is also dependent on the presence of a large 1.4 kb operator region which is at least 1.5 kb upstream of the promoter.

2.1.3 The Tet-on and Tet-off system

The P_{Tet} system i.e the tetracycline inducible promoter system is a versatile system as it has shown to regulate gene expression both *in vitro* and *in vivo* (Blokpoel MC et al, 2005, Carroll P et al,2005). It had been developed for use in Gram-positive and Gram-negative bacteria like *Escherchia coli, Staphylococcus aureus, Streptococcus pneumoniae*, few eukaryotic models, and recently been used for mycobacteria related species. In the P_{Tet} system the expression of tetracycline proteins is regulated by the Tet repressor (TetR) proteins. There are two divergent promoters in the P_{Tet} system of which one is responsible for expression of the target gene whereas the other drives the expression of the Tet repressor (TetR). The P_{Tet} system also comprises two operators to which the Tet repressor (TetR) binds. In the presence

of tetracycline (Tc), also known as the Tet-on system, as tetracycline enters the cell it binds to the Tet repressor (TetR) inducing conformation changes resulting in the dissociation of the Tet repressor (TetR) from the tet operators thus resulting in the expression of the target gene. Alternatively in the absence of tetracycline (Tc), also known as the Tet-off system, the Tet repressor (TetR) binds to the tet operators which in turn suppresses the transcription of the target gene. Advantage of using tetracycline is that it easily crosses biological membranes through diffusion making bacterial cells easily accessible for these inducers. This also has an added advantage especially in mycobacteria that enables regulated gene expression in liquid cultures as well as mammalian cells infection models (Ehrt et al, 2005). The Tet-on system works with the addition of the inducer tetracycline hence leading to expression of the target gene, which is the basis for most of the gene expression systems but is not useful if the target is to silence gene expression. Ehrt et al, 2009 constructed an expression system where in, the addition of anhydrotetracycline (ATc), a derivative of tetracycline, to the culture silenced gene expression. They constructed a mutant of the Tet repressor (TetR) which harboured three amino acid mutations in the DNA binding domain. Normally when tetracycline (Tc) enters the cell it binds to the Tet repressor (TetR) causing it to dissociate from the DNA, but the mutations to the Tet repressor reversed this response to tetracycline (Tc) binding. So in the altered system, as tetracycline (Tc) enters the cell it binds to the mutated Tet repressor causing the repressor to bind to the DNA. Efficient repression by this mutated Tet repressor required high levels of TetR expression which could only be achieved if it were located on a multi copy plasmid and if the TetR gene was transcribed by the mycobacterial promoter (Ehrt et al, 2009).

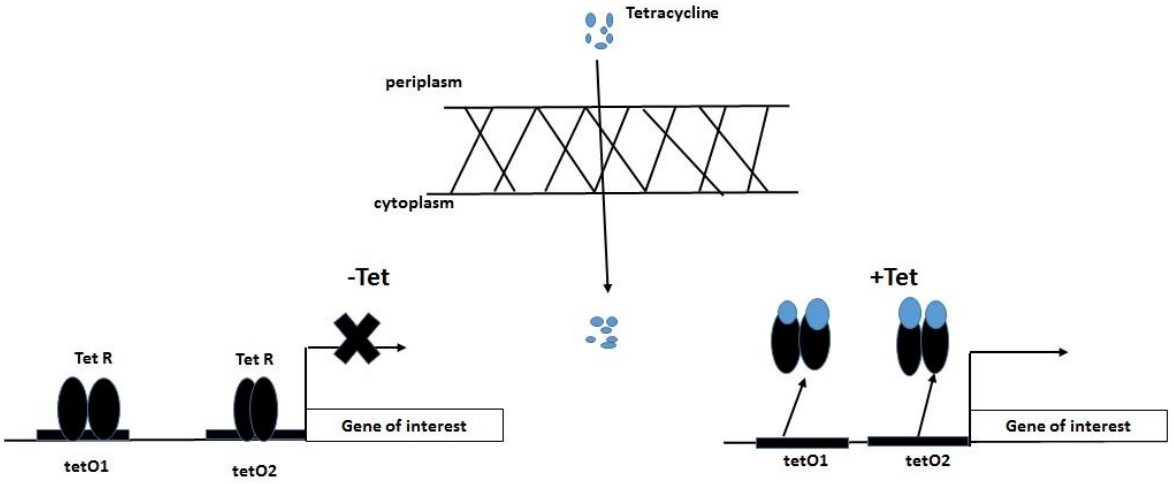

Figure 2.1: Diagram representing a general tetracycline inducible expression system.

The expression system used for the conditional mutant in this study is a modification to the original Tet system. In this system, efficient gene silencing is by the addition of anhydrotetracycline (ATc), a derivative of tetracycline, instead of removal. In the original tetracycline system regulation is mediated when the tc repressor (TetR), a single repressor protein, binds to two operators ($tetO_1$ and $tetO_2$) in the tet promoter (P_{Tet}). RNA polymerase masks the access of the tet promoter and hence initiation of transcription is inhibited (Klotzsche et al, 2009). In the modified system used in this study, the native promoter of Rv2509 in *Mycobacterium tuberculosis* and the vaccine strain *Mycobacterium bovis* BCG Mb2537 was replaced with a Tet-off promoter (P_{Tet}) which in the presence of anhydrotetracylcine (ATc) an exogenously expressed Tet repressor binds to the promoter preventing expression of the Rv2509 gene and subsequently leads to silencing of the gene and depletion of Rv2509 in the bacterial cell. For my project I worked with the conditional mutant of *M. tuberculosis* Rv2509 in *M. bovis* BCG.

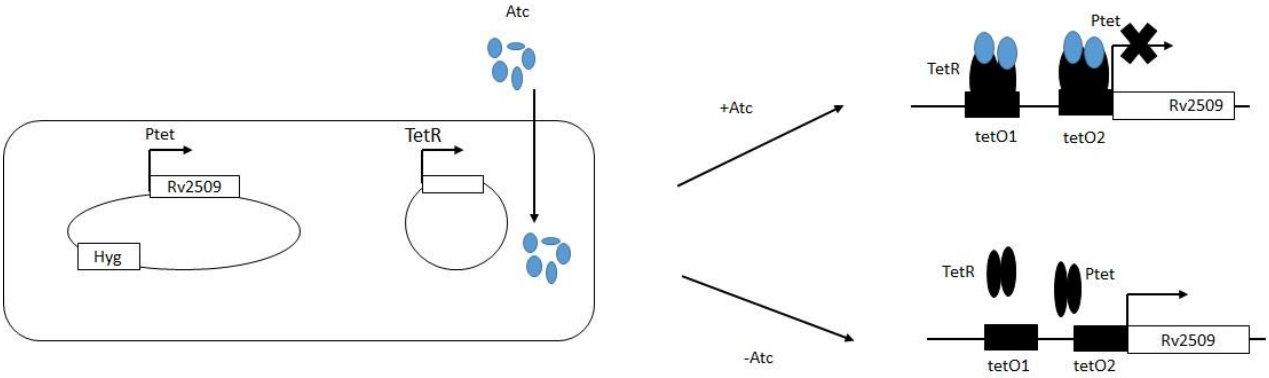

Figure 2.2 : Diagram representing of expression system used for conditional mutant in *M. tuberculosis* Rv2509 and *M. bovis* BCG Mb2537.

2.2 Materials and Methods

2.2.1 Bacterial Strains

The BCG conditional mutant of Rv2509 was got from Dr. Rainer Kalscheurer, Institue for Medical Microbiology and Hospital Hygiene, Heinrich-Heine University, Dusseldorg, Germany. These contained 1 ml aliquots of BCG-Pasteur c-Rv2509-tet-off mutant clones and a vector control strain.

Table 2.1: List of plasmids used to test essentiality of Rv2509.

Plasmids	Source
pMV261- BCG P_{Tet} Rv2509	pMV261 is an *E. coli-Mycobacterium* shuttle plasmid vector with hsp60 promoter and kanamycin resistant cassette (Stover et al,1991). pMV261-BCG P_{Tet} Rv2509 is the tet-off mutant obtained from Dr. Rainer's laboratory
pMV261-BCG P_{Tet} Rv2509 control	pMV261 is an *E. coli-Mycobacterium* shuttle plasmid vector with hsp60 promoter and kanamycin resistant cassette (Stover et al,1991). pMV261-BCG P_{Tet} Rv2509 is the control strain obtained from Dr. Rainer's laboratory

2.2.2 Culture condition and growth

The strains were grown at 37^{o}C in Difco Middlebrook 7H9 broth (Difco/VWR) and Difco Middlbrook 7H11 agar (Difco/VWR). Antibiotics for selection were added at the following concentrations: Kanamycin 20 mg/ml and Hygromycin 50 mg/ml. It is important to add Kanamycin as this is necessary to stabilize the tetR expression plasmid (a pMV261 derivative). 7H9 broth and 7H11 agar were supplemented with a growth supplement OADC (Sigma), 0.2% glycerol and 0.05% Tween 80 and anhydrotetracycline (ATc) added appropriately.

2.2.3 Conditional depletion of *M. bovis* BCG tet-off mutant

To determine the death kinetics, 1% inoculum of the vector control strain and the *M. bovis* BCG tet-off mutant (grown in the absence of ATc) were sub cultured in 10 ml of 7H9 broth and varying concentrations of ATc ranging from 0.025-0.3 mg/ml. Death kinetics in this experiment refers to determining the ideal concentration of ATc that is required to observe the depletion of the *M.bovis* BCG tet-off mutant. The initial preculture was spread on 7H11 agar plates containing varying concentrations of Atc (0.25-0.3 mg/m ATc).

2.2.4 Growth dependence on anhydrotetracycline of the BCG tet-off mutant

To observe the silencing effect at various time points, the vector control strain BCG P_{Tet} Rv2509 control and *M. bovis* BCG tet-off mutant BCG P_{Tet} Rv2509 were monitored at O.D 600 nm over a period of 0-60 hours at intervals of 12,24,36,48 and 60. The strains were grown in 7H9 broth containing OADC, 0.2% glycerol, 0.05% Tween 80, Kanamycin (20 mg/ml), Hygromycin (50 mg/ml) and 0.075 mg/ml of ATc. The starting O.D of all cultures was 0.1. Aliquots of 5 ml were taken at different time points and washed with PBS to get rid of any ATc. These were serially diluted to 10^{-6} and plated on 7H11 agar plates containing OADC, appropriate antibiotics and 0.075 mg/ml of ATc. The plates were incubated at 37^{o}C up to 4 weeks for colonies to form, after which cfu/ml calculated and graphs plotted.

2.2.5 Extraction and analysis of Fatty acyl methyl ester (FAMEs) and Methyl acyl mycolic ester (MAMEs)

For extraction of FAMEs and MAMEs, the vector control strain and BCG tet-off mutant were labelled with ^{14}C. Both the strains were grown in 10 ml of 7H9 Middlebrook broth at 37^{o}C

in a shaking incubator up to mid-logarithmic phase and then 10 µl of ^{14}C acetate (57 mCi/mmol, GE healthcare, Amersham Biosciences) was added to the culture and further incubated for 24 hours. The bacterial cells were harvested and washed with PBS and FAMEs and MAMEs were extracted and analysed by 1D TLC. Autoradiograms were produced by overnight exposure of Kodak AR films to the plates. Extraction protocol and thin layer chromatography analysis have been illustrated in detail in section 7.2 in chapter 7 of general methods and materials.

2.3 Results

2.3.1 The gene encoding the mycolic acid reductase is an essential gene in *M. tuberculosis* and *M. bovis* BCG

It was previously shown that the mycolic acid reductase gene that was involved in the final steps of mature mycolic acid motif synthesis was non-essential in *M. smegmatis*. A null mutant for this gene was identified in *M. smegmatis* but even after repeated attempts a knockout mutant was not generated in *M. tuberculosis* and *M. bovis* BCG. With the help of Dr. Rainer Kalscheurer, a conditional mutant was constructed in *M. tuberculosis* Rv2509 and *M .bovis* Mb2537. I worked with the *M. bovis* BCG Mb2537 strain whereas all the *M. tuberculosis* work was done at Dr. Rainer Kalscheurer lab. In the conditional mutant, the native promoter of Rv2509 in the vaccine strain *Mycobacterium bovis* BCG was replaced with a Tet-off promoter (P_{Tet}). In the presence of anhydrotetracylcine (ATc) an exogenously expressed Tet repressor binds to the promoter preventing expression of the Rv2509 gene and subsequently leads to silencing of the gene and depletion of Rv2509 in the bacterial cell. The

recombinant strain *M. bovis* BCG Mb2537 was grown on agar plates and in broth with varying ATc concentrations to conditionally deplete gene function. Figure 2.3 represents the liquid culture of the vector control strain growing in varying concentrations of ATc. The vector control strain grew well in the absence of ATc as well as in increasing concentrations of ATc ranging from as low as 0.05 mg/ml to 0.3 mg/ml of ATc. This showed that the control strain growth was not influenced by the presence or absence of ATc. Figure 2.4 represents the liquid culture of *M. bovis* BCG Mb2537, the conditional mutant when grown in the absence of ATc where it grew well as shown in the first tube and depletion of growth in the following tubes with the presence of ATc in the media. Absence of growth was seen right from the lowest concentration of ATc at 0.05 mg/ml.

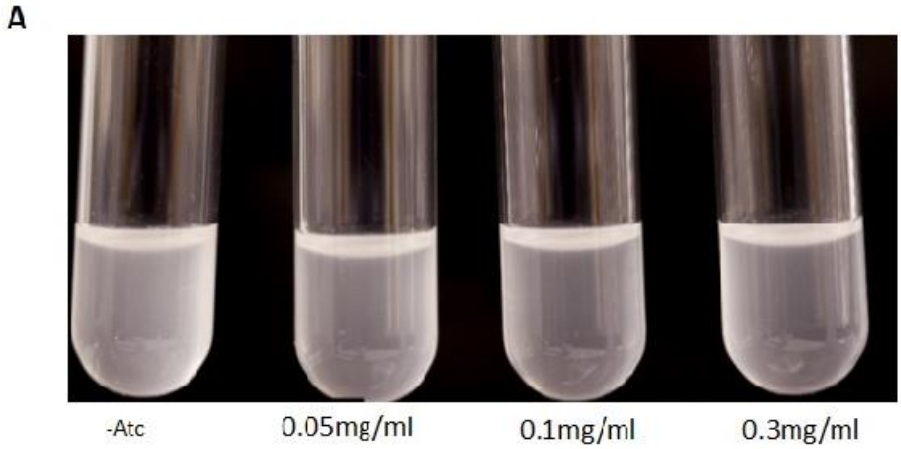

Figure 2.3: Liquid culture of vector control grown in varying ATc concentrations. Growth of control vector strain in the absence of Atc and presence of varying concentration of Atc in 7H9 broth+ OADC+0.2% glycerol+0.05% Tween 80.

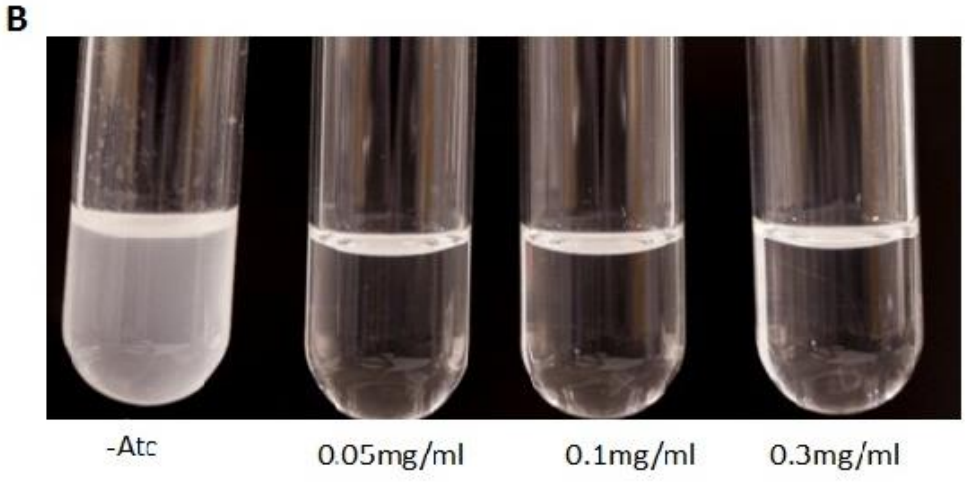

Figure 2.4: Liquid culture of conditional mutant in *M. bovis* Mb2537 grown in varying ATc concentrations. Growth of BCG P_{Tet} Rv2509 in absence of Atc and presence of varying concentrations of Atc in 7H9 broth+OADC+0.2% glycerol+0.05% Tween 80.

2.3.2 Effect of conditional mutant *M. bovis* BCG P_{Tet} Rv2509 on growth in liquid media

The growth of the mutant strain *M. bovis* BCG P_{Tet} Rv2509 in the presence and absence of 0.075 mg/ml of ATc were compared. *M. bovis* BCG P_{Tet} Rv2509 was grown up to 60 hours in 7H9 broth supplemented with OADC, 0.2% glycerol and 0.05% Tween 80 and O.D measured at regular intervals. The *M. bovis* BCG P_{Tet} Rv2509 grown in the absence of ATc grew up to an O.D of 0.4 at 24 hours and then growth declined to 0.2 at 60 hours. When *M. bovis* BCG P_{Tet} Rv2509 was grown in the presence of ATc it grew up to an O.D of 0.2 at 12 hours after which growth declined to 0.1 at 60 hours. The addition of ATc affected the growth of the mutant strain.

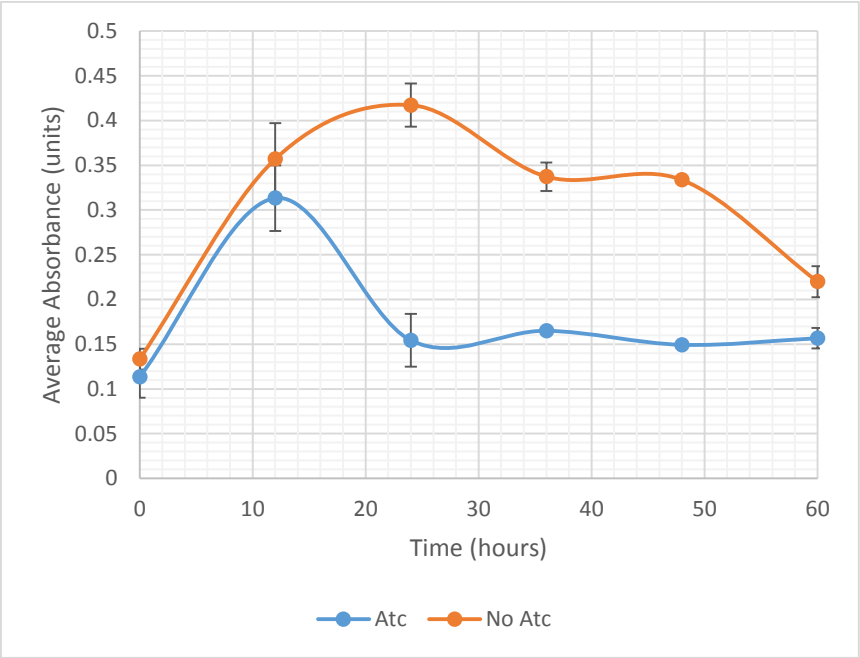

Figure 2.5: Growth curve of conditional mutant BCG P_{Tet} Rv2509 in 7H9 broth with the addition of OADC+0.2% glycerol+0.05% Tween 80 at 12 hour intervals up to 60 hours.

2.3.3 Effect of condition mutant *M. bovis* BCG P_{Tet} Rv2509 when grown on agar plates

The effect of growth of the conditional mutant *M. bovis* BCG Mb2537 was monitored first in liquid cultures. Experiments were set up first to optimise the concentration of ATc to be added to liquid cultures in order to visualise depletion in growth. But with liquid cultures depletion in growth was seen with even a low concentration of 0.05 mg/ml of ATc when added to the medium. A similar approach was used when growing the conditional mutant on agar plates. The conditional mutant and the vector control strain were spread on agar plates with no addition of ATc and varying concentrations of ATc ranging from 0.05 mg/ml to 0.3 mg/ml. Figure 2.6 represents growth of the vector control strain grown on agar plates in the

presence and absence of ATc. There was confluent growth of the control strain in the presence and absence of ATc showing that the growth was not influenced by ATc. Figure 2.7 represents growth of the conditional mutant grown on agar plates in the absence of ATc and the presence of 0.075 mg/ml of ATc. After some standardisation experiments, 0.075 mg/ml of ATc seemed to be the right concentration to grow the conditional mutant on. While there was confluent growth on agar plates without ATc, there were single colonies on the ATc containing plates. These single colonies were most likely spontaneous non regulated mutants with basal level of Rv2509 expression even in the presence of ATc.

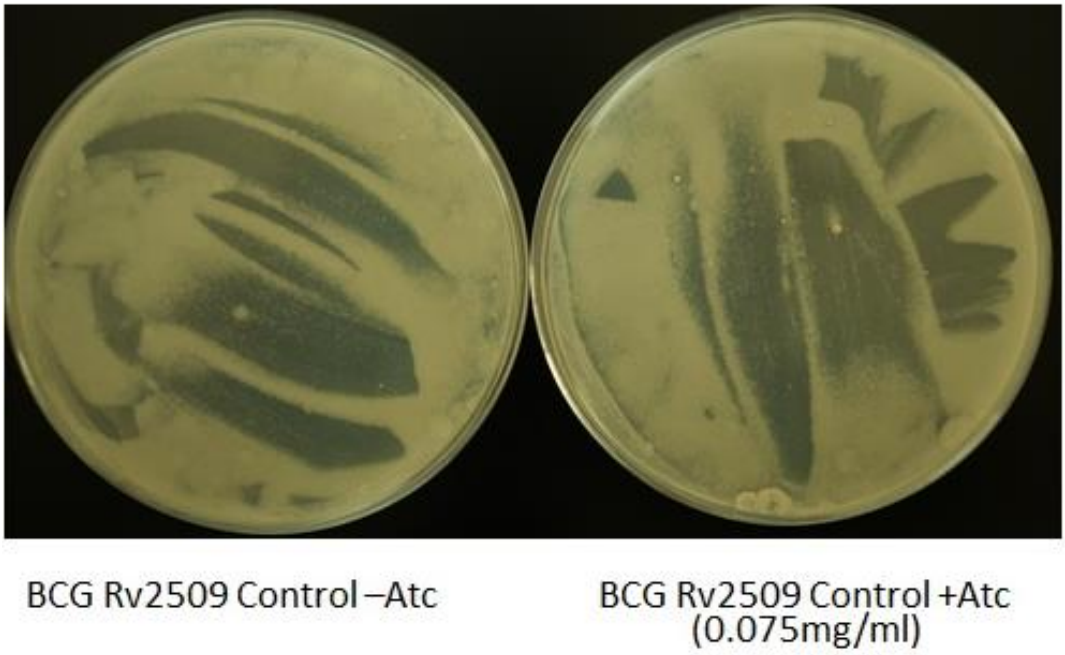

Figure 2.6: Growth of the vector control strain on 7H11 agar plates with and without ATc.

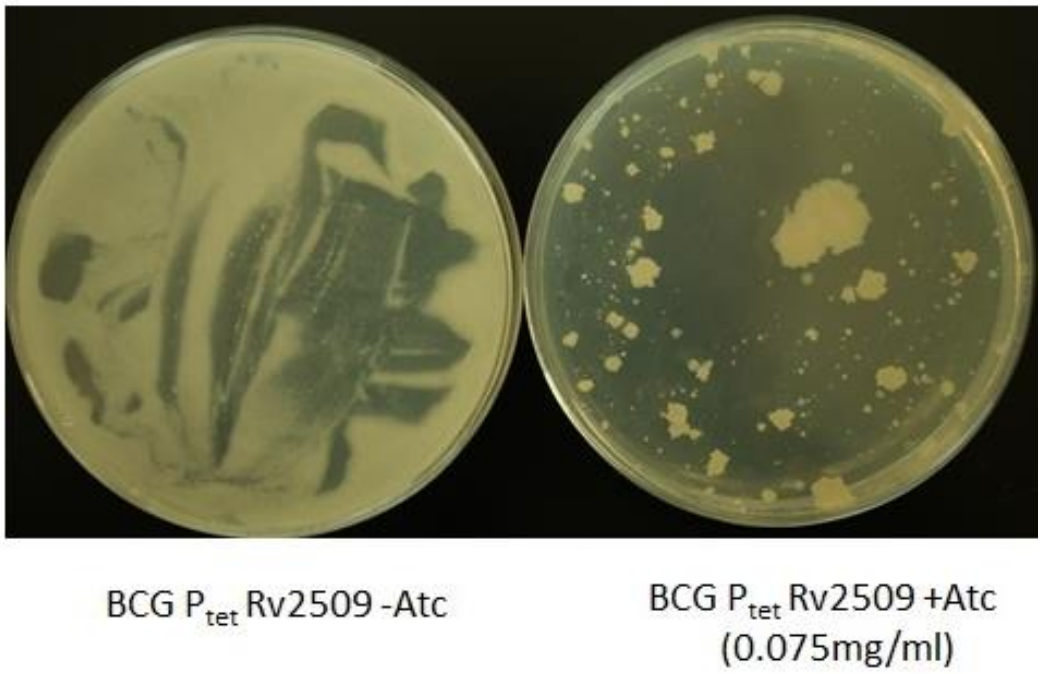

Figure 2.7: Growth of conditional mutant *M. bovis* BCG Mb2537 with no ATc and 0.075 mg/ml of ATc.

2.3.4. Depletion of Rv2509 leads to accumulation of premycolates in BCG-P_{Tet} Rv2509

To further characterise the *M. bovis* BCG Mb2537, I grew cultures of the strain on agar plates similar to the one shown in figure 2.6 and figure 2.7 but supplemented it with ^{14}C acetate. The conditional mutant depicted a slow growth rate which was similar to that seen when the mutant strain ΔMSMEG4722 was grown on plates. Mycolic acids were extracted from the cultures as methyl esters of mycolic acids (MAMEs) and analysed by thin layer chromatography shown in figure 2.8. Previously it was shown that alkaline hydrolysis of the parental strain *M. smegmatis* mc^2155 released the α, α' and epoxy mycolates whereas the mutant strain ΔMSMEG4722 upon alkaline hydrolysis showed a reduction in the mycolates

and an accumulation of the degradation products of the premycolates as these were unstable. Alkaline hydrolysis of the conditional mutant *M. bovis* BCG Mb2537 showed a similar phenotype as the mutant ΔMSMEG4722 when grown in the presence of ATc. Figure 2.8 shown below shows that alkaline hydrolysis of the control strain releases α and keto mycolates when grown in the presence and absence of ATc. It also shows that the alkaline hydrolysis of the conditional mutant grown in the presence of ATc shows a clear accumulation of the degradation products of premycolates marked as Y in the figure and reduction of mycolates, a phenotype similar to ΔMSMEG4722. The conditional mutant released α and keto mycolates when grown in the absence of ATc.

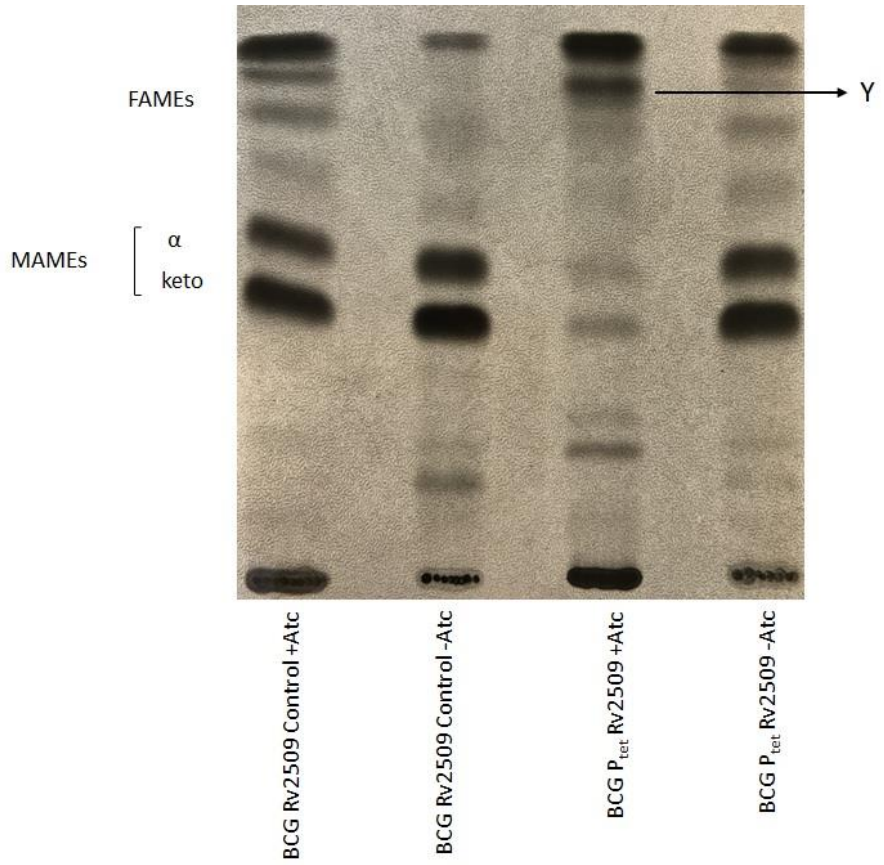

Figure 2.8: 1D TLC of FAMEs and MAMEs of the conditional mutant *M. bovis* BCG Mb2537.

2.4 Discussion

Mycolic acids play an important role in the cell wall composition and any alternation in genes involved in their synthesis makes the strain susceptible to antibiotics. It has been shown previously that the gene involved in the final production of mature mycolic acids is non-essential in *C. glutamicum* and *M. smegmatis* (Lea-Smith et al,2007 and Bhatt et al,2008). Bhatt et al, 2008 were unable to generate mutants of the reductase gene in *M. bovis* BCG and *M. tuberculosis*, hence indicating that the gene was likely to be essential. The work in this

chapter describes working with a conditional mutant of the reductase gene in *M. bovis* BCG Mb2537 termed as BCG P_{Tet} Rv2509. Biochemical characterisation of the mutant showed a reduction in mycolic acids and an accumulation of the degradation products of the premycolates, a phenotype similar to the one obtained for *M. smegmatis* (Bhatt et al, 2008).

The BCG P_{Tet} Rv2509 showed a clear decrease in growth when grown in the presence of 0.075 mg/ml of ATc (Figure 2.4). When the mutant was streaked on 7H11 agar plates without ATc there was confluent growth whereas in the presence of ATc single colonies were obtained. These could be spontaneous non-regulated mutants obtained in culture. Since the experiment involved dealing with an essential gene, there is a strong selection pressure for deregulation. It is shown with some conditional mutants that they acquire secondary mutations for some unknown reason leading to deregulation very rapidly. With liquid cultures it was difficult to recognise when deregulated mutants were present but on plates they were obvious. BCG P_{Tet} Rv2509 showed the reduction of mycolic acids (Figure 2.8) from both liquid cultures as well as mass extracted from ^{14}C labelled plates.

In conclusion I was able to show that Rv2509 is an essential gene in *M. tuberculosis* through a series of biochemical characterisations. The findings from this chapter state that Rv2509 has similar functions as the *M. smegmatis* homologue. Further chapters look into the structural characterisation and interaction with other mycolic acid intermediates.

Chapter 3

Structure Function Relationships of

Rv2509

3.1 Introduction

The essentiality of the mycolic acid reductase Rv2509 in *M. tuberculosis* and its homologue in BCG Mb2357 were demonstrated in the previous chapter. Following the confirmation of essentiality, I further investigated the potential mechanisms of Rv2509 in mycolyl moiety reduction using a combination of *in vitro* and *in vivo* approaches. The first stage involved bioinformatics studies in collaboration with Dr.Vassily Bavro (School of Biological Sciences, University of Essex) and his PhD student Robert Marshall (School of Biosciences, University of Birmingham). Using bioinformatics, Lea Smith et al, 2007 and Bhatt et al, 2008 identified Rv2509 in *M. tuberculsosis* as the homolog of NCgl2385 in *C. glutamicum* and MSMEG4722 in *M. smegmatis* respectively. Bioinformatic studies done on *M. tuberculosis* Rv2509 showed that the proteins were structurally similar to those of short chain dehydrogenase/reductase family. There are a number of members belonging to the short chain reductase (SDR) family within mycobacteria. Within *M. tuberculosis* genome *mab*A and *inh*A, genes encoding the fatty acid synthase 2 (FAS-11) reductases belong to short chain dehydrogenases/ reducatses (SDR) family.

3.1.1 Short Chain Dehydrogenases

Short chain dehydrogenases/reductases (SDR), are a large enzyme super family of NADH/NADPH dependent enzymes, present in prokaryotes as well as eukaryotes. The first members of the short chain dehydrogenase/reductase (SDR) were *Drosophila* ADH and a prokaryotic ribitol dehydrogenase (Jornvall et al, 1995). At present there are about 3000 highly conserved, distinct, well studied members to this family (Jornvall et al, 1995).

Members of this family show low amino acid/nucleotide identity of 15-30%. Despite this low sequence identity the members depict a highly conserved α/β fold and largely superimposable peptide back bones (Favia et al, 2008). SDR family consisting of isomerases, oxidoreductases and lyases classes of enzymes plays an important role in metabolic processes. Functions mainly carried out by the SDR family are oxidation/reduction reactions mediated by the nicotinamide ring of NADH/NADPH, which acts as an electron carrier (Kavanagh et al, 2008). Some examples include utilization and detoxification of ethanol and xenobiotics, regulation of signalling molecules and hormones.

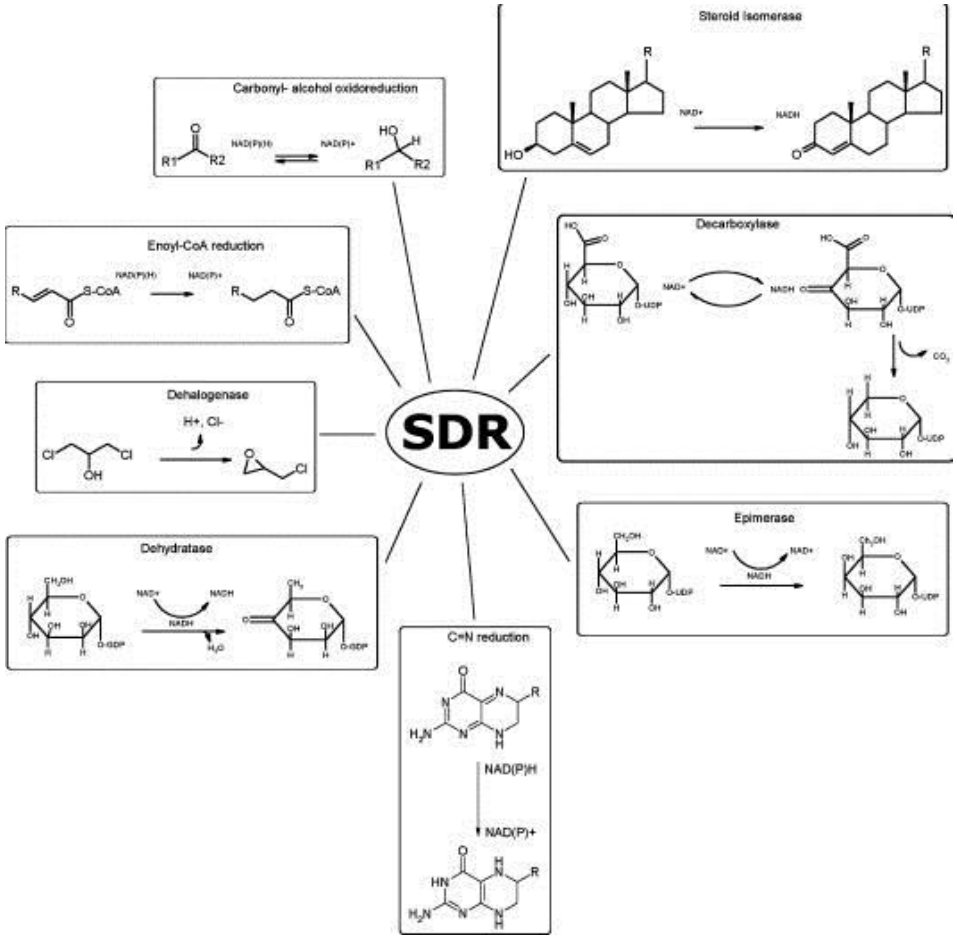

Figure 3.1: Representative reactions characterised by SDR enzymes.

The SDRs can mainly be divided into classical SDR and extended SDR families. The two families differ in the number of residues with classical having 250+ residues and extended having 350+ residues. They also differ in the glycine residue pattern in the co-enzyme binding region. This glycine rich pattern is important for structural integrity and accommodation and binding of the pyrophosphate portion of the nucleotide cofactor (Persson et al, 2003).

3.1.2 Rossmann Fold

Common characteristic features of the SDR family is the occurrence of Rossmann fold dinucleotide cofactor binding motif and a conserved Try-X-X-X-Lys segment essential for catalytic activity of SDR proteins. The Rossmann fold is composed of a central twisted β-sheets consisting of six to seven strands flanked by three to four α-helices from each side. The nicotinamide binding site resides in the β-β motif of the Rossmann fold. For NADH binding enzymes there is an acidic residue present in the second β-strand whereas for NADPH preferring enzymes there are two basic residues present in the β-strand. The members of SDR family generally display a simple one domain architecture where in the coenzyme binds to the N terminal part and the substrate binds to the C terminal part of the protein. The active site for the SDR family is generally a triad or tetrad comprising of serine, tyrosine, lysine and asparagine residues. Through sequence similarities it has been shown that the common criterion for SDRs is the Rossmann fold scaffold and the ability to bind either NADH/NADPH dinucleotides. The structure of majority of SDRs shows a tyrosine based catalytic centre with adjacent serine and lysine residues. Kinetic studies on classical SDRs show that SDR reactions proceed through a bi-bi mechanism, wherein the coenzyme

binds first and is the last to dissociate. The general scheme for the mechanistic aspects of SDR enzymes is as follows though variations have been seen amongst the other families. The SDR chemical groups mainly constitute hydroxyl/carbonyl groups and catalyse reduction of C=C and C=N double bonds. In SDRs the central acid-base catalyst is a hydroxyl-tyrosinate ion that either donates or abstracts a proton to or from the substrate. The mechanism of the tyrosine and lysine active site residues is as follows. The tyrosine residues acts as a catalytic acid or base that is enhanced by an adjacent lysine residue which along with the oxidised positively charged cofactor nicotinamide lowers the pKa of tyrosinehydroxyl. Apart from this, the lysine residue is also involved in nicotinamide ribose binding. The roles of active site serine is to stabilize and polarise the carbonyl substrate group where the asparagine residue produces a characteristic helical kink. The carbonyl group of the asparagine residue ligates water molecules to the distant active site lysine in the H-bonding state. This forms a proton relay system that connects the bulk solvent to the active site tyrosine residue (Kavanagh et al, 2008).

3.1.3 Aims and objectives

The main aim of this chapter was to generate an *in silico* 3D structure for Rv2509. This was done in collaboration with Dr.Vassily Bavro and his PhD student Robert Marshall. Using bioinformatics, we looked into the available crystal structures of SDR proteins to help build a model for Rv2509. The *in silico* structure of Rv2509 gave us an insight of the possible critical residues and regions that might play a role in the functionality of the protein. Using this information the aim was to test the role of these residues by complementing the *M. smegmatis* mutant ΔMSMEG4722 with the mutated copies of plasmid borne Rv2509.

3.2 Materials and Methods

3.2.1 *In Silico* analysis

Rv2509 gene sequence was obtained from the tuberculosis genome database (http://tuberculist.epfl.ch/). Protein sequence alignment of Rv2509 was done with the help of blastp (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins). Structural characterisation of Rv2509 was done in collaboration with Dr. Vassily Bavro and his student Robert Marshall. i-Tasser suite was used to determine sequence similarity between Rv2509 sequence and those available in the protein database (PDB) (https://www.rcsb.org/pdb/home/home.do). Based on these sequence similarities the software builds structural similarities with those entities.

3.2.2 Plasmid, strains and DNA manipulations

The plasmid used in this study was pMV261, kanamycin resistant. This is an *E.coli Mycobacterium* shuttle plasmid vector with *hsp60* promoter and kanamycin resistant cassette (Stover et al, 1991). All the strains were grown either in Tryptic Soy broth or Luria-Bertain broth at 37^{o}C with the addition of appropriate antibiotics. Kanamycin was added at a concentration of 50 µg/ml.

Table 3.1: List of plasmids used in this chapter.

Plasmids	Source
pMV261	pMV261 is an *E.coli-Mycobacterium* shuttle plasmid vector with hsp60 promoter and kanamycin resistant cassette (Stover et al,1991).
pMV261: Rv2509 D	Used for this work in the generation of deletion mutants
pMV261: RV2509 D1	Used for this work in the generation of deletion mutants
pMV261: Rv2509 D2	Used for this work in the generation of deletion mutants
pMV261: Rv2509 D3	Used for this work in the generation of deletion mutants
pMV261: Tyr 157	Used for this work in the generation of site directed mutants
pMV261: Lys 161	Used for this work in the generation of site directed mutants
pMV261:Gly 94	Used for this work in the generation of site directed mutants
pMV261:Pro 187	Used for this work in the generation of site directed mutants
pMV261 Phe 98	Used for this work in the generation of site directed mutants
pMV261: Ala 197	Used for this work in the generation of site directed mutants
pMV261 Ser:144	Used for this work in the generation of site directed mutants
pMV261 :Asp 116	Used for this work in the generation of site directed mutants

3.2.3 Polymerase chain reaction (PCR) of predicted transmembrane domain

The primers listed in tables (3.2 and 3.3) were designed for MSMEG4722 in *M. smegmatis* and Rv2509 in *M. tuberculosis* H37Rv.

Table 3.2: Primers for MSMEG4722 deletions

Name	Sequence (5'-3')
MSMEG4722 D F	**GCAGGATCCA**ATGAGCCGCATGCCAGTACCG
MSMEG4722 D P	**GCAGAATTCC**TATTGGCTGGCCACCGACATCG
MSMEG4722 D1 F	**GCAGGATCCA**ATGAGCCGCATGCCAGTACCG
MSMEG4722 D1 P	**GCAGAATTCC**TACACGATGGCGCGCGGAGGGTA
MSMEG4722 D2 F	**GCAGGATCCA**ATGAGCCGCATGCCAGTACCG
MSMEG4722 D2 P	**GCAGAATTCC**TAAAAGGCACCCACGATTTGGCGC
MSEMG4722 D3 F	**GCAGGATCCA**ATGAGCCGCATGCCAGTACCG
MSEMG4722 D3 R	**GCAGAATTCC**TAGCTGCCCCCAAGCCTCTTTGT

Table 3.3: Primers for Rv2509 deletions

Name	Sequence (5'-3')
Rv2509 DF	GCAGGATCCAATGCCGATACCCGCGCCC
Rv2509 DR	GCAGAATTCCTATTGGCTGGCCACCGACATCG
Rv2509 D1F	GCAGGATCCAATGCCGATACCCGCGCCC
Rv2509 D1R	GCAGAATTCCTACACGATGGCGCGCGGAGGGTA
Rv2509 D2F	GCAGGATCCAATGCCGATACCCGCGCCC
Rv2509 D2R	GCAGAATTCCTAAAAGGCACCCACGATTTGGCGC
Rv2509 D3F	GCAGGATCCAATGCCGATACCCGCGCCC
Rv2509 D3R	GCAGAATTCCTAGCTGCCCCCAAGCCTCTTTGT

PCR amplification for the MSMEG4722 and Rv2509 with deletions was performed using Phusion polymerase mix (NEB). The PCR products were purified and cloned in pMV261-kan and sequenced. Restriction sites used for cloning were *Bam*H1 and *Eco*R1. To check for functionality of the deletion constructs, the sequenced plasmids were electroporated into ΔMSMEG4722 and plated with appropriate antibiotics (kanamycin 50 µg/ml and hygromycin 70 µg/ml).

3.2.4 Site-Directed Mutagenesis

Using the predicted three dimensional model of Rv2509 certain deletion mutations were designed to analyse the change in functionality. Primers specific to these mutations were designed using the NEB base changer website (http://nebasechanger.neb.com/) and site directed mutagenesis was carried out using the Q5 site directed mutagenesis kit provided by NEB. This kit enables rapid, site-specific mutagenesis of double stranded plasmid ΔDNA. Site directed mutagenesis is an *in vitro* technique that uses custom designed oligonucleotide primers to confer a desired mutation in a double stranded DNA plasmid. This method takes advantage of a strain deficient in dUTPase and uracil deglycosylase so that the recipient *E.coli* degrades the uracil-containing wild-type DNA. According to the manufacturer's protocol once the PCR is done using the designed primers, the amplified material was directly added to Kinase-Ligase-Dpn1 (KLD) enzyme mix, placed at room temperature for circularisation and template removal. It was then transformed with high efficiency NEB 5-aplha competent *E. coli* provided with the kit and plated on specific antibiotic plates.

Table 3.4: Kit components (Site directed mutagenesis, NEB)

Component	Concentration	Storage (ºC)
Q5 hot start high fidelity 2X master mix*	2X	-20
KLD enzyme mix	10X	-20
KLD reaction buffer	2X	-20
Control SDM Primer Mix	10µM	-20
Control SDM Plasmid	5µg/ml	-20
NEB 5-alpha competent *E.coli* (High efficiency)		-80
pUC19 vector	0.05ng/ml	-20

Q5 hot start high fidelity 2x master mix*: it features a high-fidelity, thermostable, hot start DNA polymerase with 3'-5' exonuclease activity, fused to a processivity-enhancing Sso7d domain to support robust DNA amplification. The mix contains dNTPs, Mg++ and a proprietary broad-use buffer requiring only the addition of primers and DNA template for robust amplification regardless of GC content.

Table 3.5: List of primers used to generate side-directed mutants

Name	Sequence 5'-3'
Lys 161 F	TGCCGCGACCTTTGCCTTCGTGAA
Lys 161 R	TAGGTGGCGTTGTAGGGA
Ser 144 F	GATTTCTGGTTTTGCGGCCGGCAATTC
Ser 144 R	AAGATGCCGCCGGCCTTG
Asparagine 116 F	GGTGCAGTTGTTTGCCGTGGCGG
Asparagine 116 R	TGCGTCTTTTCGCCGGCA
Tyrosine 157 F	CAACGCCACCTTTGCCGCGACCA
Tyrosine 157 R	TAGGGAATCGGTGAATTGCCGG
Glysine 94 F	CGCCAACGCGTTTACCGCGACATTC
Glycine 94 R	CACAGGATCGAGATGGGC
Proline 187 F	GCTGGCCCCGTTCCCGGTTCGCAC
Proline 187 R	ACCGTGACGTGCACGCCG
Phenylalanine 98 F	TACCGCGACATGCGGCCCGATCG
Phenylalanine 98 R	CCCGCGTTGGCGCACAGG
Alanine 197 F	GCTACCGGATTCCTCCGAAGCGTCAC
Alanine 197 R	TCGGTGCGAACCGGGCCC

3.2.5 Extraction and analysis of Fatty acyl methyl esters (FAMEs) and Methyl acyl mycolic esters (MAMEs)

The plasmids from deletion constructs (section 3.2.3) and the site-directed mutants (section 3.2.4) were all grown at in 10 ml of Tryptic Soy broth (TSB) with the appropriate antibiotic at 37^{o}C in a shaking incubator up to mid-logarithmic phase and then 10 µl of ^{14}C acetate (57 mCi/mmol, GE healthcare, Amersham Biosciences) was added to the culture and incubated for 12 hours. The bacterial cells were harvested and washed with PBS and FAMEs and MAMEs were extracted and analysed by 1D TLC. Extraction protocol for FAMEs and MAMEs and TLC analysis can be found in chapter 7 general methods and materials, section 7.20. Autoradiograms were produced by overnight exposure of Kodak AR films to the plates.

3.2.6 RNA extraction and Reverse Transcription PCR (RT PCR)

50 ml cultures of wild type *Mycobacterium smegmatis* mc^2 155, ΔMSMEG4722, MSMEG4722-C, MSMEG4722-CRv, the deletion constructs of MSMEG4722 and Rv2509 (D,D1,D2 and D3) and site-directed mutants were grown to an OD_{600} of 0.8 to extract RNA from it using the Trizol method. Once grown, cultures were transferred to 15 ml polypropylene tubes (Dnase/Rnase free tubes) and centrifuged for 10 minutes at 4000 rpm, 4^{o}C. To the pellets 1 ml of freshly prepared 3:1 (v/v) chloroform:methanol was added and mixed immediately. Vortex for a minute and then to each tube 5 ml of trizol was added and tubes incubated at room temperature for 10 minutes. At this stage layers should separate. The tubes were then centrifuged for 15 minutes at 4000 rpm, 4^{o}C. The upper aqueous phase was then transferred to another set of Dnase/Rnase free tubes to which equal amounts of

isopropanol was added and kept overnight to precipitate at -20^{o}C. The following day the tubes were centrifuged for 30 minutes at 4000rpm, 4^{o}C and the pellets got were washed twice with cold freshly prepared 70% ethanol. The resulting RNA was resuspended in 100 µl DEPC treated water and stored at -20^{o}C until further use. The isolated RNA was treated with DNase to get rid of the residual genomic DNA. The purified DNA were used as templates for cDNA synthesis using the NEB kit. The protocol followed was as per the manufacturer's instructions and simultaneous control reactions were set up.

The primers used for Revere Transcription PCR are listed in the table below.

Table 3.6: Primers for Reverse Transcription PCR

Name	Sequence (5'-3')
Rv2509 RNA F	CCTACAACGCCACCTATGCC
Rv2509 RNA R	GACACCTAGAGCTGCCTCGT

For the PCR One Taq hot start 2X master mix kit from NEB was used and the cycling conditions for PCR were programmed for 25, 30 and 50 cycles respectively. The transcripts were analyzed on a 1.8% agarose gel.

Table 3.7: Kit components for Reverse Transcription PCR

Component	Concentration	Storage (ºC)
Oligo $d(T)_{23}$ VN	50uM	-20
Nuclease free water		-20
M-MuLV enzyme mix	10X	-20
M-MuLV reaction mix	2X	-20
Random primer mix	60µM	-20
One taq hot start master mix with standard buffer	2X	-20

3.2.7 Cellular localisation of Rv2509

For cellular localisation of Rv2509, constructs were made using pMV261 expression vector with the addition of his tag so it could be easily identified using his anitbodies.The pMV261 Rv2509 clones were electroporated into wild type *M. smegmatis* mc^2155 and the mutant ΔMSEMG4722 and colonies got were grown in 1 litre tryptic soy broth till it reached an OD of 0.8. The cells were pelleted and then resuspended in 20 ml of lysis buffer (50 mM Tris HCl, pH 7.5, 150 mM NaCl, 1% Igepal, 1 mM DDT, 1% sodium deoxycholate). The resuspended pellet was then French pressed and spun at 18,000 rpm for 40 minutes at 4^0C. The supernatant got was then spun for 1 hour at 40,000rpm, 4^oC. The pellets and supernatants got from both the fractions were run on SDS page gels and analysed for protein localisation using western blot. ΔMSMEG4722 was used alongside as a control.

3.3 Results

3.3.1 Bioinformatics studies for Rv2509

Mycobacterium tuberculosis Rv2509 is a 28 kDa putative short chain dehydrogenase/reductase. The closest match of Rv2509 protein in *M. smegmatis* mc^{2}155 genome is MSMEG4722 with 76% identity and in *C. glutamicum* is NCgl2385 with 56% sequence similarity. Rv2509 is also present in the highly decayed genome of *M. leprae*, ML0429 with 89% identity with Rv2509. Using NCBI BLAST amino acid similarities for the mycolic acid reductase with other related mycobacteria and related species was generated (Figure 3.2) and a phylogenetic tree generated to see similarities (Figure 3.3).

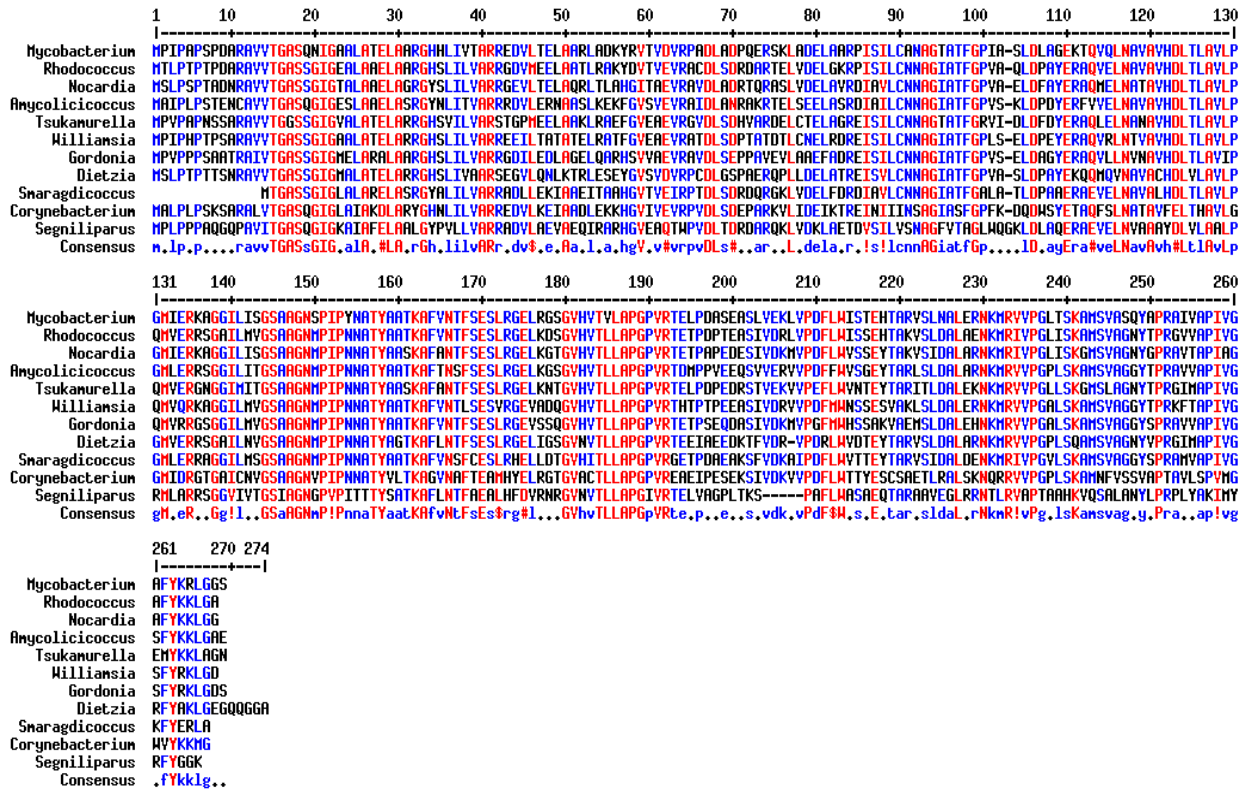

Figure 3.2: Multiple sequence alignment of the mycolic acid reductase Rv2509 with related mycobacteria and other species. The software used is ClustalW2

(https://www.ebi.ac.uk/Tools/msa/clustalw2/) and the colours are to indicate the residues according to their physiochemical properties like red is for small (small+hydrophobic (including aromatic-Y)), blue stands for acidic, magneta for basic-H, green for hydroxyl+sulfhydryl+amine+G and grey for unusual amino/imino acids.

Phylogenetic analysis can either be used to find evolutionary ties between organisms or understand relationships between an ancestral sequence and its descendants. The horizontal lines on a phylogenetic tree gives the amount of genetic change, representing evolutionary lineages changing over time. The longer the line the more changes have occurred over a period of time. The phylogenetic tree in figure 3.3 shows that the first divergence separated the lineage that gave rise to *Segniliparus rugous* which then led to further lineages. This latter lineage split into two, first one relating to *Corynebacterium glutamicum*. The second lineage further divided into branches probably depicting that they share a much recent ancestry when compared to main initial branching.

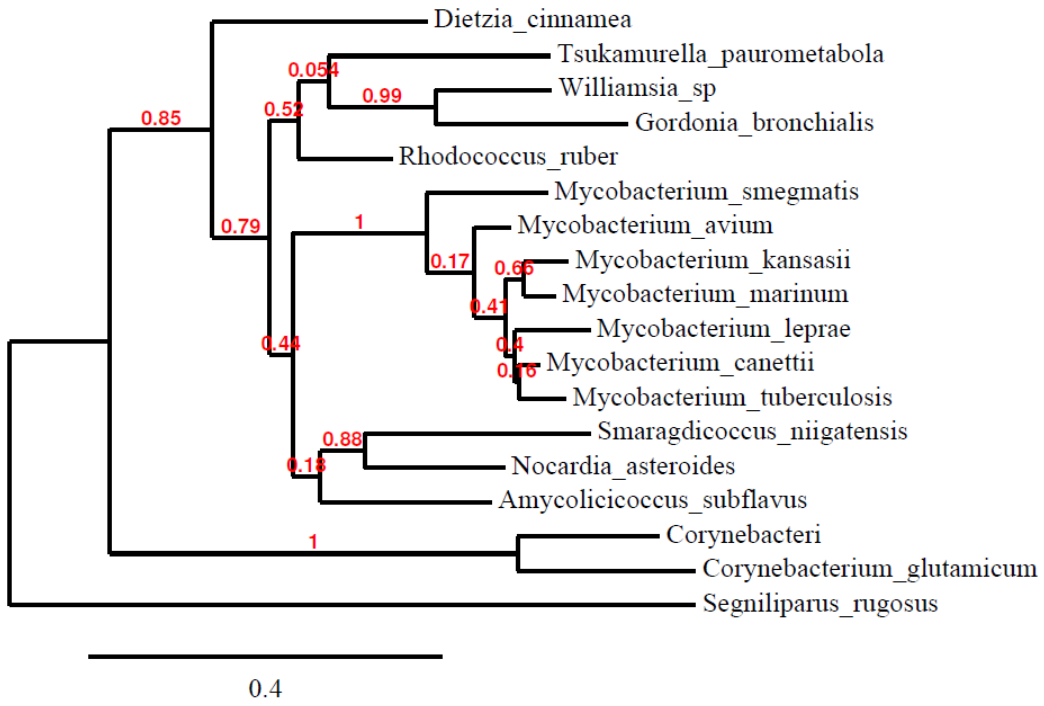

Figure 3.3: Phylogenetic tree of *M. tuberculosis* Rv2509 with related mycobacteria and other species. The scale of 0.4 is the length of branch which represents the amount of genetic change. The phylogenetic tree was generated using phyloT software. (https://phylot.biobyte.de/)

The predicted protein structure of Rv2509 showed conserved active site residues and residues for NAD/NADP binding which was similar in *C. glutamicum* NCgl 2385 and *M. smegmatis* MSMEG4722. The 3D structural prediction of Rv2509 was done using I-TASSER online server and @TOME server (http://atome.cbs.cnrs.fr/AT23/index.html) which suggested a strong match to *Vibrio vulnificus* oxidoreductase (3GUY.pdb) (36% sequence identity). All of the 3D structural work was done with the help of Robert Marshall, PhD student of Dr.Bavro and used in my thesis. The predicted structure showed a typical Rossmann fold consisting of central β sheet with flanking α helices. Proteins belonging to short chain dehydrogenases/ reductases (SDR) families have a characteristic polypeptide backbone

topology in which each subunit has s single domain with a central core containing a Rossmann fold supporting an NADH binding site (Rozwarshi et al, 1999). Using the 3GUY coordinates a 3D structure of Rv2509 was generated in silico. The prediction suggested the presence of an N-terminal TG(X)5G motif. This motif represents a potential binding site for cofactors NAD(H)/NADP(H). Presence of active site residues 157-161 was suggested and shown in figure 3.4. Further analysis of the predicted 3D structure showed the presence of an additional long C-terminal helix which appeared to be hinged from the rest of the protein. This C-terminus (38 amino acids long) consisted of hydrophobic residues which could potentially be a transmembrane domain. Structural analysis also showed the presence of tunnel and cleft like structure present in Rv2509 and also gave an indication of the potential active site residues. These active site residues could potentially be present within the tunnel and cleft like structures. Also these tunnel and cleft like structure could form the pathway for the passage of the substrate. Targeted mutations with the help of site directed mutagenesis was also suggested to see if it affected functionality of the protein. Figure 3.4 shows the predicted structure of RV2509 using the 3GUY template. The green portion represents the superimposed regions of the *Vibrio* reductase with the predicted Rv2509 structure. The purple region indicates the unique C-terminal helix present in the predicted Rv2509 structure containing the hydrophobic residues.

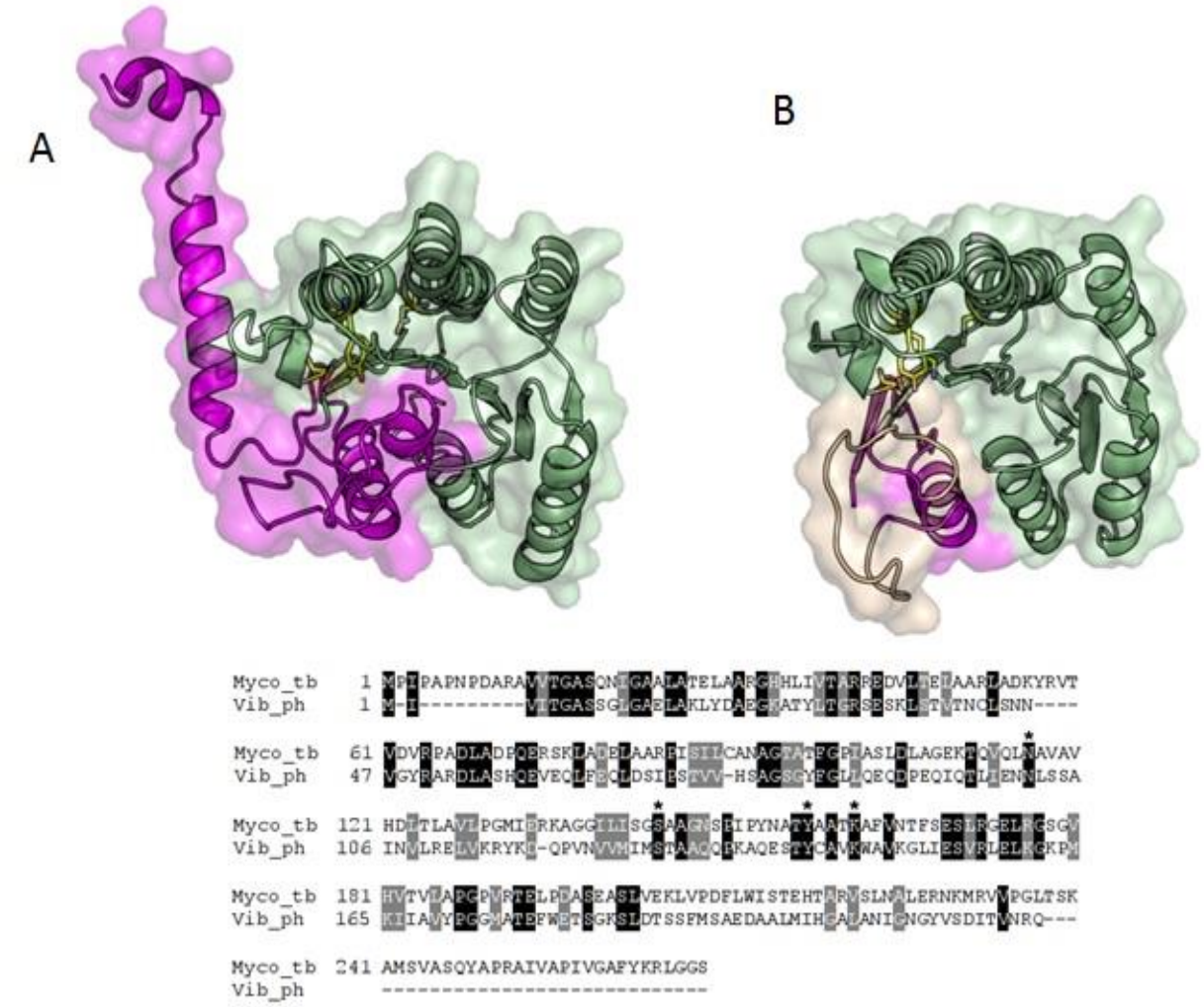

Figure 3.4: 3D structural prediction of Rv2509 using 3GUY template. A. Predicted 3D structure of Rv2509 with an extra C-terminal portion. B. Crystal structure of *Vibrio* reductase. The figure also shows sequence aliment of M.tuberculosis Rv2509 with the *Vibrio* reductase and the conserved active site residues (157-161). Robert Marshall, student of Dr.Bavro helped generate these images.

3.3.2 Deletion analysis of the hydrophobic C-terminal of Rv2509

The 3D structural prediction of Rv2509 showed similarities with a *Vibrio* reductase (*Vibrio vulnificus*, oxidoreductase) and superimposed well with it except for a C-terminal helix

(Figure 3.5). This C-terminal helix is about 38 amino acid (694-807bp) long and showed to have hydrophobic residues. Deletions were made to this helix to see if it at all it had any role in the functionality of the protein. Deletions were made in such a way that in the first deletion as shown in figure 3.5, D represented retention of 25% of the residues. The same applied to the other deletions where in D1 represented 50% retention, D2 represented 75% and D3 represented 100% retention of the residues.

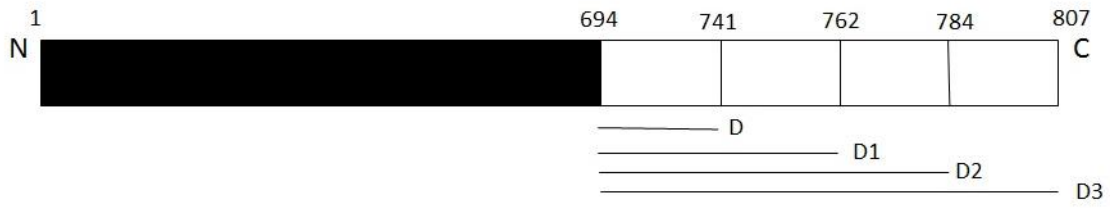

Figure 3.5: Representation of deletions made to the C-terminal helix of MSMEG4722 and Rv2509. The figure represents the deletions made to the C-terminus hydrophobic residues with D3 showing 100%, D2 75%, D1 50% and D 25% of retention of residues.

All these deletion constructs were made in MSMEG4722 and Rv2509 to see if they revealed different phenotypes for the deletions. The deletion constructs were cloned in pMV261 and transformed into the *M. smegmatis* ΔMSMEG4722. When *M. smegamtis* ΔMSMEG4722 undergoes alkali treatment the phenotype obtained is the reduction of mycolic acids and the accumulation of the degradation products of premycolates as they are unstable. When the wild type *M. smegmatis* mc^2155 was transformed into *M. smegmatis* ΔMSMEG4722 it resulted in complementation with the formation of mature mycolic acids. The rational behind the following experiment was to obtain residues important in functionality of Rv2509. If the

residues were important for funtionality upon transformation in ΔMSMEG4722 they would not complement resulting in a phenotype similar to that of the ΔMSEMG4722 and if the residues were not important for funtionality they would result in phenotype with the production of the mature mycloic acids as seen with the wild type *M. smegmatis* mc^2155. All the strains were grown to mid log phase and then labelled with ^{14}C acetate and further incubated for 12 hours. The cells were harvested and fatty acid methyl esters (FAMEs) and methyl acid mycolic esters (MAMEs) were extracted and analysed.

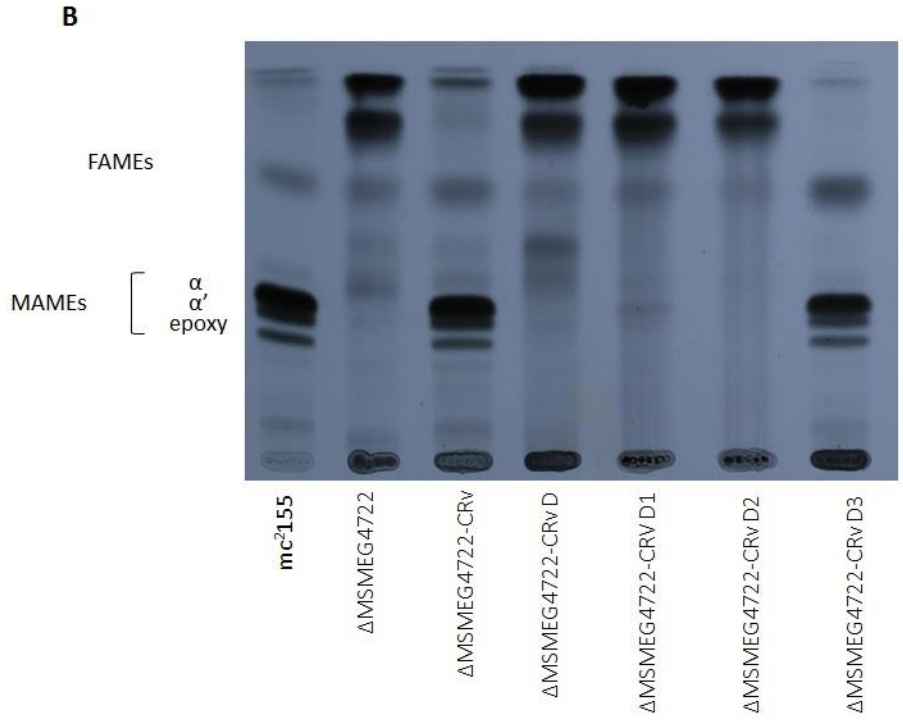

Figure 3.6:1D TLC analysis of FAMEs and MAMEs of the deletions made to the C-terminal helix. This figure is a representation of deletions constructs made in RV2509. A similar phenotype was got with the constructs made in MSMEG4722.

The above figure (3.6) is a 1D TLC image of FAMEs and MAMEs extracted from the ^{14}C labelled deletions made to the C-terminal helix. Deletions D, D1 and D2 showed a similar phenotype to the mutant strain ΔMSMEG4722 which shows the absence of mycolates and the accumulation of the degradation products of premycolates. This suggested that deletions made to part of the helix was important in the funtionality of the protein. Deletion D3 restored mycolate biosynthesis in the mutant strain ΔMSMEG4722 suggesting that the terminal residues (784-807bp) did not play a role in the funationality of Rv2509.

3.3.3 Effect of mutagenesis of key residues on Rv2509 funtion

Short chain dehydrogenases/reductases family generally have a triad or tetrad of active site residues which are tyrosine, lysine, serine and aspargine. Similarities were found in the modelling of Rv2509 with the available crystal structure of *Vibrio* reductase (*Vibrio vulnificus*, oxidoreductase). The tetrad of active site residues were also seen in the predicted 3D structure of Rv2509. Apart from this a tunnel and cleft like structure were also identified. It was suggested that the active sites are either in the tunnel or cleft like structure.

In total nine mutations were genetrated with the help of Q5 site directed mutagenesis which are shown in figure 3.7. Once the mutants obtained, the resultant pMV261 plasmid clones were introduced into the *M. smegmatis* mutant strain ΔMSMEG4722 to check for funtionality. All the strains were grown to mid-log phase and then labelled with ^{14}C acetate and grown for a further 12 hours. The cells were harvested and FAMEs and MAMEs extracted and analysed by thin layer chromatography (TLC). Using site-directed mutagenesis active site residues Tyr157, Asn116, Lys161 and Ser144 were changed to phenylalanine.

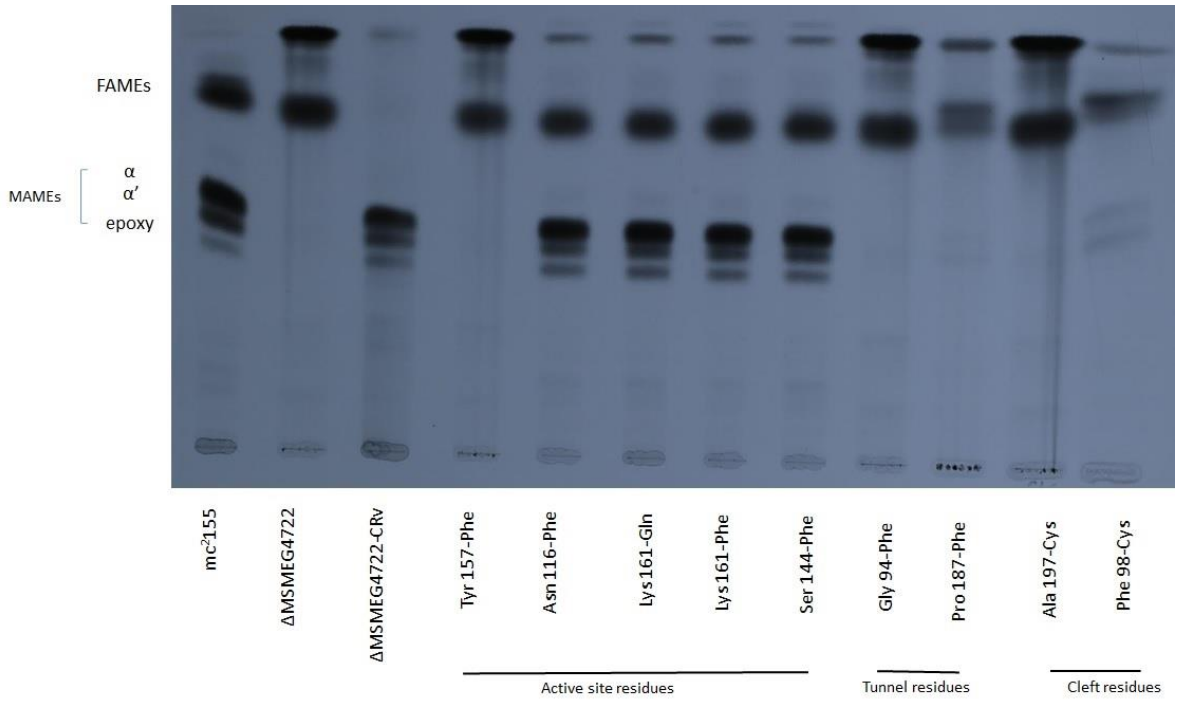

Figure 3.7: 1D TLC analysis of FAMEs and MAMEs of site directed mutants.

Fatty acyl methyl esters (FAMEs) and methyl acyl mycolic esters (MAMEs) analysis of these active sites suggested that the change made to Try157 was the only potentially identified active site residue that was important for funtionality of the protein and the mutated plasmid did not complement the mutant strain ΔMSMEG4722. It was the only one that showed the accumulation of premycolates similar to that seen in the mutant strain ΔMSMEG4722 whereas the rest had a phenotype similar to the wild type *M. smegmatis* mc^2155. The tunnel like structure harboured residues Gly94 and Pro187 which were also changed to phenylalanine. It was suggested that the active sites are either in the tunnel or cleft like structure and for this access was targeted via mutation of residues to phenylalanine which should ideally occlude the ends of the tunnel like structure. FAMEs and MAMEs analysis suggested that changing Gly94 to phenylalanine disrupted function as the transformed strain

depicted the same phenotype as the mutant strain MSMEG4722, whereas the change of Pro187 to phenylalanine showed the partial reduction of mycolic acids suggesting that it may have a potential role in the functionality of the protein. The residues Ala 97 and Phe98 were found in the cleft like structure and were changed to cysteine. The disulfide bond formed between two cysteines is important for the stabilization of the tetriary and quaternary structures of the protein. FAMEs and MAMEs analysis of Ala97 and Phe98 to cysteines also showed the same phenotype as the mutant strain ΔMSEMG4722 i.e accumulation of degradation products of premycolates and reduction of mycolic acids, suggesting that they play a role in the funtionality of the protein.

3.3.4 Cellular localisation of Rv2509

It is important to understand the role of Rv2509 not only at the molecular level but also at the cellular level. For this pMV261 Rv2509 his tagged was grown in a litre of tryptic soy broth, lysed and fractionated and analysed by western blot. The mutant strain ΔMSMEG4722 was used as a control and was fractionated in the same way as pMV261 Rv2509. To fractionate the cells and isolate the compartments, the whole cell was lysed and centrigued at 27,000 g. The supernantant labelled as pMV261 Rv2509 sup 1 in figure 3.8 corresponds to the supernantant got from this spin. This mainly forms the cytosolic fraction. The pellet got from this initial first spin which still contains some unbroken cells was lysed once again and centrifuged at 100,000 g. The pellet and supernatant obtained from this spin, labelled as pMV261 RV2509 sup2 and pellet 2 correspond to cytosolic portion in the supernatant and the cell membranes in the pellet. Expression of Rv2509 was found in both the cytosolic

fraction as well as the membrane fraction. This result indicates that Rv2509 is a soluble protein and can be co-localised with some membrane proteins as part of it function.

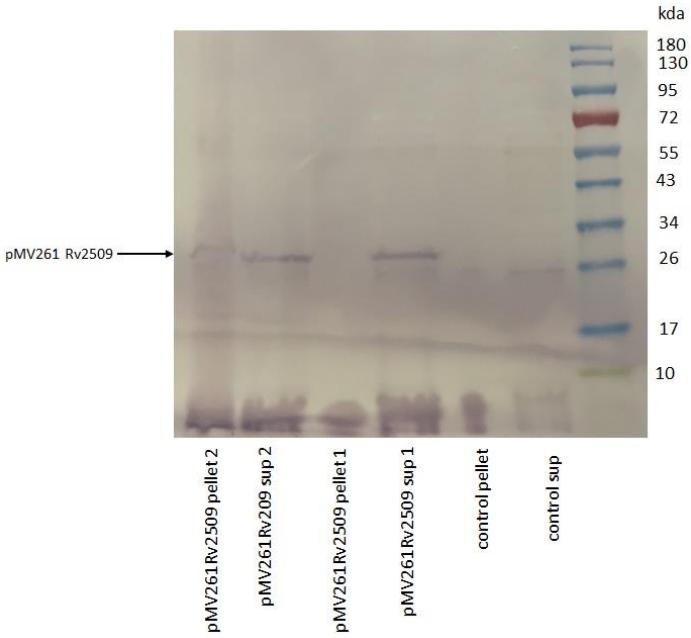

Figure 3.8: Cellular localisation of Rv2509. pMV261 Rv2509 sup 2 corresponds to the cytosolic fraction and pMV261 Rv2509 pellet 2 corresponds to the cell membranes.

3.3.5 Comparison of expression levels of transcripts produced by wild type *M. smegmatis* mc^{2}155, MSMEG4722, deletions constructs and site directed mutants

The deletion constructs and site-directed mutants explained in section 3.3.2 and 3.3.3 were transformed in the mutant ΔMSMEG4722 and checked for funtionality via complementation. Complementation is visualised with thin layer chromatography (TLC) and it tells us if the residues or deletions are important in the funtionality of the protein but gives no information if the plasmid pMV261 is introduced into the bacterial cell as it is dependent on kanamycin. The expression levels was thus checked with the help of RT PCR and this was the rational

behind setting up this experiment. The entire gene length of MSMEG4722 and Rv2509 is 807bp and deletions were made from 694bp to 807bp. These segment (694-807bp) corelated with the C-terminal helix consisting hydrophobic residues and each deletion construct comprised of aroung 50 bp deleted. Using site-directed mutagenesis certain mutations were made to the active site residues and a tunnel and cleft like structure residues from the predicted 3D structure. All the above mentioned mutants and deleted constructs were made in order to analyse their effects on the funtionality of the protein. Using RT PCR, the gene expression levels of wild type *M. smegamtis* mc^2155, ΔMSMEG4722, the deletion constructs and the site-directed mutants were determined. Since no specific antibodies have been synthesised for RV2509, western blot was not an option for checking expression levels of the above. Therefore, RT PCR was used for the evaluation of gene expression with normalisation to the house keeping gene *sigA*. This sigma factor *sigA* is present in all mycobacterial species and is the initiation factor that promotes attachment of RNA polymerase to specific intiation sites. The results obtained from RT PCR show levels of transcription with the wild type *M. smegmatis* mc^2155, deletion constructs and site-directed mutants.

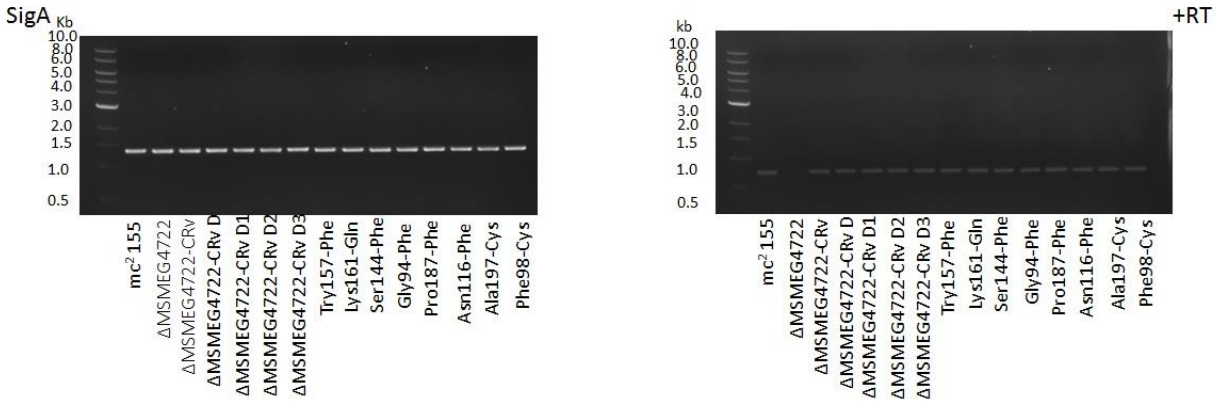

Figure 3.9: Comparison of expression levels of transcripts from *M. smegmatis* mc^{2}155, MSMEG4722, deletion constructs and site directed mutants.

3.4 Discussion

Studying the 3D structure of proteins is not only important to understand its biological activity but can also be useful for identifying new targets in the field of drug discovery. In the previous chapter it was shown that Rv2509 is an essential gene in *M. tuberculosis* and in this chapter we illustrated a 3D predicted model of the mycolic acid reductase, Rv2509. Structural modelling gave us an insight that Rv2509 is a putative short chain dehydrogenase/reductase, contained an extra C-terminal helix composed of hydrophobic residues, which could be a potential transmembrane domain. The modelling also illustrated the potential acitve site residues, a tunnel and cleft like structure which could be the pathway through which the subtrate passes. Experiments on the C-terminal helix were conducted by various deletions made to it to see if it played any role in the functionality of the protein. In a similar way site-directed mutants were made to see if they too played any role in the

functionality of the protein. Mutations can lead to the loss of function of the protein with or without drastic changes to the three dimensional structure of the protein. We were able to successfully construct nine site-directed mutants out of which not all were important in the funtionality of the protein. Due to time restriction we were not able to perfom circular dichroism on these mutants. Circular dichroism would have checked for gross effects on the secondary structure of the protein caused by the mutations. For any outcome the following would be the explanation. If these mutated residues showed loss or change due to the mutation but did not affect the secondary structure of the protein they would have played a direct role in the catalysis or binding activities of the protein. And if the mutated residues resulted in a change in the protein structure then those residues were involved in the overall structural maintenance of the protein and an indirect role in the function of the protein. It was observed that out of all the active site residues changed to phenylalanine it was just Try157 which was important in the functionality of the protein. It was even observed that the changes made to the residues of the tunnel and cleft like structure were important for functionality. The possible explanation could be that the change led to the loss of functional groups essential for catalysis or binding of the subtrate.

Additionally if time permitted, the site-directed mutants proteins could have been extracted and purified and subjected to size exclusion chromotography. This could be have been further used for biochemical and biophysical characterisation.

It was important to determine the function of Rv2509 not only at the molecular level but cellular level too. Subcellular localisation is important for determining the cellular functions of newly found proteins and hypothetical proteins. It also gives us information of the function

of protein. Fractionation of Rv2509 indicated that it was a soluble protein but was also co-localised with some membrane proteins as part of its function.

The following chapter looks into expression and purification of Rv2509 as it is essential in *M. tuberculosis* and is shown to be involved in the final step of formation of mature mycolic acids.

Chapter 4

Expression and Purification of Rv2509

4.1 Introduction

Gerhardus Johannes Mulder in 1893 was the first to coin the word ‘protein’ to describe the substance he analysed from plants and animals. Protein research is important as it gives information about the functions of the protein, shape, size and charge. Amino acid sequences and evolutionary relationships between proteins amongst a diverse group of organisms can be determined by pure protein (Brondyk WH, 2009). Purification of protein is not only useful for understanding the biochemical function but also crystals grown from pure protein can provide the protein’s tertiary structure through x-ray data, which is the actual functional unit. *Mycobacterium tuberculosis* is a highly pathogenic organism resulting in nearly 1.3 million deaths per year. Determining the 3D structures of proteins of various mycobacterial species can help build a foundation for tuberculosis diagnosis and treatment (Bashiri and Baker, 2014). This requires the use of recombinant technologies using an appropriate host expression system to produce large quantities of protein for functional and structural studies. Apart from *Escherichia coli*, which is the common host for expression systems, different expression hosts have been used for expression of proteins in *M. tuberculosis*.

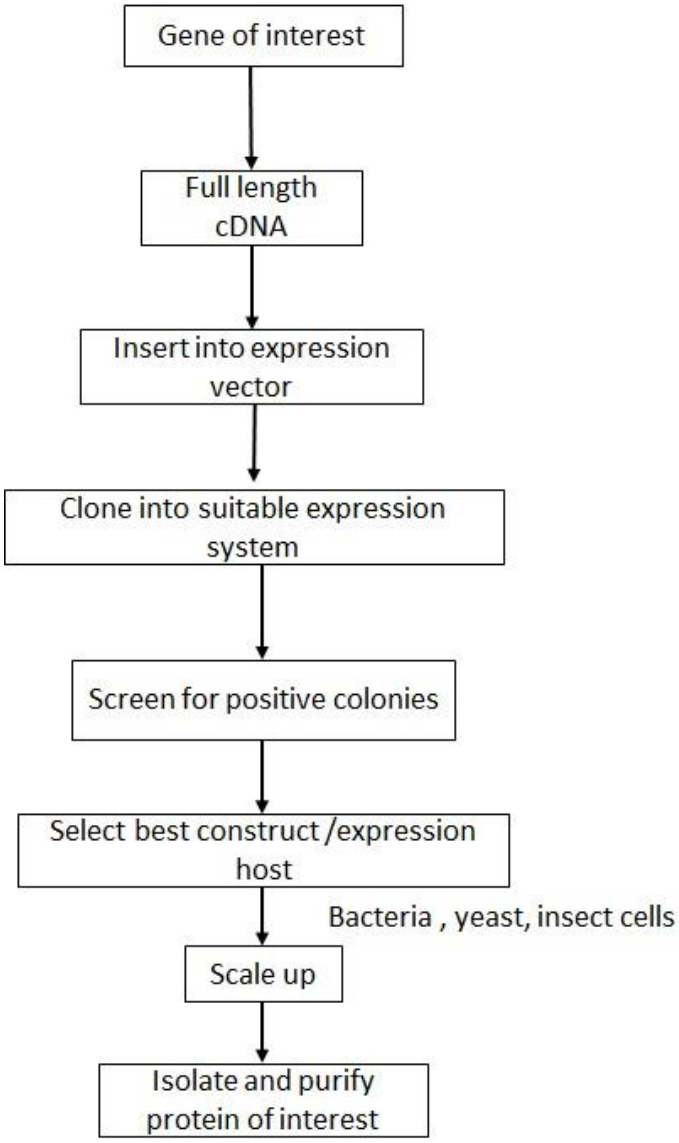

Figure 4.1: Diagram representing the steps involved in optimizing protein expression system to produce desired recombinant protein.

Proteins are purified on the basis of the size, solubility, charge and specific binding affinity. The common techniques involved in the purification of a protein are salting out, dialysis, gel-filtration chromatography, ion-exchange chromatography, high pressure liquid chromatography and affinity chromatography. The commonly used salting out technique

involves the use of high salt concentrations to precipitate proteins and can also be used for concentrating dilute protein solutions in the active form. Dialysis incorporates the separation of proteins through a cellulose membrane with pores. This technique is especially useful to get rid of the smaller molecules that pass through the membrane leaving the bigger molecules corresponding to the protein of interest with the membrane bag. Dialysis can also be used to get rid of the remaining salts from the salting out technique. Another commonly used technique for protein purification is ion-exchange chromotagraphy, in which proteins are separated based on their net charge. In this technique the protein is passed through a column with diethylaminoethyl cellulose (DEAE), and if the protein has a net positive charge the protein binds to the cellulose and eluted with increasing concentrations of sodium chloride or any other salt.

Rv2509 is 28 kDa, short chain dehydrogenase/reductase protein and cellular localisation studies discussed in chapter 3 showed that the protein might be cytoplasmic though it demonstrated association with the membrane. In this chapter, a few expression vectors have been used for the purification of full length Rv2509 and various optimisation conditions employed for extracting full length Rv2509 in the soluble form. Attempts were made to purify the protein of full length Rv2509 and used in an assay to check the mechanism of action. The rationale behind the assay is illustrated in the following section.

4.1.1 Rv2509 activity assay

Mycolic acid synthesis involves two fatty acid synthases, a type 1 fatty acid synthase initially synthesis as long chain fatty acids which are then elongated by the multi enzyme type 11

fatty acid synthase complex. After a series of additional functionalisation the α branch and the meromycolic acid are condensed by the polyketide synthase Pks13. This forms a precursor 2-alkyl-3-keto fatty acid which is then reduced by a mycolic acid reductase (Lea-Smith et al, 2007) (Bhatt et al,2008) to form the mature mycolic acids. The mature mycolic acid that is formed is linked to 6-hydroxyl of disaccharide trehalose which forms trehalose monomycolate (TMM) (mycobacteria) or trehalose monocorynomycolate (TMCM) (corynebacteria). The steps that are involved between the synthesis of the mycolyl chains and the formation of trehalose monomycolate (TMM) were unknown until recently (Gavalda et al. 2014). The α-alkyl branch and the mermycolic acid are condensed by the ketosynthase domain of Pks13 which results in an α-alkyl-β ketoacyl chain which is linked to ACP domain C-terminus. Studies by Gavalde et al, 2014 demonstrated that this α-alkyl-β ketoacyl is directly transferred to a hydrophilic head which is trehalose and this transfer is not dependent on mycolyl transferases which were previously proposed (Besra et al, 1994, Takayama et al,2005). The TE domain of Pks13 catalyses the transfer of the α-alkyl-β ketoacyl chain onto trehalose. Following reduction by the mycolic acid reductase, the resultant trehalose monomycolate (TMM) is translocated by MmpL3 protein (Varela et al, 2012) to the Antigen 85 complex. Trehalose monomycolate (TMM) is then used as a substrate by the enzymes of Antigen 85 complex for the biosynthesis of mycolate containing lipids located in the mycomembrane and the arabinogalactan of the cell wall.

Based on these findings a functional assay was developed to test the mechanism of action of Rv2509. For the assay the dried lipids especially non-polar lipids of the mutant ΔMSMEG4722 were mixed with the purified protein of Rv2509 and subjected to alkaline

hydrolysis. The non polar lipids apart from a mixture of other lipids contains the trehalose monomycolates (TMM) and trehalose dimycolates (TDM). The mutant strain ΔMSMEG4722 has shown to accumulate trehalose premonomycolates and trehalose predimycolates. The interaction of the non polar lipids and the pure form of Rv2509 should result in the reduction of the β-oxo intermediate to β-hydroxyl group if the trehalose premycolates were the substrate of Rv2509. This would no longer be sensitive to alkaline hydrolysis and hence would result in the release of α, α' and epoxy mycolates.

4.1.2 Aims and objectives

The main aim of this chapter was to optimize conditions for the expression and purification of full length Rv2509 which could then be used in the functional assay to check for mechanism of action.

4.2 Materials and methods

4.2.1 Plasmid, strains and DNA manipulation

The plasmids and the strains used have been listed in the table 4.1 below. *E. coli* strains used in this study were grown in Luria-Bertani (LB) broth routinely in the laboratory at 37ºC shaking. The media was supplemented with antibiotics for *E. coli* strains, kanamycin at 50 µg/ml and ampicillin at 100 µg/ml. For protein expression studies Rv2509 was cloned into all the plasmids mentioned in table 4.1 to check for expression and then used for purification.

Table 4.1: List of plasmids used for expression of protein of Rv2509.

Plasmids	Description
pMV261	*E. coli-Mycobacterium* shuttle plasmid vector with *hsp*60promoter and kanamycin resistant cassette (Stover et al,1991)
pET28a	Bacterial expression plasmid, kanamycin resistant and has a His tag at N terminus and C terminus
pMAL-p2X	Bacterial expression vector with a maltose binding protein tag at the N terminus, ampicillin resistant.
pET26b	Bacterial vector for expressing proteins in the periplasm, has a His tag in the C termini and kanamycin resistant
pET28a	*E. coli* expression vector with a histidine tag
pET41c	*E. coli* expression vector with a histidine tag
pGEX-4T-1	*E. coli* expression vector with glutathione tag

4.2.2 Generation of expression constructs of Rv2509

The full length of Rv2509 was PCR amplified using *M. tuberculosis* H37Rv genomic DNA. The PCR products were extracted from the gel and purified using the Qiagen PCR purification kit, restriction digested with restriction enzymes and then ligated with the vectors

mentioned in table 4.2. The ligation mix was then transformed with *E. coli* Top 10 cells and spread on plates with anitbiotics. The transformants obtained were then analysed by sequencing and restriction digestion. The entire procedure from PCR amplification to ligation has been explained in detail in Chapter 7 section 7.11.

Table 4.2: List of primers used for generating expression constructs of Rv2509

Name	Sequence 5'-3'
pET26b:Rv2509 F	**TTTACAT**ATGATGCCGATACCCGCGCCC
pET26b: Rv2509 R	**ATTACTC**GAGCGACGGGGGTTCGGAGAA
pMAL-p2X: Rv2509 F	**TTTACAT**ATGATGCCGATACCCGCGCCC
pMAL-p2X: Rv2509 R	**ATTACTC**GAGCGACGGGGGTTCGGAGAA
pETSUMO: Rv2509 F	**TAATGGA**TCCATGCCGATACCCGCCCCC
pETSUMO: Rv2509 R	**ATTAAAG**CTTGCTGCCCCCAAGCCTCCTG
pGEX4T-1: Rv2509 F	**TAATGGA**TCCATGCCGATACCCGCCCCC
pGEX4T-1: Rv2509 R	**ATTAAAG**CTTGCTGCCCCCAAGCCTCCTG
pET41C: Rv2509 F	**TATACAT**ATGCCGATACCCGCGCCC
pET41C: Rv2509 R	**TTTAAAG**CTTGCTGCCCCCAAGCCTCTTG
pET28a: RV2509 F	**TTTAGAA**TTCATGCCGATACCCGCGCCC
Pet28a: Rv2509 R	**ATTAAAG**CTTGCTGCCCCCAAGCCTCTTG

4.2.3 Expression of Rv2509 protein

For expression studies the expression constructs obtained using the list of primers from table 4.2 were transformed into BL21 (DE3) *E. coli* expression host cells. The recombinant strains obtained were grown in 5 ml of LB broth with the addition of antibiotics at 37^{o}C shaking. This initial culture was then scaled up to 1 litre cultures and grown to an O.D of 0.6 followed by IPTG induction. Optimisation of expression of full length Rv2509 protein was done under varying temperature conditions and varying IPTG concentrations. Temperatures used were 16^{o}C, 20^{o}C and 37^{o}C and IPTG concentrations ranged from 0.1 mM, 0.25 mM, 0.5 mM and 1 mM. The cell lysates and pellets were run on a SDS page gel to check for expression. Once conditions were optimised the recombinant strains were grown up to 6 litres, induced with IPTG and pellets stored for further purification. Protein expression of the Rv2509 constructs were monitored by western blot.

4.2.4 Western Blot Analyses

Western blot helps identification of the protein with specific antibodies. The procedure involves transfer of the protein from the gel to the membrane with the application of electric charge. This membrane is now the replica of the gel's protein pattern and stained with the appropriate antibodies. The SDS PAGE gel and the membrane are sandwiched between sponge and clamped ensuring no air bubbles are there. This is then placed in a transfer buffer to which electrical field is applied. The transfer buffer used was 1X Tris-Glycine buffer with the addition of methanol to a final concentration of 20%. The transfer was done for 4 hours. After 4 hours the membrane was blocked for an hour with 3% non-fat milk which prevents

non-specific binding of the primary and secondary antibodies to the membrane and the primary antibody added (Monoclonal anti- polyHistidine antibody produced in mouse, Sigma). The primary antibody was used at a dilution of 1:500 which was made in TBST (TBS+ tween 20). This was placed at 4^{o}C overnight. Following day, membrane was washed 3 times with TBST and then secondary antibody (Anti IgG coupled to alkaline phosphatase, Sigma) at dilution of 1:1000 was added and incubated at room temperature for 1 hour. Again the membrane was washed three times with TBST and developed using BCIP/NBT detection kit.

4.2.5 Protein purification of Rv2509

The purification buffer used was 50 mM sodium phosphate (pH 7.5), 500 mM sodium chloride and 25 mM imidazole. The cell pellets were resuspended in 50 ml of the purification buffer which was also supplemented with DNase 1 and protease inhibitor tablet (Roche). The cells were sonicated using a probe sonicator (MSE Soniprep 150, 12 micron amplitude, 30s ON and 30s OFF repeated for 15 cycles on ice). The sonicated cells were then centrifuged at 18,000 rpm for 45 mins at 4^{o}C to separate the clarified supernatant from pellet. The clarified supernatant obtained was then passed through a Ni^{2+}NTA HiTrap column (GE healthcare, Amersham, UK) which was equilibrated with the purification buffer containing 25 mM of imidazole. 5 ml fractions of the equilibrated buffer containing 50 mM, 100 mM, 150 mM, 200 mM, 300 mM and 1 M imidazole were eluted. The fractions were analyzed using SDS-PAGE and the fractions with protein of interest were pooled and further purified using an ion-exchange chromatography.

4.2.6 Anion-exchange protocol

For ion-exchange chromatography two buffers were used, Buffer A which comprised of 50 mM Tris.Cl (pH 7.5) and 10% glycerol and Buffer B comprised 50 mM Tris.Cl (pH7.5), 10% glycerol and 1 M NaCl. The column was equilibrated with Buffer A and then the protein fractions collected were passed through this column which passed through a resin and were collected as 10 ml fractions. Then these 10 ml fractions were passed through a Q HP column (GE Healthcare, Amersham, UK) which was equilibrated by passing 10 ml of Buffer B and then 10 ml of Buffer A. The protein eluted was estimated using Bradford assay and the washes were collected until there was no protein detected in the flow through. Anion-exchange was performed from the fractions eluted from Q HP column over an increasing sodium chloride gradient which was analysed by SDS-PAGE and the purified protein was stored at -20^{o}C for further analysis.

4.2.7 SDS-PAGE

For analysis of proteins, 8-12% precast SDS-PAGE gels (Biorad) were used. The samples to be analysed were added to SDS gel sample buffer and boiled for 10 minutes. Prior to loading the boiled samples were centrifuged at low speed for 5 minutes. The buffer used for running the samples was Tris-glycine-SDS buffer at a current of 35-45 mA and potential difference 200 V. For analysis the gels were stained with either instant blue or coomassie brilliant blue stain.

4.2.8 Rv2509 activity assay

The ^{14}C labelled non polar lipids of *M. smegmatis* mc^2155 and the mutant ΔMSMEG4722 were extracted following the protocol illustrated in chapter 7 section 7.19. Equal counts of the non polar lipids of mc^2155 (3 tubes) and ΔMSMEG4722 (3 tubes) corresponding to 5 µl were placed in glass tubes and dried. The following amounts stated corresponded to a single reaction in one tube. A buffer containing 250 µl of phosphate buffered saline (PBS) and 0.2 nM of the coenzyme NADH and NADPH was prepared and placed on ice. This buffer was added to the tubes as depicted in the table below:

Table 4.3: List of buffers used for Rv2509 assay activity.

Lipid	Only Buffer	Buffer+NADH	Buffer+NADPH
mc^2155	Non polar lipid and buffer	Non polar lipid and buffer containing NADH	Non polar lipid and buffer containing NADPH
ΔMSMEG4722	Non polar lipid and buffer	Non polar lipid and buffer containing NADH	Non polar lipid and buffer containing NADPH

Once the buffers were added to the respective tubes were sonicated in a water bath and then aliquots of purified Rv2509 (0.1 µg) were added to the tubes and left on ice. This was followed by alkaline hydrolysis of all the strains. Fatty acyl methyl esters (FAMEs) and mycolic acyl methyl esters (MAMEs) were extracted and analyzed by thin layer

chromatography (TLC). The procedure for extraction of FAMEs and MAMES and TLC analysis has been illustrated in detail in chapter 7 section 7.20.

4.2.9 Autoinduction media and protocol

The autoinduction media comprises of the following components and is for 1 litre of culture:

4 g typtone

2 g yeast extract

1.42 g Na_2HPO_4

1.36 g KH_2PO_4

1.07 g NH_4Cl

Make up 100 ml of the following separately

1 g glucose

4 g lactose

40 mM $MgSO_4$

Prepare 60% glycerol separated to a volume of 50 ml

Trace metal mixture (1000x): To make up 100 ml

Table 4.4: Components and concentration of trace metals added to autoinduction media

Trace element	**Volume**	**Concentration (1X)**
0.1 M $FeCl_3$ in 0.12 M HCl	50ml	50 µM Fe
1 M $CaCl_2$	2 ml	20 µM Ca
1 M $MnCl_2$-$4H_2O$	1 ml	10 µM Mn
1 M $ZnSO_4$-$7H_2O$	1 ml	10 µM Zn
0.2 M $CoCl_2$-$6H_2O$	1 ml	2 µM CO
0.1M $CuCl_2$-$2H_2O$	2 ml	2 µM Cu
0.2 M $NiCl_2$-$6H_2O$	1 ml	2 µM Ni
0.1 M Na_2MoO_4-$2H_2O$	2 ml	2 µM Mo
0.1 M Na_2SeO_3-$5H_2O$	2 ml	2 µM Se
0.1 M H_3BO_3	2 ml	2 µM B
Water	36 ml	

Typtone, yeast and the other components were mixed along with 3.2 ml of 60% glycerol, 20 ml of glucose, lactose and magnesium mixture, antibiotic and 1 ml of trace metals in a sterile 2 litre flask and to this 5 ml of the overnight culture was added and placed in a shaking incubator at 37^{o}C. The cells were grown to an O.D of 0.6 for up to 3 hours and then switched

to grow at 20^{o}C overnight. The following day the cells were spun down and pellets obtained were either used directly for protein purification or kept frozen for later use.

4.3 Results

4.3.1 Protein expression optimisation

Varoius constructs for protein expression of full length Rv2509 using different expression vectors were used, for example, pET28a, pET26b, pET41C, pMAL-p2x and pGEX4T-1. The recombinant strains obtained with each of these expression vectors were sequenced and analysed for protein expression using western blot. Once confirmed with sequencing and western blot the strains were grown in LB at 37^{o}C for 4 hours till it reached an O.D of 0.6 and then induce with IPTG and then grown at different temperatures. For optimisation of protein expression the recombinant strains were grown at varying temperatures of 37^{o}C, 25^{o}C and 16^{o}C and varying concentrations of IPTG like 0.1 mM, 0.25 mM,0.5 mM and 1 M. The samples were centrifuged and the pellet obtained was resuspended in a purification buffer, the cells sonicated and centrifuged to separate the clarified lysate which corresponded to the soluble form of protein and the pellet which was the insoluble form. The samples were then boiled and run on SDS PAGE gels and the gel images are represented below. The recombinant strains were grown in different media too like terrific broth and an autoinduction media and expression of full length Rv2509 monitored with these media. Expression of protein did not vary much with different media.

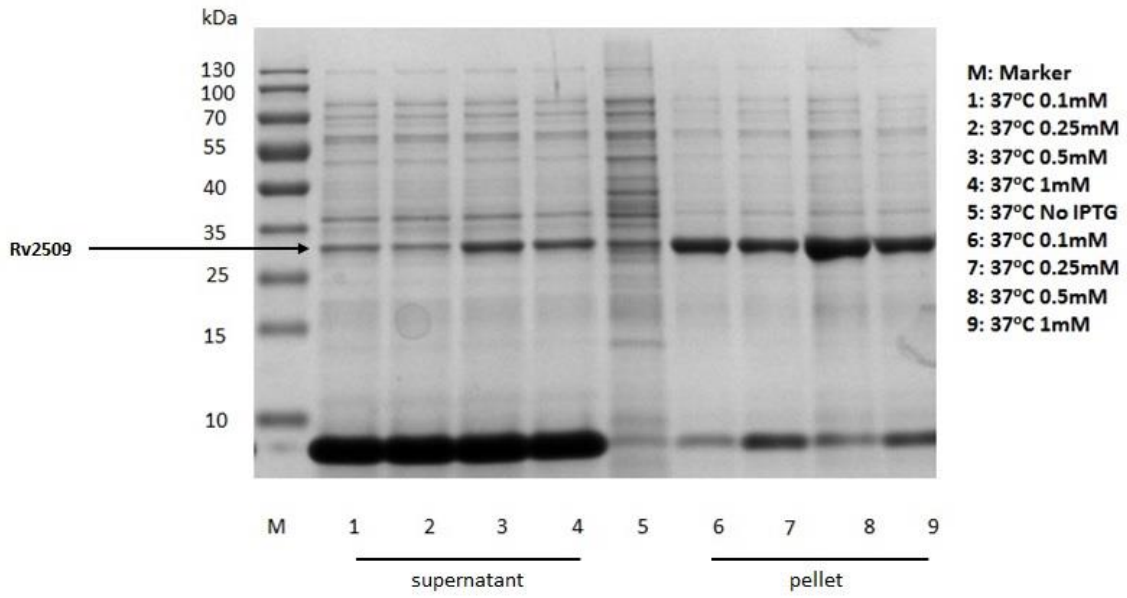

Figure 4.2: SDS PAGE gel of pET28a:Rv2509 induced at 37ºC with varying concentrations of IPTG

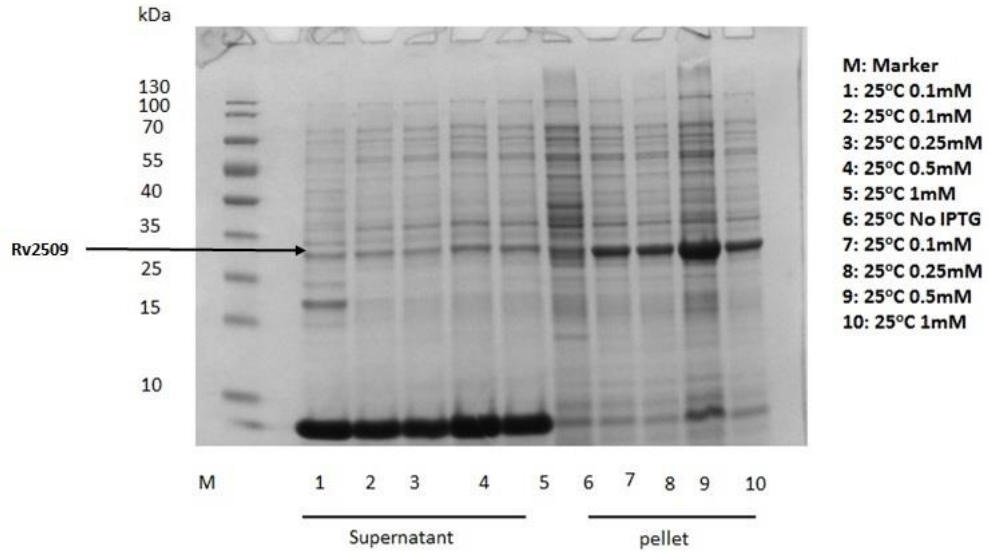

Figure 4.3: SDS PAGE gel of pET28a: Rv2509 induced at 25ºC with varying concentrations of IPTG.

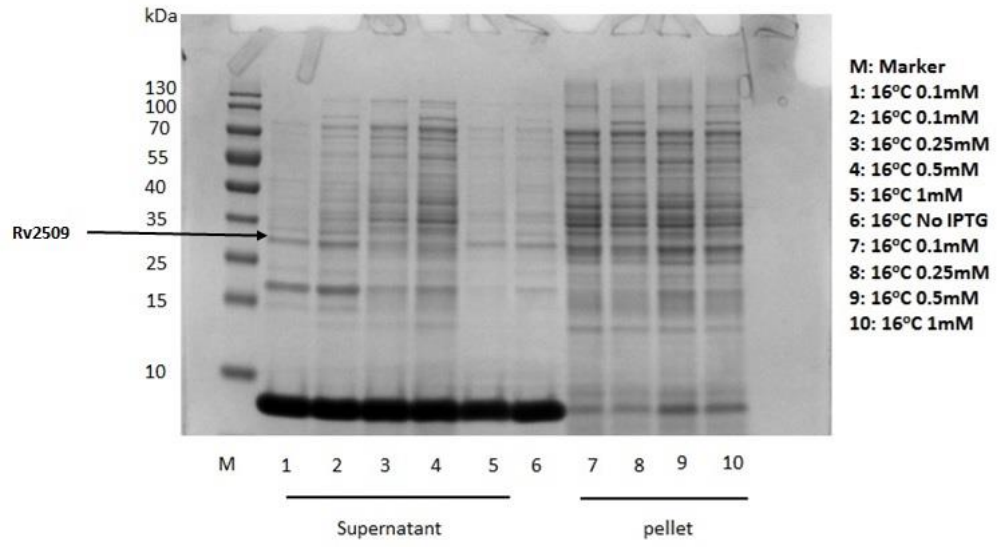

Figure 4.4: SDS PAGE gel of pET28a: Rv2509 induced at 16°C with varying concentrations of IPTG.

Figures 4.1, 4.2 and 4.3 are SDS PAGE gels of one expression construct, pET28a Rv2509. The recombinant strain was grown at 37°C, 25°C and 16°C after IPTG induction at concentrations of 0.1 mM, 0.25 mM, 0.5 mM and 1 mM. Similar optimisation procedure was used with all the recombinant strains using different expression vectors. The best suited condition for expression of Rv2509 was induction at 1 mM IPTG concentration at 37°C and grown for 8 hours after induction, demonstrated in Figure 4.1. This condition was chosen on the basis of having fairly equal amounts of soluble (clear lysate/supernatant) and insoluble (pellet) forms of the protein. When induced at varying temperatures the yield of the protein was not enough like in the case of 16°C where there was not enough expression of protein neither in the supernatant nor pellet at all the concentrations of IPTG. With 25°C most of the protein at varying IPTG concentrations was obtained in the pellet fraction suggesting that the protein was not folding properly.

4.3.2 Western blot analysis of the expression constructs

The expression levels of protein in the recombinant strain using different expression vectors was monitored using western blot shown the figures below. For western blot analysis the clear lysate and pellet of samples were first boiled and then run on a SDS PAGE gel which was then transferred on a membrane, blocked and then stained with specific antibodies to target the protein of interest.

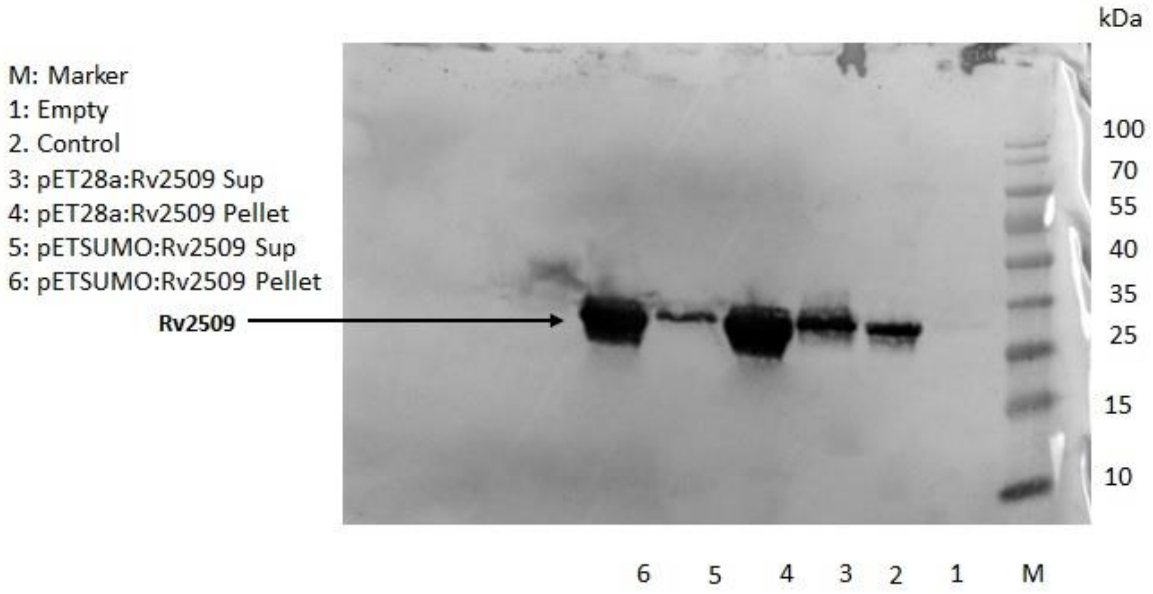

Figure 4.5: Western blot analysis of pET28a:Rv2509 and pETSUMO:Rv2509.

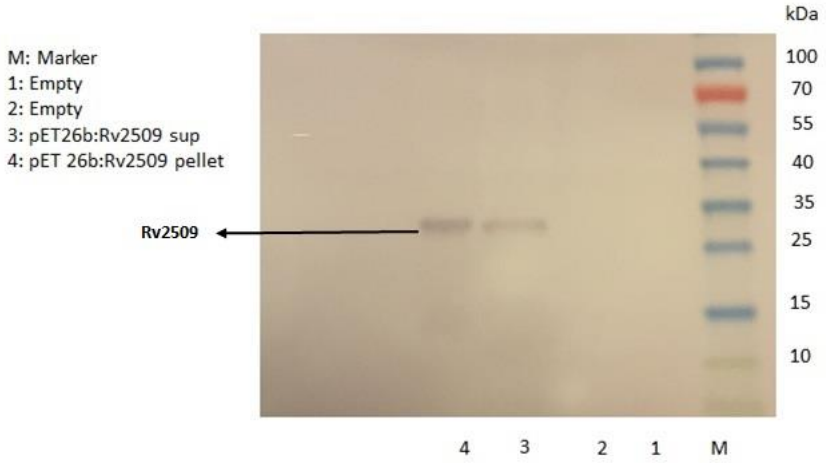

Figure 4.6: Western blot analysis of construct pET26b:Rv2509.

Figure 4.5 and 4.6 are western blot analysis of pET28a:Rv2509, pETSUMO:Rv2509 and pET26b:Rv2509 depicting that the size of Rv2509 with the addition of the expression vectors is 34kDa which was also confirmed by sequencing. All vectors have a histidine tag which is easily recognized by anti-his antibodies. Western blot analysis of pET28a:Rv2509 and pMAL-p2x:Rv2509 showed that majority of the protein was found in the pellet which is in the insoluble state when compared to supernatant or the clear lysate which corresponds to the soluble state. This was seen with other expression vectors and after several trial and errors and attempts of getting more protein in the soluble form pET26b which is mainly used for periplasmic proteins was used. The western blot analysis seen in figure 4.6 is of pET26b:Rv2509 which shows fairly equal amounts of expression of protein in the clear lysate/supernatant fraction and the pellet. This construct was used for purification of Rv2509 protein.

4.3.3 Purification of Rv2509

Following optimisation, pET26b:Rv2509 expression construct was used for further purification steps. The buffer that best suited purification of Rv2509 was a combination of sodium phosphate and sodium chloride, composition has been detailed in section 4.2.5. The cells were resuspended in the purification buffer along with imidazole, probe sonicated and then centrifuged to separate the clear lysate from the pellet. The clear lysate was then passed through a Ni^{2+}NTA HiTrap column which was already equilibrated with the purification buffer containing 25 mM of imidazole. As the protein is His tagged when passed through the column the polyhistidine tag binds to the nickel in the column. This gets rid of the untagged proteins and the protein of interest can be eluted using different concentrations of imidazole. 5 ml fractions of the equilibrated buffer containing 50 mM, 150 mM, 200mM, 300 mM and 1 M imidazole were collected.

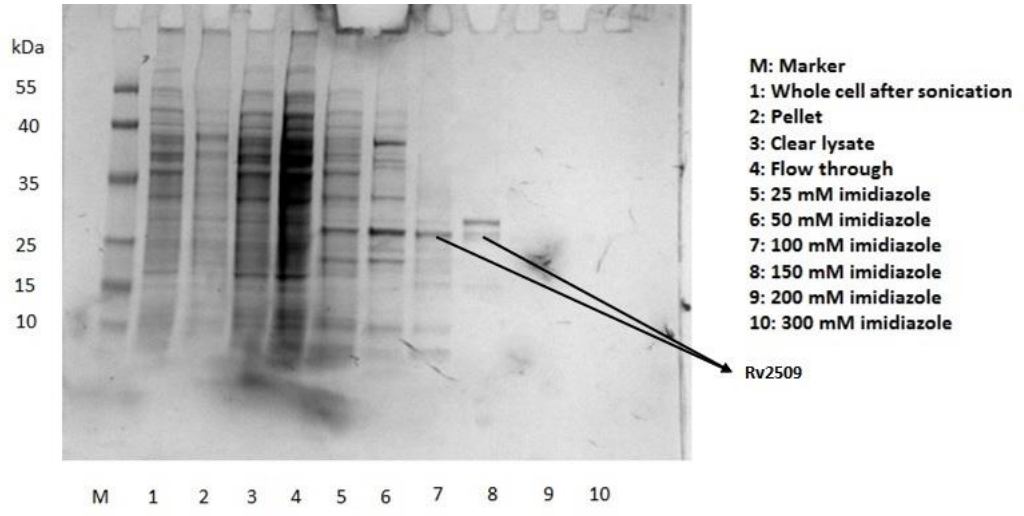

Figure 4.7: SDS PAGE gel for purification of RV2509 eluted with varying imidazole concentrations.

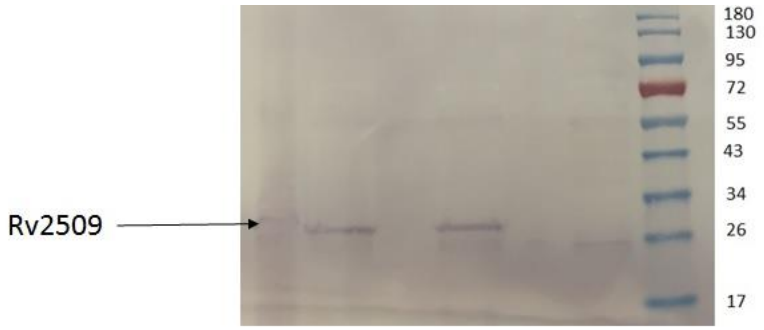

Figure 4.8: A western blot to show the purified fraction for figure 4.7 SDS PAGE gel.

The fractions were then run on a SDS PAGE gel as shown in Figure 4.7. The protein started eluting in fractions of 50 mM, 100 mM and 150 mM imidazole. Along with the protein of interest there were accompanying protein bands and to eliminate those another round of purification was required. The fractions corresponding to 50 mM, 100 mM and 150 mM imidazole were subjected to further purification using anion-exchange.

4.3.4 Anion-exchange

For further purification of the protein eluted in fractions 50 mM, 100 mM and 150 mM imidazole, anion-exchange chromatography was used. For this, the fractions were combined and passed through a column containing a resin, DEAE-Sephadex. This column was equilibrated with a no salt buffer, Buffer A (section 4.2.6) prior to passing the protein. Once the protein was passed through the column 10 ml fractions were collected. These 10 ml fractions where then passed through a Q HP column. The column was equilibrated with Buffer B which contained salt, then Buffer A and then the 10 ml fractions were passed through. The fractions eluted from the Q HP column were subjected to Bradford assay for protein estimation. The fractions with protein are then finally passed through a BioLogic LP

which is a low pressure chromatography system for protein purification. Using this system pure protein is eluted with increasing salt concentrations which is demonstrated in the graph presented in Figure 4.9. SDS PAGE gels were run on the samples to confirm a single band of the pure protein and protein concentration was measured using a nano drop. The protein concentration obtained for Rv2509 was 0.1 µg/ml which was not enough to set up crystal trays but instead used in an activity assay.

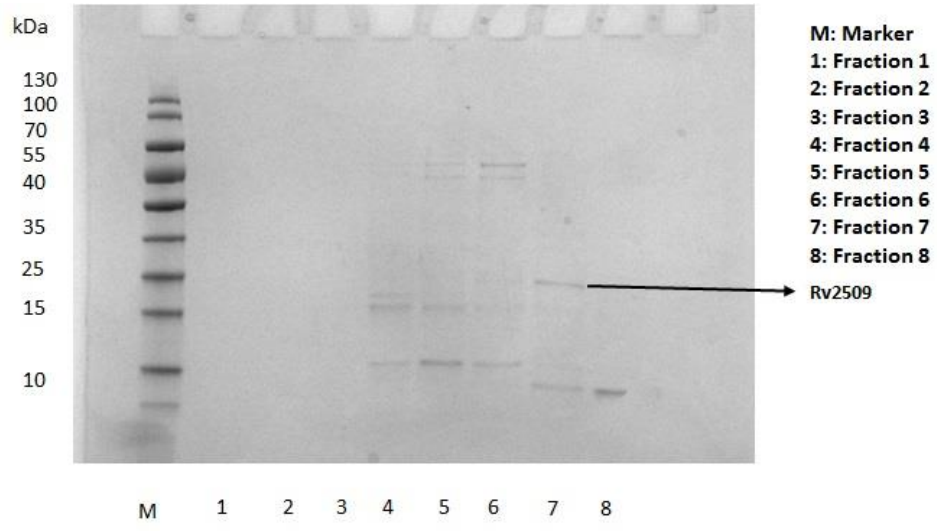

Figure 4.9: SDS PAGE gel for purification of Rv2509 protein using anion-exchange chromatography. The protein was eluted with increasing salt concentrations.

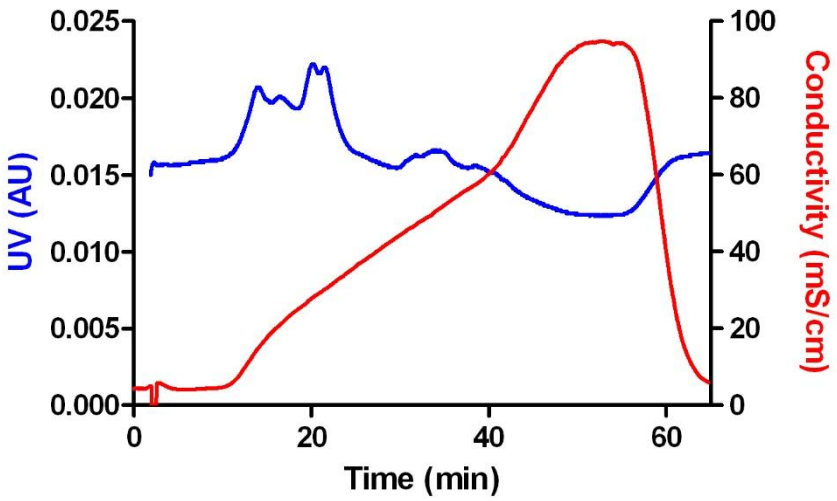

Figure 4.10: Graph representing elution of protein of Rv2509 with increasing salt concentrations. The peaks in blue represent the protein being eluted which is then checked by running on a SDS PAGE gel.

4.3.5 Rv2509 acitivity assay

Rv2509 is the mycolic acid reductase that is involved in the final steps of mycolic acid biosynthesis. Deletion of Rv2509 has been associated with reduction in mature mycolates and an accumulation of the premycolates. Rv2509 belongs to a short chain dehydrogenase/ reductase family as like other members of the family Rv2509 would require NADH or NADPH as a coenzyme in any enzymatic assay. A study suggested that the TE domain of Pks13 catalyses the transfer of the α-alkyl-β ketoacyl chain onto trehalose and trehalose monomycolate (TMM) is then used as a substrate by the enzymes of Antigen 85 complex for the biosynthesis of mycolate containing lipids located in the mycomembrane and the arabinogalactan of the cell wall. On the basis of this a potential funtional assay was used to

see the meachnism of action of Rv2509. An assay using pure protein of Rv2509 and the non-polar lipids of ΔMSMEG4722 was set up and a control *M. smegmatis* mc^{2}155 was used. The non-polar ^{14}C labelled lipids of mc^{2}155 and ΔMSMEG4722 which constitute trehalose monomycolates were mixed with buffer containing NADH or NADPH sonicated, and then Rv2509 protein added. These mixtures were treated with tetrabutyl ammoniumhydroxide (TBAH) for the release of mycolic acids. FAMEs and MAMEs were extracted as explained in chapter 7 section 7.20 and analysed by thin layer chromatography (TLC) which are shown in figure 4.11.

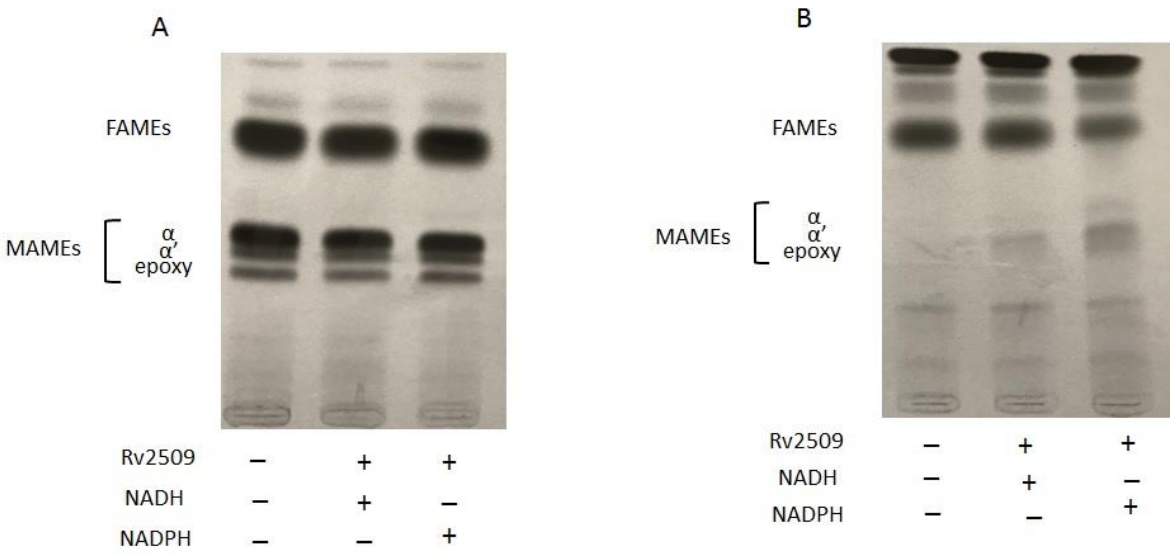

Figure 4.11: TLC analysis of FAMEs and MAMEs of *M. smegmatis* mc^{2}155 and ΔMSMEG4722 with the addition of Rv2509

TLC analysis shown in figure 4.11 of *M. smegmatis* mc^{2}155 did not show any difference in the synthesis of mycolic acids with the addition of Rv2509 whereas the TLC analysis of

FAMEs and MAMEs extracted from ΔMSMEG4722 with the addition of Rv2509 showed a partial release of α, α' and epoxy mycolates. This showed that the trehalose monopremycolate accumulated in the mutant ΔMSMEG4722 was the substrate for RV2509 which resulted in the partial formation of mature mycolic acids.

4.4 Discussion

Recombinant technologies for producing large amounts of protein for structural and functional studies especially for genes in *Mycobacterium tuberculosis* can be very useful and informative. For this the choice of an appropriate expression method is crucial for the production of proteins in high quantity and good quality. High expression of protein is based on the right choice of the expression hosts and then the choice of expression vectors which can produce protein in a soluble form which is also active. In this chapter, *E. coli* was used as an expression host and a number of expression vectors were used for increasing the expression of Rv2509 protein in the soluble form. Studies have shown that maltose binding protein and glutathione fusion tags help in increasing the expression of protein of interest but when used for Rv2509 resulted in more protein expression in the pellet which corresponds to the insoluble form. Getting more protein in the pellet could also be due to the protein not folding properly so to avoid this and let the protein fold properly the recombinant strain was induced and moved to a lower temperature of 16^{o}C. Lowering the temperature leads to proper folding of the protein and hence increase in the expression of soluble form but this was not the case with Rv2509. Lowering the temperature did not make much of a difference to the expression of Rv2509 again resulting with more in the insoluble form. Different growth media were also used including an auto induction media which does not require IPTG

induction. This led us to believe that Rv2509 was probably a membrane protein and would require the uses of detergents and folding and refolding techniques to obtain more of it in the soluble form. Cellular localisation studies of Rv2509 discussed in chapter 3 showed that the protein was a cytoplasmic protein probably being associated with some membrane functions. For this reason it was observed in both the cytoplasmic fraction as well as the membrane fraction. After several attempts at using different expression vectors for Rv2509, pET26b was the only vector that led to the equal expression of RV2509 in both the soluble and insoluble forms. pET26b has shown to be used for the expression of periplasmic proteins and since Rv2509 has been suggested to be cytoplasmic associated it helped enhance the expression.

The next hurdle was to select a purification buffer for the purification of Rv2509 protein. The buffer that best suited purification for Rv2509 was a combination of sodium phosphate and sodium chloride. Rv2509 protein obtained from the first purification contained accompanying proteins which needed to be removed and for this another purification method was used. Dialysis did not seem to be a useful means of purifying protein so anion-exchange was used. This method eluted the protein with increasing concentrations of salt. The protein ontained was confirmed with Bradford assay and concentration checked using a nano drop. The concentration of protein ontained was too low to carry out any enzymatic assay or set up crystal trays for crystallography. Instead the protein obtained was used for a potential functional assay based on the studies that showed the TE domain of Pks13 catalyses the transfer of the α-alkyl-β ketoacyl chain onto trehalose and that TMM was the substrate used by the enzymes of Antigen 85 for the biosynthesis of mycolates to the cell wall. The deletion

of the orthologue of Rv2509 in *M. smegmatis* MSMEG4722 has shown a reduction of mature mycolic acids and an accumulation of trehalose monopremycolates. If the tehalose monopremycolates in ΔMSMEG4722 was the substrate of Rv2509 then it would lead to the formation of mature mycolic acids in the activity assay which was observed. These results could also be confirmed using mass spectrometry which would produce peaks corresponding to the mycolic acids formed.

All these experiments were done on a small scale from an initial one litre culture of the recombinant strain and after several attempts of purifying protein which could be used in an assay. To increase the yield of pure protein upscale of the initial culture would be required. Even after the second purification there were certain bands still present along with Rv2509 which led to the concentration of Rv2509 to eliminate them. In order to avoid all this gel filtration could have been used if time permitted. Crystallography studies would give more information on the structure and function of Rv2509 and its potential of being used as a drug target, which could be looked into next.

Chapter 5

Studies on the cell envelope hydrophobicity of the ΔMSMEG4722 mutant

5.1 Introduction

Previous studies have shown that any disruption to the mycolic acids in the cell wall of mycobacteria results in a change in the hydrophobicity of the strain (Etienne et al, 2002). Increase in hydrophobicity has been attributed to strains that have a rough colony morphology. The colony morphology of *ΔMSMEG4722* resulted in colonies with a rough surface when compared to the smooth colonies obtained from wild type *Mycobacterium smegmatis* mc^2155, especially on Tween 80 containing plates. The rough morphology could attribute to altered hydrophobicity of the cell wall of the mutant *ΔMSMEG4722* altered. Increased hydrophobicity has also been attributed to affect the internalisation of the bacteria and also results in the cells growing as large aggregates (Daffe and Draper, 1998). All this promoted the study of hydrophobicity in the mutant ΔMSMEG4722.

Altered hydrophobicity could be driven by altered packing of the premycolates in the cell wall of the mutant strain and as a result altered ability to retain the 'free' lipids found on the outer envelope of the mutant. It was thus interesting to study the lipids extracted from the outer membrane of *ΔMSMEG4722*. The mycobacterial cell wall constitutes three structural components which are the plasma membrane, a cell wall and an outer layer (Daffe and Draper, 1998). In 1982, Minnikin was the first to propose that mycobacterial cell wall consisted of a second lipid bilayer which were covalently attached to the cell wall mycolate inside and an outer leaflet of free lipids. Analysis of the lipids present in the outer membrane would give an insight to the changes in the structure of cell wall.

5.1.1 Aims and objectives

The change in the colony morphology of ΔMSMEG4722 promoted the following aims of this chapter.

1. Accessing biochemically the changes in the structure of the cell wall by extracting polar and apolar lipids from liquid cultures and colonies on plates of ΔMSMEG4722
2. Hydrophobicity studies to access the change effect of the colony morphology by hexadecane partitioning.

5.2 Materials and methods

5.2.1 Bacterial strains

M. smegmatis mc^2155, ΔMSMEG4722 and ΔMSMEG4722-C were grown in 20 ml of tryptic soy broth (TSB) with the addition of hygromycin (70 mg/ml) and kanamycin (50 mg/ml) and tween 80 at 37ºC in a shaking incubator. When the cultures reached mid-log phase 10 ml of this culture was radiolabelled with ^{14}C acetate for extraction of polar and apolar lipids and the remaining 10 ml used for hydrophobicity assay.

5.2.2 Hexadecane aqueous buffer partitioning

10 ml cultures of *M. smegmatis* mc^2155, ΔMSMEG4722 and ΔMSMEG4722-C were grown to mid-log phase, collected in tubes and centrifuged. The cells were washed twice with PUM buffer (22 g K_2HPO_4, 7.26g of KH_2PO_4, 1.8 g of urea, 0.2 g of $MgSO_4.7H_2O$, made up to 1 litre with water, pH7.1) and finally suspended to an $O.D_{600}$ of 0.7. 3 ml aliquots of each strain

were transferred to glass tubes to which 2.4 ml of hexadecane added. The tubes were mixed by inversion and incubated for 8 minutes at 37°C. Then the tubes were placed at 22°C for 15 minutes for phase separation. Hydrophobicity index was defined as aqueous phase $O.D_{600}$ expressed as a percentage of that of the bacterial suspension in PUM buffer alone. For the statistical analysis, the results from at least three independent experiments were expressed as mean±SD.

5.2.3 Labelling of lipids with ^{14}C acetate for liquid cultures and agar plates

M. smegmatis mc^2155, ΔMSMEG4722 and ΔMSMEG4722-C were grown up to mid-log phase (10ml) were labelled with 1μCi/ml of ^{14}C acetic acid-sodium salt (50 mCi/ml, Amersham Pharmcia Biotech). The cells were incubated for 12 hours at 37°C. The labelled cells were then harvested by centrifugation and washed with PBS before drying. ^{14}C acetate was added to agar plates and *M. smegmatis* mc^2155, ΔMSMEG4722 and ΔMSMEG4722-C were spread to obtain well isolated colonies. Since the lipids from the outer membrane were subject to analysis, the cells obtained from the liquid cultures and plates were resuspended in petroleum ether and the supernatant obtained was subject to lipid extractions.

5.2.4 Polar and Apolar lipid extraction

The petroleum ether extracts of *M. smegmatis* mc^2155, ΔMSMEG4722 and ΔMSMEG4722-C were mixed with 2ml of methanol:0.3%NaCl (10:1)and 2ml of petroleum ether (b.p 60-80°C), mixed and centrifuged at 3000 rpm for 5 minutes. The upper phase was transferred to a new tube and lower phase again resuspended in 2 ml of petroleum ether (bp 60-80°C), mixed and spun down for 5 minutes. The upper phase was collected along with the previous

upper phase, dried and resuspended in 200 µl of chloroform:methanol (2:1). This formed the apolar fraction of the extract, which was dried under liquid nitrogen and analysed. For the polar fraction extraction the lower phase from the previous step was resuspended in 2.3 ml of chloroform:methanol:0.3%NaCl (9:10:3), mixed for 1 hour and then centrifuged for 5 minutes at 3000 rpm. The supernatant was transferred to a new tube and to the pellet 750 µl of chloroform:methanol:0.3%NaCl (5:10:4) was added, mixed for 30 minutes and centrifuged. This step was repeated twice and the resulting supernatants were collected in the same tube as the previous. To the collected supernantant 1.3 ml of chloroform and 1.3 ml of 0.3% NaCl was added, tubes mixed for 5 minutes and then spun down. The lower phase was transferred to a fresh tube, dried and resuspended in 200 µl of chloroform:methanol (2:1). 5 µl of the dissolved polar and apolar lipids were dried in scintillation vials and 5ml of scintillation fluid added, mixed and radioactivity measured as counts per minute. 1D TLC were run for the polar and apolar lipids extracted from *M. smegmatis* mc^2155, ΔMSMEG4722 and ΔMSMEG4722-C and the system used was chloroform: methanol: water (60:16:2).

5.3 Results

5.3.1 Hydrophobicity data

To understand the hydrophobicity of the mutant ΔMSMEG4722 because of the difference in colony morphology which demonstrated that the mutant colonies had a rough surface when compared to the smooth surface of the colonies of the wild type, cells were subjected to hexadecane partitioning (Rossenberg et al, 1980). The mechanism of hexadecane partitioning is based on the ability of the hydrocarbon degrading cells to maintain contact with their water

soluble alkane substrates. The method depends on the level of adherence of cells to hydrocarbons after brief mixing. A comparison was made between the wild type mc^2 155, the mutant ΔMSMEG4722 and the complemented strain ΔMSMEG4722-C. The cells were grown to mid-log phase and then washed with PUM buffer and then resuspended in the same buffer till the cells to reach an O.D of 0.7. Hexadecane was added to the cells and mixed for phase separation. The upper layer formed was denser when compared to the lower aqueous phase. The upper layer also consisted of emulsions of oil in water which consisted of droplets of hexadecane. The lower layer was not dense when compared to the upper layer and the decrease in absorbance was used as a measure of cell surface hydrophobicity. Tween was added to ensure growth of all strains without clumping. The results from at least three independent experiments were expressed as mean±SD. For statistical analysis, the student's unpaired t-test (with equal variance assumed) was used to evaluate difference between the samples. Two tailes P-values below 0.01 were considered statistically significant.The graph below suggests that the mutant ΔMSMEG4722 was more hydrophobic when compared to wild type mc^2155 whereas the complemented strain ΔMSMEG4722-C did not show a significant difference and was more similar to the wild type when compared to the mutant. This data suggests that the disruption of MSMEG4722 which plays a role in mycolic acid synthesis resulted in an increased level of hydrophobicity. Increase in hydrophobicity and lower absorption in the lower aqueous phase of the mutant ΔMSMEG4722 can be due to the increased interaction of the inner regions with the lipooligasaccharide.

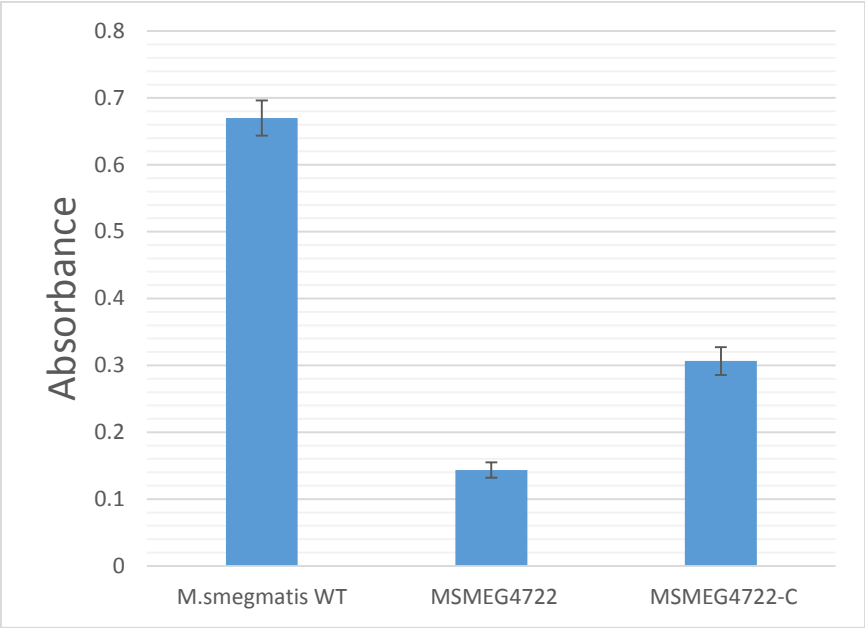

Figure 5.1: Graph representing the assessment of hydrophobicity in ΔMSMEG4722.

5.3.2 Analysis of lipids from the outer membrane

To study the lipids from the outer membrane of the mutant ΔMSMEG4722, cultures of mc^2155, ΔMSMEG4722 and ΔMSMEG4722-C were grown to mid-log phase and then labeled with ^{14}C acetate and grown for another 12 hours. For plates, ^{14}C acetate was added to the plates, mc^2155, ΔMSMEG4722 and ΔMSMEG4722-C spread on plates and left to grow until well isolated colonies were obtained. To the cells collected from liquid cultures and plates petroleum ether was added, mixed and centrifuged and the resulting supernatant was used for lipid extractions. Polar and non-polar lipids were extracted from both liquid cultures and from plates which are shown in the figures below.

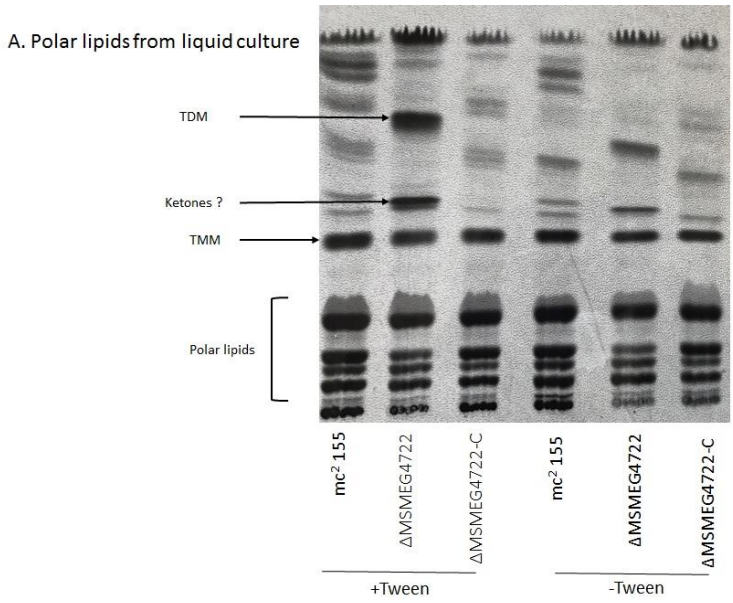

Figure 5.2: 1D TLC analysis of polar lipids from liquid culture.

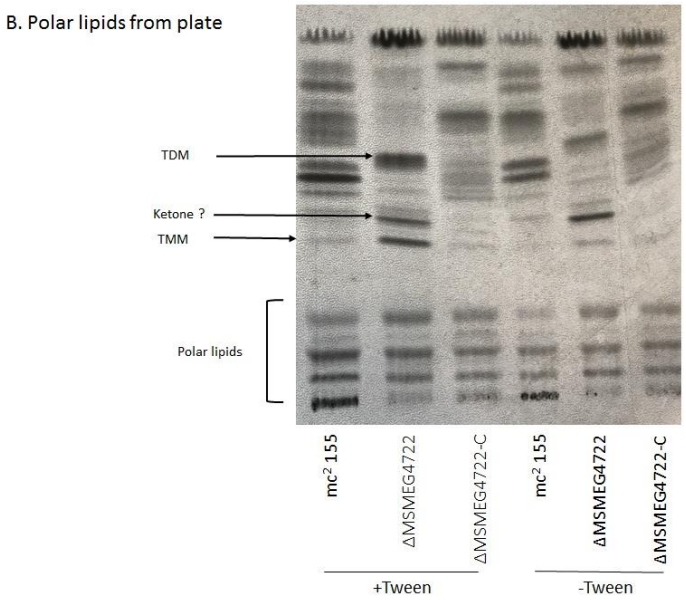

Figure 5.3: 1D TLC analysis of polar lipids from plates

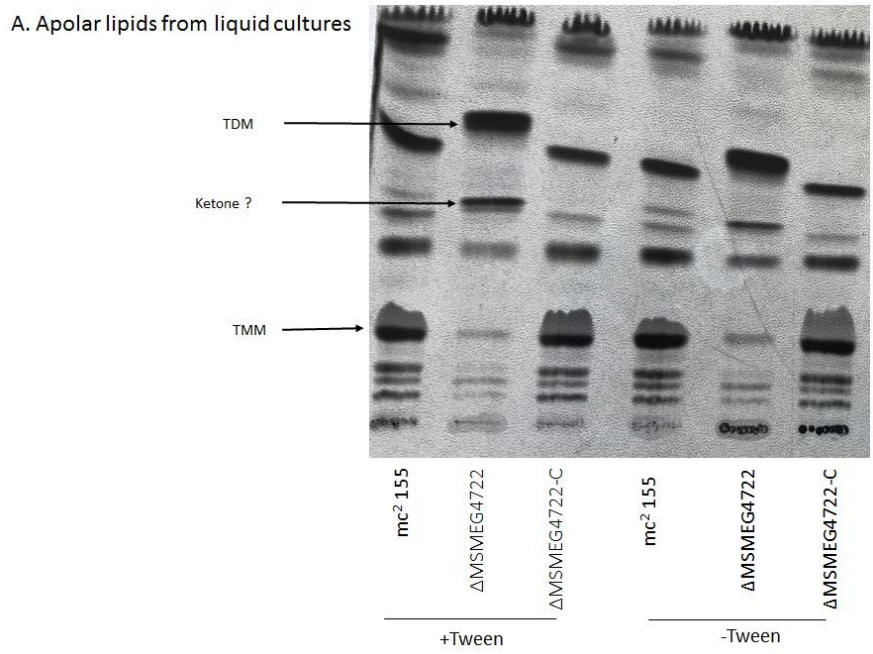

Figure 5.4: 1D TLC analysis of apolar lipids from cultures

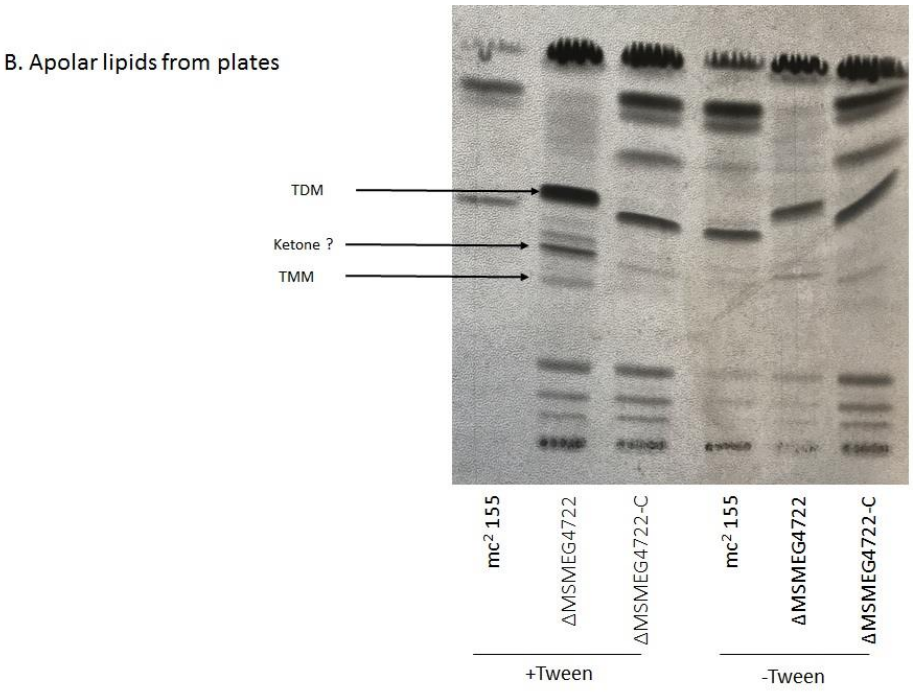

Figure 5.5: 1D TLC analysis of apolar lipids from plates

The TLC analysis of the mutant ΔMSMEG4722 of polar and non-polar lipids from plates and liquid cultures apart from the other lipids shows the presence of trehalose monomycolates (TMM) which is common to the lipid analysis of mc^2155 and *ΔMSMEG4722*-C. mc^2155, *ΔMSMEG4722* and *ΔMSMEG4722*-C lipids show the presence of trehalose dimycolates (TDM) but the TDM of *ΔMSMEG4722* migrates higher when compared to the TDM of mc^2155 and *ΔMSMEG4722*-C which could be because the mutant *ΔMSMEG4722* accumulates the premycolates. There is a presence of a unique lipid labelled as 'Ketone?' in *ΔMSMEG4722* which is missing in mc^2155 and ΔMSMEG4722-C. The free α-alkyl-β oxo precursors found in *ΔMSMEG4722* undergo decarboxylation to form ketones which corresponds to ketone obtained in the lipid extracts of *ΔMSMEG4722*.

5.4 Discussion

Studies have shown that disruption of mycolic acids in the cell wall results in increased hydrophobicity which reflects on the alterations of the composition of the cell envelope (Etienne at al, 2002). Pathogenic members of the *Mycobacterium tuberculosis* complex portray enhanced hydrophobicity as an important characteristic feature. Strains with an altered colony morphology depicting rough surface are more hydrophobic when compared to the smooth variants. Cells growing in aggregates is also related to increased hydrophobicity (Daffe and Draper, 1998). Increased cell wall hydrophobicity can also influence the internalization of the bacteria.

Previous studies on the colony morphology of *ΔMSMEG4722* depicted that the mutant had rough surfaced colonies when compared to the smooth colonies of wild type. This promoted

the need to check if this altered colony morphology had an effect on hydrophobicity. For this hexadecane partitioning was used and the results obtained showed that the mutant *ΔMSMEG4722* was more hydrophobic when compared to the wild type. Studies in *M.canettii* have shown that the rough variants were more hydrophobic and readily transmittable through aerosols. Further studies are required to test hydrophobicity of the mycolic acid reductase Rv2509 in *M. tuberculosis*.

The next section of this chapter looked into the lipids of the outer membrane of *ΔMSMEG4722*. The mutant *ΔMSMEG4722* apart from the presence of other lipids showed the presence of tehalose monomycolates which was also present in the wild type and also the presence of trehalose dimycolates which migrated above the trehalose dimycolates of the wild type. This corresponded to the accumulation of premycolates a phenotype similar to the lipids extracted from the cell wall. The mutant *ΔMSMEG4722* also showed the presence of ketones which is formed by the decarboxylation of the free α-alkyl-β oxo precursors found in *ΔMSMEG4722*. This was also seen with the lipids extracted from the cell wall. Studies in *M. tuberculosis* would give an insight on the lipids found in the outer membrane.

Chapter 6

General Discussion

6.1 Discussion

Tuberculosis is one of the leading killers amongst the bacterial pathogens and *Mycobacterium tuberculosis* is the causative agent of this disease. This disease causes nearly two million deaths every year and recently there has been a 60 % rise in multi-drug resistant (MDR) and extensively-drug resistant (XDR) cases. There has been continuous research for finding new drug targets that can be useful in the developing new drugs and new treatment therapies. Most of the drugs developed so far target the genes involved in the biosynthesis of mycolic acids which is the major component of the cell wall.

Mycolic acids are short α-alkyl long β-hydroxyl fatty acids ranging from 60 to 80 carbons per chain. They are mainly found as esters of the non-reducing arabinan terminus of the arabinogalactan and also present as free lipids within the cell wall associated to trehalose to form trehalose dimycolates (TDM). The synthesis pathway of mycolates involves two fatty acid synthases synthesised by the repetitive addition of two carbon units to the growing end of a hydrocarbon chain. The two types involve fatty acid synthase (FAS) type I and type II. A type I fatty acid synthase initially synthesis as long chain fatty acids which are then elongated by the multi enzyme type II fatty acid synthase complex. After a series of additional functionalisation the α-branch and the meromycolic acid are condensed by the polyketide synthase Pks13. This forms a precursor 2-alkyl-3-keto fatty acid which is then reduced by a mycolic acid reductase (Lea-Smith et al, 2007) (Bhatt et al,2008) to form the mature mycolic acids. The mycolic acid reductase was found to be non-essential in *C. glutamicum* and *M. smegmatis*. Several attempts of generating a knock-out in *M. tuberculosis* and *M. bovis* BCG were unsuccessfully promoting that it might be essential for the

functionality of these species. This chapter summarises the essentiality of the reductase gene in *M. bovis* BCG and *M. tuberculosis* using a conditional mutant that silences the expression of the gene on the addition of anhydrotetracycline (ATc). The chapter also focuses on *in silico* structure of Rv2509 highlighting some of critical residues and regions that play a role in the functionality of the protein, localization of the protein and finally the purification strategies used to obtain Rv2509 in soluble active form.

To study the essentiality of *M. tuberculosis* Rv2509 and *M. bovis* BCG Mb2537 a conditional mutant was constructed with the help of Dr.Rainer Kalscheurer, Institute for Medical Microbiology and Hospital Hygiene, Heinrich-Heine University, Dusseldorf, Germany. They constructed a conditional mutant in both *M. tuberculosis* and *M. bovis* BCG where the native promoter of the gene was replaced by a Tet-off promoter. The system used was a modification to the original Tet-off system where the addition of anhyrotetracycline (ATc) led to the silencing of the gene and the subsequent depletion of it in the bacterial cell. The most commonly used expression systems in mycobacteria for silencing of genes is the inducible system which involves the addition of an inducer which have disadvantages. To avoid these the best suited expression system for studying silencing of genes are the systems which can be turned off by the addition of a corepressor which was the approach used for studying the essentiality of the mycolic acid reductase Rv2509. Biochemical characterisation of the mutant showed a reduction in mycolic acids and an accumulation of the degradation products of the premycolates, a phenotype similar to the one obtained for *M. smegmatis* (Bhatt et al, 2008). The biochemical studies suggested that Rv2509 is essential in *M. tuberculosis* and that it showed functional similarities with its homologue in *M. smegmatis*.

Rv2509 is a 28kDa putative protein belonging to a family of short chain dehydrogenases/reductases. Using bioinformatics, a model was developed for Rv2509 from the solved crystal structures of other members of short chain reductases. The *in silico* analysis of the structure of Rv2509 gave us insights into the critical residues and regions which play an important role in the functionality of the protein. Structural modelling gave us an insight that Rv2509 is a putative short chain dehydrogenase/reductase, contained an extra C-terminal helix composed of hydrophobic residues, which could be a potential transmembrane domain. The modelling also illustrated the potential acitve site residues, a tunnel and cleft like structure which could be the pathway through which the subtrate passes. Deletion analysis of the C-terminal residues showed that this terminal C helix played a role in the funtionality of the protein. Apart from this site directed mutagenesis was used to produce mutants of the active site residues and residues for the tunnel and cleft like structures. It was observed that out of all the active site residues changed to phenylalanine it was just Try157 which was important in the functionality of the protein. It was even observed that the changes made to the residues of the tunnel and cleft like structure were important for functionality. The possible explanation could be that the change led to the loss of functional groups essential for catalysis or binding of the subtrate.

It was important to determine the function of Rv2509 not only at the molecular level but cellular level too. Subcellular localisation is important for determining the cellular functions of newly found proteins and hypothetical proteins. It also gives us information of the function of protein. Fractionation of Rv2509 indicated that it was a soluble protein found in the cytosol but was also co-localised with some membrane proteins as part of its function.

Different stratgies were employed to optimise the expression of Rv2509 protein and even to purify it. The conditions were finally optimised by using a pET26b expression vector and a purification buffer which is a combination of sodium phosphate and sodium chloirde. The yield of the protein was 0.1mg/ml which was not enough for crystallisation process. The yield of the protein was not enough to develop functional assays which would demostrate the mechanism of action of the protein. Low yield of the protein could be attributed to the fact that nearly half of the protein was expressed in the insoluble form and to avoid using harsh detergents and refolding techniques I used an expression vector that gave sufficient expression of the protein in the soluble state.

So far no enzymatic assays have been developed for the mycolic acid reductase and since the yield was low it was not enough to be used in developing an assay. . Instead the protein obtained was used for a potential functional assay based on the studies that showed the TE domain of Pks13 catalyses the transfer of the α-alkyl β-ketoacyl chain onto trehalose and that TMM was the substrate used by the enzymes of Antigen 85 for the biosynthesis of mycolates to the cell wall. The deletion of the orthologue of Rv2509 in *M. smegmatis* MSMEG4722 has shown a reduction of mature mycolic acids and an accumulation of trehalose monopremycolates. If the tehalose monopremycolates in ΔMSMEG4722 was the substrate of Rv2509 then it would lead to the formation of mature mycolic acids in the activity assay which was observed. These results could also be confirmed using mass spectrometry which would produce peaks corresponding to the mycolic acids formed.

The change in colony morphology promoted studies to look into the lipids extracted in the outer membrane of the mutant ΔMSMEG4722. Previous studies on the colony morphology

of *ΔMSMEG4722* depicted that mutant had rough surfaced colonies when compared to the smooth colonies of wild type. This promoted the need to check if this altered colony morphology had an effect on hydrophobicity. For this hexadecane partitioning was used and the results obtained showed that the mutant *ΔMSMEG4722* was more hydrophobic when compared to the wild type. Studies in *M. canettii* have shown that the rough variants were more hydrophobic and readily transmittable through aerosols. Further studies are required to test hydrophobicity of the mycolic acid reductase Rv2509 in *M. tuberculosis*. The extraction of lipids in the outer membrane of the mutant *ΔMSMEG4722* showed the presence of tehalose monomycolates which was also present in the wild type and the presence of trehalose dimycolates which migrated above the trehalose dimycolates of the wild type. This corresponded to the accumulation of premycolates a phenotype similar to the lipids extracted from the cell wall. The mutant *ΔMSMEG4722* also showed the presence of ketones which is formed by the decarboxylation of the free α-alkyl β-oxo precursors found *ΔMSMEG4722*. This was also seen with the lipids extracted from the cell wall. Studies in *M. tuberculosis* would give an insight on the lipids found in the outer membrane.

In conclusion, it was found that Rv2509 is essential in *M. tuberculosis* and *M. bovis BCG*. *In silico* analysis of structure of Rv2509 revealed a C-terminus with hydrophobic residues which was essential for the functionality of the protein, and could possibly be a transmembrane domain. The strucutre also gave an insight on the potential acitve site residues which are important for the functionality of the protein and also residues that formed the pathway for the passage of the subtrate which were important for the functionality of the protein. Cellular localisation studies showed that Rv2509 was a soluble protein but could be

assoicated with some membrane proteins for funtion. Apart from this the conditions for purification of protein were optimised and future work would require upscaling the yield of the protein to develop functional assays to study the mechanism of action and for cyrstallisation process for gaining the structure of the protein. These findings also identified pre trehalose monomycolate as the subtrate for Rv2509 which when used in a potential funtional assay resulted in the formation of mature mycolic acids in the mutant. The essentiality of Rv2509 in *M. tuberculosis* and its deletion resulting in a reduction of mature mycolic acids highlight the potential of Rv2509 being used as a secondary drug target.

The available drug treatment regimen according to the WHO guidelines for the treatment of TB susceptible patients is the adminstration of two months of isoniazid (INH), rifampicin (RIF), ethambutol (EMB) and pyrazinamide (PZA) which is followed by four months of isoniazid and rifampicin (WHO, 2010). Treatment for multi-drug resistant for now involves administration of pyrazinamide along with isoniazid and rifampicin during the intial two months of treament followed by the four second line drugs (Millard et al, 2015). Extensively-drug resistant patients are difficult to treat because the bacteria is resistant to the potent second line drugs (WHO, 2014).

The cell wall of *M.tuberculosis* is the layer that forms the barrier and the protection against drugs, but any defect in the proteins or the enzymes involved in the cell wall integrity results in the bacteria being susceptible to multiple drugs. From previous studies on the proteins of the antigen 85 complex (Ag85) which are involved in the biogenesis of trehalose dimycolates (TDM) (Jankute et al, 2015) it was shown that inactivation of the Antigen 85 gene resulted in the depletion of mycolates which directly resulted in the cell wall of *M.tuberculosis* being

more permeable. This change in permeability of the cell envelope of the bacteria means a change in the drug resistance phenotype. Production of TDM by Ag85, from recent studies, has been shown to be essential in antibiotic resistance in some mycobacteria and the inactivation of the gene can lead to the treatment of TB and other mycobacterial diseases with the use of either Ag85-specific inhibitors on its own or in combination with other antibiotics (Nguyen et al, 2005). Apart from the genes in Ag85 complex few important proteins like GLmU (Mill et al, 2004), MurX (Philalay et al, 2004), Ddl (Calvanese et al, 2014), accD6 (Alderwick et al, 2015) and Pks12 (Jankute et al, 2015) have been shown to be essential and resulted in being drug targets for the development of new antibacterial agents. In a similar way, further studies into Rv2509 can lead its way into becoming a secondary drug target for the treatment of mycobacterial infections.

Chapter 7

General Materials and Methods

7.1 7H9 Middlebrook broth

To prepare 1 litre of 7H9 broth, 4.7 g of Middlebrook 7H9 broth mix (BD) was dissolved in 900 ml of water. To this 2 ml of glycerol was added along with 0.05% Tween 80. The mix was either filter sterilized prior to use. When growing BCG 10% OADC (BD) was added, and stored at 4^{o}C. The Middlebrook 7H9 broth mix is composed of the components stated in the table below

Table 7.1: Components of Middlebrook 7H9 broth mix

Components	Weight (g/L)
Ammonium sulfate	0.5
Disodium phosphate	2.5
Monopotassium phosphate	1.00
Sodium citrate	0.10
Magnesium sulfate	0.05
Calcium chloride	0.0005
Zinc sulfate	0.001
Copper sulfate	0.001
Ferric ammonium citrate	0.04
L-glutamic acid	0.50
Pyridoxine	0.001
Biotin	0.0005

7.2 7H11 Middlebrook agar

For 1 litre of 7H11 agar, 4.7 g of Middlebrook 7H11 agar mix (BD) was dissolved in 900 ml of water. 2 ml of glycerol is added and the mix is autoclaved. The agar mix is the supplemented with 10%OADC (BD). The components of 7H11 Middlebrook agar mix are listed in the table below.

Table 7.2: Components of Middlebrook 7H11 agar mix

Components	Weight (g/L)
Casein enzymatic hydrolysate	1.00
Ammonium sulfate	0.50
Monopotassium phosphate	1.50
Disodium phosphate	1.50
Sodium citrate	0.40
Magnesium sulfate	0.05
L-Glutamic acid	0.50
Ferric ammonium citrate	0.04
Pyridoxine	0.001
Biotin	0.005
Malachite green	0.001
Agar	15

7.3 Tryptic Soy Broth (TSB)

To prepare 1 litre tryptic soy broth, 30 g of TSB mix (Sigma Aldrich) was dissolved in 1 litre of distilled water and sterilized by autoclaving. Tyrptic soy broth mix is composed of the components listed in the table below.

Table 7.3: Components of Tryptic soy broth mix (TSB)

Components	Weight (g/L)
Casein peptone (pancreatic)	17.00
Dipotassium hydrogen phosphate	2.5
Glucose	2.5
Sodium chloride	5.00
Soya peptone (papain digest)	3.00

7.4 Tryptic Soy Agar (TSA)

For 1 litre of TSA, 15 g of agar was added to 1 litre of TSB and the mix sterilized by autoclaving. The agar mix was cooled down to 55^{o}C and then the appropriate antibiotics added and poured into plates aseptically (25 ml). Tryptic soy agar is composed of the following components listed in the table below.

Table 7.4: Components of Tryptic soy agar

Components	Weight (g/L)
Agar	15.00
Casein peptone (pancreatic)	15.00
Sodium chloride	5.00
Soya peptone (papainic)	5.00

7.5 Luria-Bertain Broth (LB)

To prepare 1 litre 25 g of LB (Tryptone-Yeast extract NaCl 2:1:2 w/w/w/) (Sigma Aldrich) was dissolved in 1 litre of distilled water and autoclaved.

7.6 Luria-Bertain Agar (LBA)

To prepare 1 litre of LBA, either 37 g of LBA agar mix (Tryptone-Yeast extract NaCl-agar 2:1:2:3 w/w/w/w) (Sigma Aldrich) is added to 1 litre of water and autoclaved or 15 g of agar is added to 1 litre of LB broth and autoclaved. The agar mix was allowed to cool to 55^{o}C and appropriate antibiotics added if necessary. 25 ml of the mix was poured into plates aseptically.

7.7 List of antibiotics and supplements

Table 7.5: List of antibiotics used, concentrations and storage conditions

Antibiotics	Stock concentration	Storage conditions
Kanamycin	50 mg/ml	-20^{o}C, dissolved in water, filter sterilized
Hygromycin B	50 mg/ml	4^{o}C, protected from light
Anhydrotetracycline	500 mg/ml	-20^{o}C,dissolved in ethanol, filter sterilized, protected from light

Table 7.6: List of supplements used, concentrations and storage conditions.

Supplements	Stock concentration	Storage condition
OADC (Oleic acid Albumin Dextrose catalase)	10%	4^{o}C
Tween 80	10%	Room temperature, protected from light
IPTG	1 M	-20^{o}C, protected from light

7.8 Agarose Gel Electrophoresis

PCR products, genomic DNA and restriction digested plasmids were analyzed routinely by gel electrophoresis. Agarose gels (0.7-1.0% gels) were prepared by melting molecular biology grade agarose (Bioline) in 1X Tris acetate EDTA (TAE) buffer. Samples were

separated under a horizontal electric field (100-140 V, 400mA). The gel was stained with ethidium bromide and visualized under UV light (Bio-Rad Gel Doc System).

7.9 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) was carried out using Phusion polymerase mix provided by NEB. The components of the mix are listed in the table below.

Table 7.7: Components of Phusion polymerase mix (NEB)

Component	Reaction volume (µl)	Stock concentration	Final concentration
GC Buffer	20	5X	1x
DMSO (optional)	5	100%	5%
$MgCl_2$	2	50 mM	1 mM
Polymerase	1	100 units	1 unit
dNTP	0.8	2 mM	200 µM
Forward Primer	1	10 µM	0.5 µM
Reverse Primer	1	10 µM	0.5 µM
Template DNA	1	10 ng/µl	10 ng
Milli Q Water	Made up to 100 µl		

7.10 Restriction Digestion

Restriction digestion (20 µl) reaction mix for either single or double digestion were routinely set up.

Table 7.8: Components of restricted digestion mix

Components	Volume
Plasmid DNA, Genomic DNA, PCR product (1µg)	5 µl
10X digestion buffer	2 µl
Restriction enzyme (5-10 units)	1 µl
10X BSA (Bovine Serum Albumin)	2 µl
Nuclease free water	Made up to 20 µl

The reaction mix was incubated at 37^{o}C for 60 minutes. The digested DNA was then purified using the PCR purification kit and used for ligation.

7.11 DNA Ligation

The digested DNA fragments with compatible ends can be ligated using the T4 DNA ligase (NEB). The reaction mix (20 µl) contained the vector and insert DNA fragments, 1 µl of T4 DNA ligase enzyme, 2 µl of 10X T4 DNA ligase reaction mixture and distilled water. The reaction mix was either incubated at room temperature for 30 minutes or overnight at 4^{o}C, and then transformed into *E. coli* competent cells to amplify and purify plasmids.

7.12 Preparation of *E.coli* competent cells

E. coli (Top-10 strain, BL21 (DE3)) was a streaked on LB agar plate to get well isolated colonies. A single colony was inoculated in 5 ml of LB broth and incubated overnight at 37^{o}C. This overnight culture was used to inoculate 200 ml of LB media containing 20 mM $MgSO_4$ and cells grown to an $O.D._{600nm}$ of 0.5-0.6. The cells were then centrifuged at 4500 g for 10 minutes at 4^{o}C. The cell pellets was then gently resuspended in 25 ml of ice cold TFB1, incubated on ice for 15 minutes and then centrifuged at 4500 g for 10 minutes at 4^{o}C. The cell pellet was then resuspended in 10 ml of TFB2 and incubated on ice for one hour. This was then aliquoted and flash frozen and store at -80^{o}C for further use.

TFB1 : 100 mM $RbCl_2$, 30 mM Potassium acetate, 10 mM $CaCl_2$, 50 mM $MnCl_2$ and 15% glycerol. All components filter sterilized and stored at 4^{o}C

TFB2: 10 mM MOPS/PIPES pH 6.5, 75 mM $CaCl_2$, 10 mM $RbCl_2$ and 15 % glyercol. All components filter sterilized and stored at 4^{o}C.

7.13 Transformation of *E. coli* competent cells

E. coli competent cells were thawed on ice prior transformation. To these thawed *E. coli* cells 5 µl of the ligation mix was added, mixed gently and placed on ice for 30 minutes and transformed by heat shock at 45^{o}C for 45 seconds. To the cooled transformed cells 1 ml of LB media was added for recovery. Recovery time is generally 45 minutes to 1 hour after which the cells are plated on to LB agar plates with appropriate selection markers.

7.14 Plasmid Extraction

Plasmid DNA was extracted using Qiagen plasmid purification kits. For this 5 ml of bacterial culture grown overnight at 37^{o}C was used. Cells were harvested by centrifugation at 4000 rpm for 15 minutes. To the pellet 250 µl of P1 buffer was added and mixture transferred to a fresh eppendorf tube. To this 250 µl of P2 buffer was added and tubes inverted 3 to 4 times. Then 300 µl of N3 Buffer was added, mixed immediately and inverted 4 to 6 times. The above mixture was centrifuged at 13,000 rpm for 10 minutes. The supernatant was transferred to spin columns and spun down at 13,000 rpm for 1 minute. The flow through was discarded and 500 µl of Buffer PB added to the spin column and centrifuged for 1 minute at 13,000 rpm. Again flow through was discarded and to the spin column 750 µl of PE buffer was added and centrifuged for a minute at 13,000 rpm. The spin column is was transferred to a fresh eppendorf tube and DNA eluted with addition of 50 µl of EB buffer to the column. The resulting plasmid DNA was stored at -20^{o}C for further use.

7.15 Genomic DNA extraction

A single colony was inoculated in 10 ml of broth and grown to an $O.D_{600nm}$ of 1.0 and harvested by centrifugation at 4500 g for 10 minutes. The cells were then resuspended in 500 µl of glucose Tris-EDTA (GTE) buffer. For complete lysis of cells 50 µl of lysozyme (10mg/ml) was added to the above and kept at 37^{0}C overnight. The following day 100 µl of 10% SDS and 50 µl of Proteinase K (10mg/ml) (Sigma) was added, mixed and incubated at 55^{o}C for 30 minutes. To the mix 200 µl of 5 M NaCl was added, mixed and incubated at 65^{o}C for 15 minutes. 500 µl of chloroform and chloroform:isoamyl alcohol (24:1) mix was

added to get rid of the protein and cell debris. DNA was then precipitated using 500 µl isopropanol and washed twice with 70% ethanol. DNA was air dried and dissolved in water.

7.16 Preparation of mycobacterial electrocompetent cells

Mycobacterial cells were grown to an O.D600nm of 0.5-0.6 and harvested by centrifugation at 4500 g for 30 minutes. The pellets were washed twice with ice cooled 10% glycerol (filter sterilized and stored at 4^{o}C) and harvested by centrifugation at 4500 g for 30 minutes at 4^{o}C. The cells were then resuspended in 1/10th volume of 10% glycerol, aliquoted and stored at -80^{o}C for future use.

7.17 Electroporation of mycobacteria

For electroporation, 5 µl of the plasmid was added to 200 µl of mycobacterial electrocompetent cells thawed on ice. This mix was added to 1mm ice cold electroporation cuvettes and placed on ice for further 15 minutes. The cells were electroporated using Eppendorf electroporator (model No 2510) at 1800 V. 1ml of TSB (*M. smegmatis*) or 7H9 (*M. bovis* BCG) was added to the electroporated cells and allowed to recover at 37^{o}C (4 hours for *M. smegmatis* and 24 hours for *M.bovis* BCG) (Snapper et al, 1988). The cells were harvested and resuspended in 200 µl of recovery media and plated on either TSBA or 7H11 agar plates with appropriate markers.

7.18 Labelling of lipids with ^{14}C acetate

Mycobacterial cells grown up to mid log phase (10 ml) were labelled with 1 µCi/ml of ^{14}C acetic acid-sodium salt (50 mCi/ml, Amersham Pharmcia Biotech). The cells were incubated

for 12 hours (*M. smegmatis*) or 24 hours (*M. bovis* BCG) at 37ºC. The labelled cells were then harvested by centrifugation and washed with PBS before drying.

7.19 Polar and Apolar lipid extraction

^{14}C labelled apolar and polar lipid extractions were done using methods described by Dobson et al, 1985. The dried lipids were mixed with 2 ml of methanol:0.3%NaCl (10:1)and 2 ml of petroleum ether (b.p 60-80ºC), mixed and centrifuged at 3000 rpm for 5 minutes. The upper phase was transferred to a new tube and lower phase again resuspended in 2 ml of petroleum ether (bp 60-80ºC), mixed and spun down for 5 minutes. The upper phase was collected along with the previous upper phase, dried and resuspended in 200 µl of chloroform:methanol (2:1). This formed the apolar fraction of the extract, which dried under liquid nitrogen and analyzed (Besra 1998). For the polar fraction extraction the lower phase from the previous step was resuspended in 2.3 ml of chloroform:methanol:0.3%NaCl (9:10:3), mixed for 1 hour and then centrifuged for 5 minutes at 3000 rpm. The supernatant was transferred to a new tube and to the pellet 750 µl of chloroform:methanol:0.3%NaCl (5:10:4) was added, mixed for 30 minutes and centrifuged. This step was repeated twice and the resulting supernatants were collected in the same tube as the previous. To the collected supernantant 1.3 ml of chloroform and 1.3 ml of 0.3% NaCl was added, tubes mixed for 5 minutes and then spun down. The lower phase was transferred to a fresh tube, dried and resuspended in 200 µl of chloroform:methanol (2:1). 5 µl of the dissolved polar and apolar lipids were dried in scintillation vials and 5 ml of scintillation fluid added, mixed and radioactivity measured as counts per minute. The deffated cells were used for further analysis.

7.20 Fatty acyl ethyl esters (FAMEs) and Mycolic acid methyl esters (MAMEs) extraction from deffated and whole cells

Whole cells or defatted cells were subject to alkaline hydrolysis by adding 2 ml of 5% TBAH (tetrabutyl ammonium hydroxide) and leaving to cells to boil at 100^{o}C overnight. 2 ml of dichloromethane, 4 ml of water and 500 µl of iodomethane were added, mixed for 30 minutes and centrifuged at 3000 rpm for 10 minutes. The upper aqueous phase was discarded and the lower phase was washed with water three times with intervals of 10 minutes with the upper phase being discarded in every round. The resulting lower phase was then dried and to it 4 ml of diethyl ether added, sonicated and centrifuged at 3000 rpm for 5 minutes to remove the insoluble residues. The supernatant was transferred to a new tube, dried and resuspended in 200 µl of chloroform:methanol (2:1) of which 5 µl was used for counting of labelled samples.

7.21 Thin layer chromatography (TLC)

Equal counts (25,000-50,000 cpm) of labelled lipids were spotted on TLC plates (5554 silica gel 60F524, Merck) for analysis. The TLC plates were developed using the different solvent systems mentioned in the next section. Similar procedure was done for labelled FAMEs and MAMEs. The labelled lipids and mycolic acids were detected by 24 hour exposure to Kodak X-Omat AR films. Apolar lipids are separated by systems A, B, C and D and polar lipids separated by systems D and E (Dobson et al,1985).

System A:

Direction 1:

3x with petroleum ether (b.p 60-80^{o}C):ethyl acetate- 98:2 (v/v)

Direction 2:

1x with petroleum ether (b.p 60-80^{o}C):acetone- 92:8 (v/v)

System B:

Direction 1:

3x with petroleum ether (b.p 60-80oC):acetone- 92:8 (v/v)

Direction 2:

1x with toluene:acetone- 95:5 (v/v)

System C:

Direction 1:

1x with choloform:methanol- 96:4 (v/v)

Direction 2:

1x with toluene:acetone-80:20 (v/v)

System D:

Direction 1:

1x with chloroform:methano:water- 100:13:0.8 (v/v/v)

Direction 2:

1x with chloroform:acetone:ethanol:water- 50:60:2.5:3 (v/v/v/v)

System E:

Direction 1:

1x with chloroform:methanol:water- 60:30:6 (v/v/v)

Direction 2:

1x with chloroform:acetic acid:methanol:water- 40:25:3:6 (v/v/v/v)

FAMEs and MAMEs:

Direction 1:

1x with petroleum ether (b.p 60-80ºC):acetone- 95:5 (v/v).

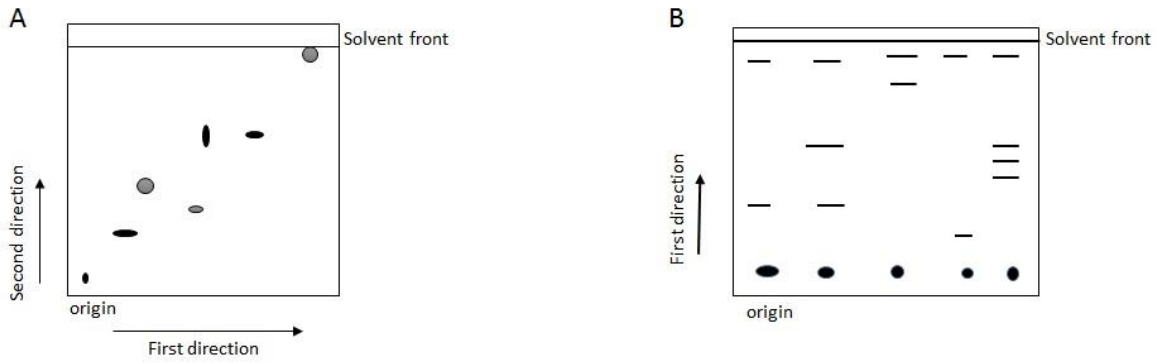

Figure 7.1: Schematic diagram of thin later chromatography (TLC). A. Diagram of 2D TLC in which 2 different solvent systems were used for developing in both dimensions. B. A diagram of 1D TLC using one solvent system for developing in the same dimension. The arrows indicate the direction of flow.

References

Amin, A. G., Goude, R., Shi, L., Zhang, J., Chatterjee, D., & Parish, T. (2008). EmbA is an essential arabinosyltransferase in *Mycobacterium tuberculosis*. *Microbiology*, *154*(1), 240–248.

Asselineau, C., Asselineau, J., Lanéelle, G., & Lanéelle, M.-A. (2002). The biosynthesis of mycolic acids by Mycobacteria: current and alternative hypotheses. *Progress in Lipid Research*, *41*(6), 501–23.

Asselineau, J., & Lederer, E. (1950). Structure of the Mycolic Acids of Mycobacteria. *Nature*, *4227*, 782–783.

Ates, L. S., Ummels, R., Commandeur, S., van der Weerd, R., Sparrius, M., Weerdenburg, E., Houben, E. N. G. (2015). Essential Role of the ESX-5 Secretion System in Outer Membrane Permeability of Pathogenic Mycobacteria. *PLoS Genetics*, *11*(5), 1–30.

Bailo, R., Bhatt, A., & Aínsa, J. A. (2015). Lipid transport in *Mycobacterium tuberculosis* and its implications in virulence and drug development. *Biochemical Pharmacology*, *96*(3), 159–167.

Banerjee, A., Dubnau, E., Quemard, A., Balasubramanian, V., Um, K. S., Wilson, T., Jr, W. R. J. (1993). inhA, Encoding Target *Mycobacterium*. *Science*, *263*(September), 227–230.

Banerjee, R., Vats, P., Dahale, S., Kasibhatla, S. M., & Joshi, R. (2011). Comparative genomics of cell envelope components in Mycobacteria. *PLoS ONE*, *6*(5).

Bañ Uls, A.-L., Sanou, A., Thi, N., Anh, V., & Godreuil, S. (2017). *Mycobacterium tuberculosis*: ecology and evolution of a human bacterium. *Journal of Medical Microbiology, 64(11)*, 1261-1269.

Barberis, I., Bragazzi, N. L., Galluzzo, L., & Martini, M. (2017). The history of tuberculosis: From the first historical records to the isolation of Koch's bacillus. *Journal of Preventive Medicine and Hygiene, 58(1),* 9-12.

Bardarov, S., Bardarov, S., Pavelka, M. S., Sambandamurthy, V., Larsen, M., Tufariello, J., Jacobs, W. R. (2002). Specialized transduction: An efficient method for generating marked and unmarked targeted gene disruptions in *Mycobacterium tuberculosis, M. bovis* BCG and *M. smegmatis*. *Microbiology, 148*(10), 3007–3017.

Bashiri, G., & Baker, E. N. (2015). Production of recombinant proteins in *Mycobacterium smegmatis* for structural and functional studies. *Protein Science*, *24*(1), 1–10.

Barry, C. E., Lee, R. E., Mdluli, K., Sampson, A. E., Schroeder, B. G., Slayden, R. a., Yuan, Y. (1998). Mycolic acids: Structure, biosynthesis and physiological functions. *Progress in Lipid Research*, *37*(2–3), 143–179.

Barry, C. E. 3rd, Crick, D. C., & McNeil, M. R. (2007). Targeting the formation of the cell wall core of *M. tuberculosis*. *Infectious Disorders Drug Targets*, *7*(2), 182–202

Barthe, P., Mukamolova, G. V., Roumestand, C., & Cohen-Gonsaud, M. (2010). The Structure of PknB Extracellular PASTA Domain from *Mycobacterium tuberculosis* suggests a Ligand-Dependent Kinase Activation. *Structure, 18*(5), 606–615.

Belardinelli, J. M., Larrouy-maumus, G., Jones, V., Pedro, L., Carvalho, S. De, Mcneil, M. R., & Jackson, M. (2014). Biosynthesis and translocation of unsulfated Acyltrehaloses in *Mycobacterium tuberculosis*. *Journal of Biological Chemistry, 289(40),* 27952-27965.

. Bellinzoni, M., & Riccardi, G. (2003). Techniques and Applications: The heterologous expression of *Mycobacterium tuberculosis* genes is an uphill road. *Trends in Microbiology*, *11*(8), 351–358

Berney, M., Hartman, T. E., & Jacobs, W. R. (2014). A *Mycobacterium tuberculosis* cytochrome bd oxidase mutant is hypersensitive to bedaquiline. *mBio*, *5(4)*, 01275-14.

Berens, C., & Hillen, W. (2003). Gene regulation by tetracyclines: Constraints of resistance regulation in bacteria shape TetR for application in eukaryotes. *European Journal of Biochemistry*, *270*(15), 3109–3121.

Besra, G. S., Khoo, K. H., Belisle, J. T., McNeil, M. R., Morris, H. R., Dell, a., & Brennan, P. J. (1994). New pyruvylated, glycosylated acyltrehaloses from *Mycobacterium smegmatis* strains, and their implications for phage resistance in mycobacteria. *Carbohydrate Research*, *251*(93), 99–114.

Bhatt, A., Kremer, L., Dai, A. Z., Sacchettini, J. C., & Jacobs, W. R. (2005). Conditional depletion of KasA, a key enzyme of mycolic acid biosynthesis, leads to mycobacterial cell lysis. *Journal of Bacteriology*, *187*(22), 7596–606.

Bhatt, A., Fujiwara, N., Bhatt, K., Gurcha, S. S., Kremer, L., Chen, B., Jacobs, W. R. (2007). Deletion of kasB in *Mycobacterium tuberculosis* causes loss of acid-fastness and subclinical latent tuberculosis in immunocompetent mice. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(12), 5157–62.

Bhatt, A., Brown, A. K., Singh, A., Minnikin, D. E., & Besra, G. S. (2008). Loss of a Mycobacterial Gene Encoding a Reductase leads to an Altered Cell Wall containing oxo- Mycolic Acid Analogs and Accumulation of Ketones. *Chemistry and Biology*, *15*(9), 930–939.

Bhowruth, V., Brown, a. K., & Besra, G. S. (2008). Synthesis and biological evaluation of NAS-21 and NAS-91 analogues as potential inhibitors of the mycobacterial FAS-II dehydratase enzyme Rv0636. *Microbiology*, *154*(7), 1866–1875.

Biswas, R., Dutta, A., Dutta, D., Hazra, D., Banerjee, D. R., Basak, A., & Das, A. K. (2015). Crystal structure of dehydratase component HadAB complex of mycobacterial FAS-II pathway. *Biochemical and Biophysical Research Communications*, *458*(2), 369–374.

Bloch, H. (1950). Studies on the virulence of tubercle bacilli; isolation and biological properties of a constituent of virulent organisms. *The Journal of Experimental Medicine*, *91*, 197–218.

Blokpoel, M. C. J., Murphy, H. N., Toole, R. O., Wiles, S., Runn, E. S. C., Stewart, G. R., Robertson, B. D. (2005). Tetracycline-inducible gene regulation in mycobacteria. *Nucleic Acid Research, 33*(2), 1-22.

Blumenthal, A., Trujillo, C., Ehrt, S., & Schnappinger, D. (2010). Simultaneous analysis of multiple *Mycobacterium tuberculosis* knockdown mutants *in vitro* and *in vivo*. *PLoS ONE, 5(12*), 1-12.

Breda, A., Valadares, N. F., Souza, O. N. De, & Garratt, R. C. (2008). Chapter A06 . Protein Structure , Modelling and Applications, (Md), 1–41.

Brennan, P. J., & Nikaido, H. (1995). The envelope of mycobacteria. *Annual Review of Biochemistry*, *64*, 29–63.

Brondyk, W. H. (2009). *Chapter 11 Selecting an Appropriate Method for Expressing a Recombinant Protein*. *Methods in Enzymology* (1st ed., Vol. 463). Elsevier Inc.

Brosch R, Gordon SV, Marmiesse M, Brodin P, Buchrieser C, Eiglmeier K, Garnier T, Gutierrez C, Hewinson G, Kremer K, Parson LM, Pym AS, Samper S, van Soolingen D, Cole ST. (2002). A new evolutionary scenario for the *Mycobacterium tuberculosis* complex. *PNAS, 99*, 3684-3689.

Brown, A. K., Bhatt, A., Singh, A., Saparia, E., Evans, A. F., & Besra, G. S. (2007). Identification of the dehydratase component of the mycobacterial mycolic acid-synthesizing fatty acid synthase-II complex. *Microbiology, 153*(12), 4166–4173.

Brown-Elliott, B. A., Nash, K. A., and Wallace, R. J. (2012). Antimicrobial susceptibility testing, drug resistance mechanisms, and therapy of infections with nontuberculous mycobacteria. *Clinical Microbiology Reviews, 25*, 545–582.

Campbell, E. a., Korzheva, N., Mustaev, A., Murakami, K., Nair, S., Goldfarb, A., Darst, S. a. (2001). Structural mechanism for rifampicin inhibition of bacterial RNA polymerase. *Cell*, *104*(6), 901–912.

Cantaloube, S., Veyron-Churlet, R., Haddache, N., Daffé, M., & Zerbib, D. (2011). The *Mycobacterium tuberculosis* FAS-II dehydratases and methyltransferases define the specificity of the mycolic acid elongation complexes. *PLoS ONE*, *6*(12).

Carpenter, C. D., Ingraham, H. A., Cochet, C., Walton, G. M., Lazar, C. S., Sowadski, J. M., Gill, G. N. (1991). Structural analysis of the transmembrane domain of the epidermal growth factor receptor. *J Biol Chem*, *266*(9), 5750–5755.

Carroll, P., Muttucumaru, D. G. N., Parish, T., Al, C. E. T., & Icrobiol, A. P. P. L. E. N. M. (2005). Use of a Tetracycline-Inducible System for Conditional Expression in *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*. *Applied Environmental Microbiology, 71(6)*, 3077–3084.

Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T. J., Higgins, D. G., & Thompson, J. D. (2003). Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Research, 31(13)*, 3497–3500.

Chhabra, A., Haque, A. S., Pal, R. K., Goyal, A., Rai, R., Joshi, S., Gokhale, R. S. (2012). Nonprocessive [2+2]e- off-loading reductase domains from mycobacterial

nonribosomal peptide synthetases. *Proceedings of the National Academy of Sciences*, *109*(15), 5681–5686.

Clark-Curtiss, J. E., & Haydel, S. E. (2003). Molecular genetics of *Mycobacterium tuberculosis* pathogenesis. *Annu. Rev. Microbiol*, *57*, 517–49.

Cole, S. T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Barrell, B. G. (1998). Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. *Nature*, *393*(6685), 537–544.

Cole, S. T. (2016). Inhibiting *Mycobacterium tuberculosis* within and without. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *371*(1707), 20150506.

Daffé, M., & Draper, P. (1997). *The Envelope Layers of Mycobacteria with Reference to their Pathogenicity*. *Advances in Microbial Physiology* (Vol. 39).

Daffé, M. (2015). The cell envelope of tubercle bacilli. *Tuberculosis*, *95*, S155–S158.

Daniel, T. M. (2006). The history of tuberculosis. *Respiratory Medicine*, *100(11),* 1862-1870.

Da Silva, P. E. A., and Palomino, J. C. (2011). Molecular basis and mechanisms of drug resistance in *Mycobacterium tuberculosis*: classical and new drugs. *Journal of Antimicrobial Chemotherapy*, *66*, 1417–1430.

Davis, E., Colston, M. J., & Hill, M. (2017). Regulation of the inducible acetarnidase gene of *Mycobacterium smegmatis. Microbiology, (1997), 143* , 2267–2276.

Ding, Q., Ying, Z., & Gómez-Pinilla, F. (2011). Exercise influences hippocampal plasticity by modulating brain-derived neurotrophic factor processing. *Neuroscience*, *192*, 773–80.

Domenech, P., Reed, M. B., & Iii, C. E. B. (2005). Contribution of the *Mycobacterium tuberculosis* MmpL Protein Family to Virulence and Drug Resistance Contribution of the Mycobacterium tuberculosis MmpL Protein Family to Virulence and Drug Resistance. *Infection and Immunity*, *73*(6), 3492–3501.

Douglas, J. D., Senior, S. J., Morehouse, C., Phetsukiri, B., Campbell, I. B., Besra, G. S., & Minnikin, D. E. (2002). Analogues of thiolactomycin: Potential drugs with enhanced anti-mycobacterial activity. *Microbiology*, *148*(10), 3101–3109.

Dragset, M. S., Barczak, A. K., Kannan, N., & Flo, T. H. (2015). Benzoic Acid-Inducible Gene Expression in Mycobacteria. *PLoS One*, *10(9)*, 1–19.

Dubnau, E., Chan, J., Raynaud, C., Mohan, V. P., Lanéelle, M.-A., Yu, K., Daffé, M. (2000). Oxygenated mycolic acids are necessary for virulence of *Mycobacterium tuberculosis* in mice. *Molecular Microbiology*, *36*(3), 630–637.

Ehrt, S., Guo, X. V, Hickey, C. M., Ryou, M., Monteleone, M., Riley, L. W., & Schnappinger, D. (2005). Controlling gene expression in mycobacteria with anhydrotetracycline and Tet repressor. *Nucleic Acid Research*, *33*(2), 1–11.

Ehrt, S., & Schnappinger, D. (2006). Controlling gene expression in mycobacteria. *Future Microbiol*, *1*(2), 177–184.

Escuyer, V. E., Lety, M. A., Torrelles, J. B., Khoo, K. H., Tang, J. B., Rithner, C. D., Chatterjee, D. (2001). The Role of the embA and embB Gene Products in the Biosynthesis of the Terminal Hexaarabinofuranosyl Motif of *Mycobacterium smegmatis* Arabinogalactan. *Journal of Biological Chemistry*, *276*(52), 48854–48862.

Etienne, G., Villeneuve, C., Billman-Jacobe, H., Astarie-Dequeker, C., Dupont, M. A., & Daffé, M. (2002). 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.

Etienne, G., Laval, F., Villeneuve, C., Dinadayala, P., Abouwarda, A., Zerbib, D., Daffé, M. (2005). The cell envelope structure and properties of *Mycobacterium smegmatis* mc2155: Is there a clue for the unique transformability of the strain *Microbiology*, *151*(6), 2075–2086.

Etienne, G., Malaga, W., Laval, F., Lemassu, a., Guilhot, C., & Daffe, M. (2009). Identification of the Polyketide Synthase Involved in the Biosynthesis of the Surface-Exposed Lipooligosaccharides in Mycobacteria. *Journal of Bacteriology*, *191*(8), 2613–2621.

Evans, J. C., & Mizrahi, V. (2015). The application of tetracycline- regulated gene expression systems in the validation of novel drug targets in *Mycobacterium tuberculosis*. *Frontiers of Microbiology, 812(6),* 1–14.

Favia, A. D., Nobeli, I., Glaser, F., & Thornton, J. M. (2008). Molecular docking for substrate identification: the short-chain dehydrogenases/reductases. *Journal of Molecular Biology*, *375*(3), 855–74.

Favrot, L. (2012). Targeting the mycrobacterial envelope for tuberculosis drug development. *Expert Review of Anti-Infective Therapy*, *10*(9), 1023–1036.

Fernandes, N. D., & Kolattukudy, P. E. (1996). Cloning, sequencing and characterization of a fatty acid synthase-encoding gene from *Mycobacterium tuberculosis* var. bovis BCG. *Gene*, *170*(1), 95–99.

Fernández, F. J., & Vega, M. C. (2013). Technologies to keep an eye on: Alternative hosts for protein production in structural biology. *Current Opinion in Structural Biology*, *23*(3), 365–373.

Feuerriegel, S., Köser, C. U., Richter, E., and Niemann, S. (2013). *Mycobacterium canettii* is intrinsically resistant to both pyrazinamide and pyrazinoic acid. *Journal of Antimicrobial Chemotherapy, 68,* 1439–1440.

Finken, M., Kirschner, P., Meier, A., Wrede, A., and Böttger, E. C. (1993). Molecular basis of streptomycin resistance in *Mycobacterium tuberculosis*: alterations of the ribosomal protein S12 gene and point mutations within a functional 16S ribosomal RNA pseudoknot. *Molecular Microbiology 9,* 1239–1246.

Flores, A. R., Flores, A. R., Parsons, L. M., Parsons, L. M., Pavelka, M. S., & Pavelka, M. S. (2005). Characterization of Novel. *Microbiology*, *187*(September), 1892–1900.

Forrellad, M. A., Klepp, L. I., Gioffré, A., Sabio y García, J., Morbidoni, H. R., de la Paz Santangelo, M., Bigi, F. (2013). Virulence factors of the *Mycobacterium tuberculosis* complex. *Virulence*, *4*(1), 3–66.

Franzblau, S. G., Witzig, R. S., Mclaughlin, J. C., Torres, P., Madico, G., Hernandez, A., Gilman, R. H. (1998). Rapid, low-technology MIC determination with clinical *Mycobacterium tuberculosis* isolates by using the microplate Alamar Blue assay. *Journal of Clinical Microbiology*, *36*(2), 362–366.

Gago, G., Diacovich, L., Arabolaza, A., Tsai, S.-C., & Gramajo, H. (2011). Fatty acid biosynthesis in actinomycetes. *FEMS Microbiology Reviews*, *35*(3), 475–497.

Gande, R., Gibson, K. J. C., Brown, A. K., Krumbach, K., Dover, L. G., Sahm, H., Eggeling, L. (2004). Acyl-CoA carboxylases (accD2 and accD3), together with a unique polyketide synthase (Cg-pks), are key to mycolic acid biosynthesis in Corynebacterianeae such as *Corynebacterium glutamicum* and *Mycobacterium tuberculosis*. *Journal of Biological Chemistry*, *279*(43), 44847–44857.

Gao, L. Y., Laval, F., Lawson, E. H., Groger, R. K., Woodruff, A., Morisaki, J. H., Brown, E. J. (2003). Requirement for kasB in Mycobacterium mycolic acid biosynthesis, cell

wall impermeability and intracellular survival: Implications for therapy. *Molecular Microbiology*, *49*(6), 1547–1563.

Gavalda, S., Bardou, F., Laval, F., Bon, C., Malaga, W., Chalut, C., Qumard, A. (2014). The polyketide synthase Pks13 catalyzes a novel mechanism of lipid transfer in mycobacteria. *Chemistry and Biology*, *21*(12), 1660–1669

Gengenbacher, M., & Kaufmann, S. H. E. (2012). *Mycobacterium tuberculosis*: Success through dormancy. *FEMS Microbiology Reviews, 36(3),* 514-532.

Georghiou, S. B., Magana, M., Garfein, R. S., Catanzaro, D. G., Catanzaro, A., and Rodwell, T. C. (2012). Evaluation of genetic mutations associated with *Mycobacterium tuberculosis* resistance to amikacin, kanamycin and capreomycin: a systematic review. *PLoS ONE, 7(3),* 1-12.

Giraud, M. F., & Naismith, J. H. (2000). The rhamnose pathway. *Current Opinion in Structural Biology*, *10*(6), 687–696.

Glickman, M. S., Cox, J. S., & Jacobs, W. R. (2000). A Novel Mycolic Acid Cyclopropane Synthetase Is Required for Cording, Persistence, and Virulence of *Mycobacterium tuberculosis*. *Molecular Cell*, *5*(4), 717–727.

Glickman, M. S. (2003). The mmaA2 gene of *Mycobacterium tuberculosis* encodes the distal cyclopropane synthase of the mycolic acid. *Journal of Biological Chemistry, 278(10),* 7844-7849.

Goate, & APP. (1991). © 19 9 1 Nature Publishing Group. *Letters To Nature*, *353*, 737–740.

Gomez, J. E., & Bishai, W. R. (2000). whmD is an essential mycobacterial gene required for proper septation and cell division. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(15), 8554–9.

Gormley, E., Corner, L. A. L., Costello, E., & Rodriguez-Campos, S. (2014). Bacteriological diagnosis and molecular strain typing of *Mycobacterium bovis* and *Mycobacterium caprae*. *Research in Veterinary Science*, *97 Suppl*, S30-43.

Goulding, C. W. (2016). MmpL membrane proteins from *Mycobacterium tuberculosis*. *Chem Biology*, *22*(8), 1098–1107.

Greendyke, R., Rajagopalan, M., Parish, T., & Madiraju, M. V. V. S. (2002). Conditional expression of *Mycobacterium smegmatis* dnaA, an essential DNA replication gene. *Microbiology*, *148*(12), 3887–3900.

Groenewald, W., Baird, M. S., Verschoor, J. A., Minnikin, D. E., & Croft, A. K. (2014). Differential spontaneous folding of mycolic acids from *Mycobacterium tuberculosis*. *Chemistry and Physics of Lipids*, *180*, 15–22.

Guo, H., Seet, Q., Denkin, S., Parsons, L., and Zhang, Y. (2006). Molecular characterization of isoniazid-resistant clinical isolates of *Mycobacterium tuberculosis* from the USA. *Journal of Medical Microbiology, 55*, 1527–1531.

Guo, X. V., Monteleone, M., Klotzsche, M., Kamionka, A., Hillen, W., Braunstein, M., Schnappinger, D. (2007). Silencing essential protein secretion in *Mycobacterium smegmatis* by using tetracycline repressors. *Journal of Bacteriology*, *189*(13), 4614–4623.

Haque, A. S., Patel, K. D., Deshmukh, M. V., Chhabra, A., Gokhale, R. S., & Sankaranarayanan, R. (2014). Delineating the reaction mechanism of reductase domains of Nonribosomal Peptide Synthetases from mycobacteria. *Journal of Structural Biology*, *187*(3), 207–214.

Hartkoorn, R. C., Sala, C., Neres, J., Pojer, F., Magnet, S., Mukherjee, R.,Cole, S. T. (2012). Towards a new tuberculosis drug: pyridomycin - nature's isoniazid. *EMBO Molecular Medicine*, *4*(10), 1032–1042.

Hawn, T. R., Day, T. A., Scriba, T. J., Hatherill, M., Hanekom, W. A., Evans, T. G., Self, S. G. (2014). Tuberculosis Vaccines and Prevention of Infection. *Microbiology and Molecular Biology Reviews*, *78*(4), 650–671.

Heep, M., Brandstätter, B., Rieger, U., Lehn, N., Richter, E., Rüsch-Gerdes, S., et al. (2001). Frequency of rpoB mutations inside and outside the cluster I region in rifampin-resistant clinical *Mycobacterium tuberculosis* isolates. *Journal of Clinical Microbiology*, *39*, 107–110.

Hett, E. C., & Rubin, E. J. (2008). Bacterial Growth and Cell Division: a Mycobacterial Perspective. *Microbiology and Molecular Biology Reviews*, *72*(1), 126–156.

Irzik, K., van Ooyen, J., Gätgens, J., Krumbach, K., Bott, M., & Eggeling, L. (2014). Acyl-CoA sensing by FasR to adjust fatty acid synthesis in *Corynebacterium glutamicum*. *Journal of Biotechnology*, *192*, 96–101.

Jackson, M., Raynaud, C., Lanéelle, M. A., Guilhot, C., Laurent-Winter, C., Ensergueix, D., Daffé, M. (1999). Inactivation of the antigen 85C gene profoundly affects the mycolate content and alters the permeability of the *Mycobacterium tuberculosis* cell envelope. *Molecular Microbiology*, *31*(5), 1573–1587.

Jackson, M. (2014). The Mycobacterial Cell Envelope-Lipids. *Cold Spring Harbour Perspective Medicine, 4*, 1–22.

Jankute, M., Grover, S., Birch, H., Besra, G. S. (2014). Genetics of Mycobacterial Arabinogalactan and Lipoarabinomannan assembly. *Microbiology Spectrum, 2(4)*, 0013-2013.

Jankute, M., Cox, J. A. G., Harrison, J., & Besra, G. S. (2015). Assembly of the Mycobacterial Cell Wall. *Annual Reviews Microbiology, 69*, 405-423.

Jankute, M., Nataraj, V., Lee, O. Y.-C., Wu, H. H. T., Ridell, M., Garton, N. J., Besra, G. S. (2017). The role of hydrophobicity in tuberculosis evolution and pathogenicity. *Scientific Reports*, *7*(1), 1315

Jomvall, H., Persson, B., Jörnvall, H., Persson, B., Krook, M., Atrian, S., Ghosh, D. (1995). Short-chain dehydrogenases/reductases(SDR). *Biochemistry*, *34*(18), 6003–13.

Kallberg, Y., Oppermann, U., Jörnvall, H., & Persson, B. (2002). Short-chain dehydrogenases/reductases (SDRs). Coenzyme-based functional assignments in completed genomes. *European Journal of Biochemistry*, *269*, 4409-4417.

Kallberg, Y., & Persson, B. (2006). Prediction of coenzyme specificity in dehydrogenases/reductases: A hidden Markov model-based method and its application on complete genomes. *FEBS Journal*, *273*(6), 1177–1184.

Kalscheuer, R., & Koliwer-brandl, H. (2014). Genetics of Mycobacterial Trehalose Metabolism. *Microbiol Spectrum*, *2*(3, NaN-0002–2013), 1–15.

Kamionka, A., Bertram, R., & Hillen, W. (2005). Tetracycline-Dependent Conditional Gene Knockout in *Bacillus subtilis*. *Society*, *71*(2), 728–733.

Karakousis, P. C., Bishai, W. R., & Dorman, S. E. (2004). *Mycobacterium tuberculosis c*ell envelope lipids and the host immune response. *Cell Microbiology*, *2* , 105–116.

Kavanagh, K. L., Jörnvall, H., Persson, B., & Oppermann, U. (2008). Medium- and short-chain dehydrogenase/reductase gene and protein families: The SDR superfamily: Functional and structural diversity within a family of metabolic and regulatory enzymes. *Cellular and Molecular Life Sciences*, *65(24)*, 3895-3906.

Keating, T. A., & Walsh, C. T. (1999). Initiation, elongation, and termination strategies in polyketide and polypeptide antibiotic biosynthesis. *Current Opinion in Chemical Biology*, *3*(5), 598–606.

Kelly, S. M., Jess, T. J., & Price, N. C. (2005). How to study proteins by circular dichroism. *Biochimica et Biophysica Acta (BBA)- Proteins and Proteomics, 1751(2)*, 119–139.

Kinatukara, P., Patel, K. D., Haque, A. S., Singh, R., Gokhale, R. S., Sankaranarayananan, R. (2016). Structural insights into the regulation of NADPH binding to reductase domains of nonribosomal peptide synthetases: A concerted loop movement model. *Journal of Structural Biology*, *194*(3), 368–374.

Kolly, G. S., Mukherjee, R., Kilacsková, E., Abriata, L. a., Raccaud, M., Blaško, J., Cole, S. T. (2015). GtrA protein Rv3789 is required for arabinosylation of arabinogalactan in *Mycobacterium tuberculosis*. *Journal of Bacteriology*, (September), JB.00628-15.

Kremer, L., Douglas, J. D., Baulard, A. R., Morehouse, C., Guy, M. R., Alland, D., Besra, G. S. (2000). Thiolactomycin and related analogues as novel anti-mycobacterial agents targeting KasA and KasB condensing enzymes in *Mycobacterium tuberculosis*. *Journal of Biological Chemistry*, *275*(22), 16857–16864.

Lea-Smith, D. J., Pyke, J. S., Tull, D., McConville, M. J., Coppel, R. L., & Crellin, P. K. (2007). The reductase that catalyzes mycolic motif synthesis is required for efficient attachment of mycolic acids to arabinogalactan. *Journal of Biological Chemistry*, *282*, 11000-11008.

Leotta, L., Spratt, J. M., Kong, C. U., & Triccas, J. A. (2015). Plasmid An inducible expression system for high-level expression of recombinant proteins in slow growing mycobacteria. *YPLAS*, *81*, 27–31.

Liu, J., Barry, C. E., Besra, G. S., & Nikaido, H. (1996). Mycolic acid structure determines the fluidity of the mycobacterial cell wall. *Journal of Biological Chemistry, 271(47)*, 29545-29551.

Luca, S., & Mihaescu, T. (2013). History of BCG Vaccine. *Maedica -a Journal of Clinical Medicine Maedica A Journal of Clinical Medicine Journal of Clinical Medicine*, *8*(81), 53–58.

Mani, V., Wang, S., Inci, F., De Libero, G., Singhal, A., & Demirci, U. (2014). Emerging technologies for monitoring drug-resistant tuberculosis at the point-of-care. *Advanced Drug Delivery Reviews*, *78*, 105–117.

Marrakchi, H., Ducasse, S., Labesse, G., Montrozier, H., Margeat, E., Emorine, L., Qumard, A. (2002). MabA (FabG1), a *Mycobacterium tuberculosis* protein involved in the long-chain fatty acid elongation system FAS-II. *Microbiology*, *148*(4), 951–960.

McNeil, M. B., Dennison, D., & Parish, T. (2017). Mutations in MmpL3 alter membrane potential, hydrophobicity and antibiotic susceptibility in *Mycobacterium smegmatis*. *Microbiology (United Kingdom)*, *163*(7), 1065–1070.

Minnikin DE. Lipids: Complex lipids, their chemistry, biosynthesis and roles. In: Ratledge C, Stanford J, editors. The biology of the mycobacteria: Physiology, identification and classification. *Academic Press, 1982,* 95–184.

Minnikin, D. E. (1988). Chemical principles in the organization of lipid components in the mycobacterial cell envelope. *Research in Microbiology, 142(4),* 423-427.

Minnikin, D. E., Lee, O. Y.-C., Wu, H. H. T., Besra, G. S., Bhatt, A., Nataraj, V., Donoghue, H. D. (2015). Ancient mycobacterial lipids: Key reference biomarkers in charting the evolution of tuberculosis. *Tuberculosis*, *95*, S133–S139.

Nasiri, M. J., Haeili, M., Ghazi, M., Goudarzi, H., Pormohammad, A., Fooladi, A. A. I., & Feizabadi, M. M. (2017). New insights in to the intrinsic and acquired drug resistance mechanisms in mycobacteria. *Frontiers in Microbiology, 681(8),* 1-19.

Nataraj, V., Pang, P. C., Haslam, S. M., Veerapen, N., Minnikin, D. E., Dell, A., Bhatt, A. (2015). MKAN27435 is required for the biosynthesis of higher subclasses of lipooligosaccharides in *Mycobacterium kansasii*. *PLoS ONE*, *10*(4), 1–10.

Nataraj, V., Varela, C., Javid, A., Singh, A., Besra, G. S., & Bhatt, A. (2015). Mycolic acids: Deciphering and targeting the Achilles' heel of the tubercle bacillus. *Molecular Microbiology*, *98*(1), 7–16.

Nobre, a., Alarico, S., Maranha, a., Mendes, V., & Empadinhas, N. (2014). The molecular biology of mycobacterial trehalose in the quest for advanced tuberculosis therapies. *Microbiology*, *160*(Pt_8), 1547–1570.

North, E. J., Jackson, M., & Lee, R. E. (2014). New approaches to target the mycolic acid biosynthesis pathway for the development of tuberculosis therapeutics. *Current Pharmaceutical Design*, *20*(27), 4357–78.

Onaka, H., Mori, Y., Igarashi, Y., & Furumai, T. (2011). Mycolic Acid-Containing Bacteria Induce Natural-Product Biosynthesis in Streptomyces Species. *Applied and Environmental Microbiology*, *77*(2), 400–406.

Oppermann, U. C. T., Filling, C., Berndt, K. D., Persson, B., Benach, J., Ladenstein, R., & Jörnvall, H. (1997). Active Site Directed Mutagenesis of 3/17-Hydroxysteroid Dehydrogenase Establishes Differential Effects on Short-Chain Dehydrogenase/Reductase Reactions. *Biochemistry 36(1)*, 34-40.

Orme, I. M. (2015). Tuberculosis Vaccine Types and Timings. *Clinical and Vaccine Immunology*, *22*(3), 249–257.

Palomino, J. C., and Martin, A. (2014). Drug Resistance Mechanisms in *Mycobacterium tuberculosis*. *Antibiotics*, *3*, 317–340.

Pardeshi, P., Rao, K. K., & Balaji, P. V. (2017). Rv3634c pefrom *Mycobacterium tuberculosis* H37Rv encodes an enzyme with UDP-Gal / Glc and UDP-GalNAc 4-epimerase activities. *PLoS One, 12(4),* 1–24.

Parikh, A., Kumar, D., Chawla, Y., Kurthkoti, K., Khan, S., Varshney, U., & Nandicoori, K. V. (2013). Development of a new generation of vectors for gene expression, gene replacement, and protein-protein interaction studies in mycobacteria. *Applied and Environmental Microbiology*, *79*(5), 1718–1729.

Parish, T., Roberts, G., Laval, F., Schaeffer, M., Daffé, M., & Duncan, K. (2007). Functional complementation of the essential gene fabG1 of *Mycobacterium tuberculosis* by *Mycobacterium smegmatis* fabG but not Escherichia coli fabG. *Journal of Bacteriology*, *189*(10), 3721–3728.

Persson, B., Kallberg, Y., Oppermann, U., & Jörnvall, H. (2003). Coenzyme-based functional assignments of short-chain dehydrogenases/reductases (SDRs). *Chemico-Biological Interactions*, *143–144*, 271–278.

Peterson, D. (1977). *Mycobacterium smegmatis* Fatty Acid Synthetase length. *The Journal of Biological Chemistry*, *252*(16), 5135–5139.

Philley, J. V., and Griffith, D. E. (2015). Treatment of slowly growing Mycobacteria. *Clinical Chest Medicine, 36,* 79–90.

Portevin, D., De Sousa-D'Auria, C., Houssin, C., Grimaldi, C., Chami, M., Daffé, M., & Guilhot, C. (2004). A polyketide synthase catalyzes the last condensation step of mycolic acid biosynthesis in mycobacteria and related organisms. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(1), 314–9.

Procop, G. W. (2017). HIV and mycobacteria. *Seminars in Diagnostic Pathology 34(4),* 332-339.

Radmacher, E. (2005). Two functional FAS-I type fatty acid synthases in *Corynebacterium glutamicum*. *Microbiology*, *151*(7), 2421–2427.

Ramaswamy, S. V., Reich, R., Dou, S.-J., Jasperse, L., Pan, X., Wanger, A., et al. (2003). Single nucleotide polymorphisms in genes associated with isoniazid resistance in *Mycobacterium tuberculosis. Antimicrobial Agents Chemotherapy*, *47*, 1241–1250.

Rao, V., Gao, F., Chen, B., Jacobs, W. R, Glickman, M. S. (2006). Trans-cyclopropanation of mycolic acids on trehalose dimycolate suppresses *Mycobacterium tuberculosis*-induced inflammation and virulence. *The Journal of Clinical Investigation, 116(6),* 1660-1667.

Rath, P., Saurel, O., Czaplicki, G., Tropis, M., Daffé, M., Ghazi, A., Milon, A. (2013). Cord factor (trehalose 6,6′-dimycolate) forms fully stable and non-permeable lipid bilayers required for a functional outer membrane. *Biochimica et Biophysica Acta - Biomembranes*, *1828*(9), 2173–2181.

Rezácová, P., Borek, D., Moy, S. F., Joachimiak, A., & Otwinowski, Z. (2008). Crystal structure and putative function of small Toprim domain-containing protein from *Bacillus stearothermophilus*. *Proteins*, *70*(2), 311–319.

Rezwan, M., Lanéelle, M.-A., Sander, P., & Daffé, M. (2007). Breaking down the wall: Fractionation of mycobacteria. *Journal of Microbiological Methods*, *68*(1), 32–39.

Roberts, D. M., Personne, Y., Ollinger, J., & Parish, T. (2013). Proteases in *Mycobacterium tuberculosis* pathogens: potential as drug targets. *Future Microbiology, 8(5),* 621–631.

Rodríguez, D. M., Ocampo, M., Curtidor, H., Vanegas, M., Patarroyo, M. E., & Patarroyo, M. A. (2012). *Mycobacterium tuberculosis* surface protein Rv0227c contains high activity binding peptides which inhibit cell invasion. *Peptides*, *38*(2), 208–216.

Rodriguez-Campos, S., Smith, N. H., Boniotti, M. B., & Aranaz, A. (2014). Overview and phylogeny of *Mycobacterium tuberculosis* complex organisms: Implications for diagnostics and legislation of bovine tuberculosis. *Research in Veterinary Science*, *97*, S5–S19.

Rosenberg, M., Gutnick, D., & Rosenberg, E. (1980). No Title, *9*, 29–33.

Rozwarski, D. A., Vilchèze, C., Sugantino, M., Bittman, R., & Sacchettini, J. C. (1999). Crystal structure of the *Mycobacterium tuberculosis* enoyl-ACP reductase, InhA, in complex with NAD+ and a C16 fatty acyl substrate. *Journal of Biological Chemistry*, *274*(22), 15582–15589.

Sacco, E., Covarrubias, A. S., O'Hare, H. M., Carroll, P., Eynard, N., Jones, T. A., Quémard, A. (2007). The missing piece of the type II fatty acid synthase system from *Mycobacterium tuberculosis*. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(37), 14628–33.

Sandhu, P., & Akhter, Y. (2015). The internal gene duplication and interrupted coding sequences in the MmpL genes of Mycobacterium tuberculosis: Towards understanding the multidrug transport in an evolutionary perspective. *International Journal of Medical Microbiology*, *305*(3), 413–423.

Sassetti, C. M., Boyd, D. H., & Rubin, E. J. (2001). Comprehensive identification of conditionally essential genes in mycobacteria. *PNAS, 98(22),* 12712-12717.

Sassetti, C. M., Boyd, D. H., & Rubin, E. J. (2003). Genes required for mycobacterial growth defined by high density mutagenesis. *Mol Microbiol*, *48*(1), 77—84.

Schnappinger, D., & Ehrt, S. (2014). Regulated Expression Systems for Mycobacteria and Their Applications. *Microbiology Spectrum, 2(1),* 1–13.

Scholz, O., Henßler, E. M., Bail, J., Schubert, P., Bogdanska-Urbaniak, J., Sopp, S., Hillen, W. (2004). Activity reversal of Tet repressor caused by single amino acid exchanges. *Molecular Microbiology*, *53*(3), 777–789.

Schroeder, E. K., de Souza, N., Santos, D. S., Blanchard, J. S., & Basso, L. a. (2002). Drugs that inhibit mycolic acid biosynthesis in *Mycobacterium tuberculosis*. *Current Pharmaceutical Biotechnology*, *3*(3), 197–225.

Schreuder, L. J., Carroll, P., Muwanguzi-Karugaba, J., Kokoczka, R., Brown, A. C., & Parish, T. (2015). *Mycobacterium tuberculosis* H37Rv has a single nucleotide polymorphism in PhoR which affects cell wall hydrophobicity and gene expression. *Microbiology (Reading, England)*, *161*(2015), 765–773.

Seeliger, J. C., Topp, S., Sogi, K. M., Previti, M. L., Gallivan, J. P., & Carolyn, R. (2012). A Riboswitch-Based Inducible Gene Expression System for Mycobacteria. *PLoS One*, *7(1*), 3–8.

Segal, W., & Bloch, H. (1955). Differentiation grown *in vivo* and *in vitro*. *Journal of Bacteriology, 72(2)*, 132–141.

Shah, B. S., Tetu, S. G., Harrop, S. J., Paulsen, I. T., & Mabbutt, B. C. (2014). Structure of a short-chain dehydrogenase/reductase (SDR) within a genomic island from a clinical strain of *Acinetobacter baumannii*. *Acta Crystallographica Section F:Structural Biology Communications*, *70*, 1318–1323.

Shi, J., Blundell, T. L., & Mizuguchi, K. (2001). FUGUE: sequence-structure homology recognition using environment-specific substitution tables and structure-dependent gap penalties. *Journal of Molecular Biology*, *310*(1), 243–257.

Shi, W., Zhang, X., Jiang, X., Yuan, H., Lee, J. S., Barry, C. E., et al. (2011). Pyrazinamide inhibits trans-translation in *Mycobacterium tuberculosis*. *Science, 333*, 1630–1632.

Singh, K., Kumar, M., Pavadai, E., Naran, K., Warner, D. F., Ruminski, P. G., & Chibale, K. (2014). Synthesis of new verapamil analogues and their evaluation in combination with rifampicin against *Mycobacterium tuberculosis* and molecular docking studies in the binding site of efflux protein Rv1258c. *Bioorganic & Medicinal Chemistry Letters*, *24*(14), 2985–2990.

Slayden, R. a., & Barry, C. E. (2002). The role of KasA and KasB in the biosynthesis of meromycolic acids and isoniazid resistance in *Mycobacterium tuberculosis*. *Tuberculosis*, *82*(4–5), 149–160.

Smith NH, Gordon SV, de la Rua-Domenech R, Clifton-Hadley RS, Hewinson RG. (2006). Bottlenecks and broomsticks: the molecular evolution of *Mycobacterium bovis*. *Nature Rev Microbiol , 4,* 670-681.

Smith, I. (2003). *Mycobacterium tuberculosis* pathogenesis and molecular determinants of virulence. *Clinical Microbiology Reviews*, *16*(3), 463–96.

Snapper, S. B., Lugosi, L., Jekkelt, A., Meltont, R. E., Kiesert, T., Bloom, B. R., & Jacobs, W. R. (1988). Lysogeny and transformation in mycobacteria : Stable expression of foreign genes. *PNAS, 85(18),* 6987–6991.

Snapper, S. B., Melton, R. E., Mustafa, S., Kieser, T., & Jr, W. R. J. (1990). Isolation and characterization of efficient plasmid transformation mutants of *Mycobacterium smegmatis. Molecular Microbiology*, *4*(11), 1911–1919.

Somoskovi, A., Parsons, L. M., and Salfinger, M. (2001). The molecular basis of resistance to isoniazid, rifampin, and pyrazinamide in *Mycobacterium tuberculosis*. *Respiratory Research, 2(3),* 164-168.

Stover, C. K., Warrener, P., VanDevanter, D. R., Sherman, D. R., Arain, T. M., Langhorne, M. H., et al. (2000). A small-molecule nitroimidazopyran drug candidate for the treatment of tuberculosis. *Nature, 405,* 962–966.

Tahlan, K., Wilson, R., Kastrinsky, D. B., Arora, K., Nair, V., Fischer, E., Boshoff, H. I. (2012). SQ109 targets MmpL3, a membrane transporter of trehalose monomycolate involved in mycolic acid donation to the cell wall core of *Mycobacterium tuberculosis*. *Antimicrobial Agents and Chemotherapy*, *56*(4), 1797–1809.

Takase, R., Mikami, B., Kawai, S., Murata, K., & Hashimoto, W. (2014). Structure-based conversion of the coenzyme requirement of a short-chain dehydrogenase/reductase involved in bacterial alginate metabolism. *Journal of Biological Chemistry*, *289(48)*, 333198-33214.

Takayama, K., Wang, C., & Besra, G. S. (2005). Pathway to Synthesis and Processing of Mycolic Acids in *Mycobacterium tuberculosis* GENETIC ANALYSIS OF SYNTHESIS AND PROCESSING OF MYCOLIC ACID. *Society*, *18*(1), 81–101.

Tran, V., Liu, J. U. N., & Behr, M. A. (2014). BCG Vaccines. *Microbiol. Spectrum*, *2*(1), 1–11.

Ventura M, Canchaya C, Tauch A, Chandra G, Fitzgerald GF, Chater KF, van Sinderen D. (2007). Genomics of *Actinobacteria*: tracing the evolutionary history of an ancient phylum. *Microbial Mol Biol Rev, 71(3),* 495-548.

Verma, J. S., Gupta, Y., Nair, D., Manzoor, N., Rautela, R. S., Rai, A., et al. (2014). Evaluation of gidB alterations responsible for streptomycin resistance in *Mycobacterium tuberculosis*. *Journal of Antimicrobial Chemotherapy 69,* 2935–2941.

Verschoor, J. A., Baird, M. S., & Grooten, J. (2012). Progress in Lipid Research towards understanding the functional diversity of cell wall mycolic acids of *Mycobacterium tuberculosis*. *Progress in Lipid Research*, *51*(4), 325–339.

Veyrier, F. J., Dufort, A., & Behr, M. A. (2011). The rise and fall of the *Mycobacterium tuberculosis* genome. *Trends in Microbiology*, *19*(4), 156–161.

Veyron-Churlet, R., Zanella-Cléon, I., Cohen-Gonsaud, M., Molle, V., & Kremer, L. (2010). Phosphorylation of the *Mycobacterium tuberculosis* β-ketoacyl-acyl carrier protein reductase MabA regulates mycolic acid biosynthesis. *Journal of Biological Chemistry*, *285(17)*, 12714–12725.

Veyron-Churlet, R., Brust, B., Kremer, L., & Blanc-Potard, A.-B. (2011). Expression of OmpATb is dependent on small membrane proteins in *Mycobacterium bovis* BCG. *Tuberculosis*, *91*(6), 544–548.

Vilchèze, C., Morbidoni, H. R., Weisbrod, T. R., Iwamoto, H., Kuo, M., Sacchettini, J. C., & Jacobs, W. R. (2000). Inactivation of the inhA-encoded fatty acid synthase II (FASII) enoyl- acyl carrier protein reductase induces accumulation of the FASI end products and cell lysis of *Mycobacterium smegmatis*. *Journal of Bacteriology*, *182*(14), 4059–4067.

Vilchèze, C., & Jr, W. R. J. (2014). Resistance to Isoniazid and Ethionamide in *Mycobacterium tuberculosis* : Genes , Mutations , and Causalities. *Microbiol Spectrum*, *2*(4, NaN-0014–2013), 1–21.

Watanabe, M., Aoyagi, Y., Ridell, M., & Minnikin, D. E. (2001). Separation and characterization of individual mycolic acids in representative mycobacteria. *Microbiology*, *147(pt7)*, 1825-1837.

Watanabe, M., Aoyagi, Y., Mitome, H., Fujita, T., Naoki, H., Ridell, M., & Minnikin, D. E. (2002). Location of functional groups in mycobacterial meromycolate chains; the recognition of new structural principles in mycolic acids. *Microbiology*, *148(pt6)*, 1881-1902.

Wick, L. Y., Wattiau, P., & Harms, H. (2002). Influence of the growth substrate on the mycolic acid profiles of mycobacteria. *Environmental Microbiology*, *4*(10), 612–616.

Woods, G. L., & Washington Ii, J. A. (1987). Mycobacteria Other than *Mycobacterium tuberculosis*: review of Microbiologic and Clinical Aspects. *Reviews of Infectious Diseases, 9(2),* 275-294.

Wu, Q., Zhou, P., Qian, S., Qin, X., Fan, Z., Fu, Q., Pei, H. (2011). Cloning, expression, identification and bioinformatics analysis of Rv3265c gene from *Mycobacterium tuberculosis* in *Escherichia coli*. *Asian Pacific Journal of Tropical Medicine*, *4*(4), 266–270.

Yamaryo-botte, Y., Rainczuk, A. K., Lea-smith, D. J., Brammananth, R., Peet, P. L. Van Der, Meikle, P., Mcconville, M. J. (2015). Acetylation of Trehalose Mycolates Is Required for E ffi cient MmpL- Mediated Membrane Transport in Corynebacterineae. *ACS Chemical Biology*, *10*, 734–746.

Yuan, Y., Leet, R. E., Besra, G. S., Belislet, J. T., & Barry Iii, C. E. (1995). Identification of a gene involved in the biosynthesis of cyclopropanated mycolic acids in *Mycobacterium tuberculosis*. *Microbiology*, *92*, 6630–6634.

Zaunbrecher, M. A., Sikes, R. D., Metchock, B., Shinnick, T. M., and Posey, J. E. (2009). Overexpression of the chromosomally encoded aminoglycoside acetyltransferase eis confers kanamycin resistance in *Mycobacterium tuberculosis. PNAS, 106*, 20004–20009.

Zhang, M., Yue, J., Yang, Y. P., Zhang, H. M., Lei. J. Q., Jin, R. L., et al. (2005). Detection of mutations associated with isoniazid resistance in *Mycobacterium tuberculosis* isolates from China. *Journal of Clinical Microbiology*, *43*, 5477–5482.

Zhang, S., Chen, J., Shi, W., Liu, W., Zhang, W., and Zhang, Y. (2013). Mutations in panD encoding aspartate decarboxylase are associated with pyrazinamide resistance in *Mycobacterium tuberculosis*. *Emerging Microbes Infections, 2(6),* 1-5.

Zhang, Y., Shi, W., Zhang, W., and Mitchison, D. (2014). Mechanisms of pyrazinamide action and resistance. *Microbiology Spectrum, 2(4)*, 1-12.

Zimhony, O., Schwarz, A., Raitses-Gurevich, M., Peleg, Y., Dym, O., Albeck, S., Shakked, Z. (2015). AcpM, the Meromycolate Extension Acyl Carrier Protein of *Mycobacterium tuberculosis* is activated by the 4′-Phosphopantetheinyl Transferase PptT, a Potential Target of the Multistep Mycolic Acid Biosynthesis. *Biochemistry, 54(14)*, 2360-2371.

Zor, T., & Selinger, Z. (1996). Linearization of the {Bradford} {Protein} {Assay} {Increases} {Its} {Sensitivity}: {Theoretical} and {Experimental} {Studies}. *Analytical Biochemistry*, *236*(2), 302–308.

Zumla, A. I., Gillespie, S. H., Hoelscher, M., Philips, P. P. J., Cole, S. T., Abubakar, I., Nunn, A. J. (2014). New antituberculosis drugs, regimens, and adjunct therapies: needs, advances, and future prospects. *The Lancet Infectious Diseases, 14*(4), 327–340.