Mycobacterial Glycolipids – Pathways to Synthesis and Role in Virulence

by

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Abstract

Mycobacterial diseases are responsible for numerous deaths worldwide, the major pathogens being *Mycobacterium tuberculosis* and *Mycobacterium leprae.* Also, in recent years threats from opportunistic pathogens, such as *Mycobacterium marinum* and *Mycobacterium kansasii* have been on the rise. These mycobacteria possess a unique lipid-rich cell wall with an array of mycolic acids and species-specific antigenic glycolipids, like the lipooligosaccharides. Some of these solvent extractable lipids possess immunomodulatory properties and play an important role during infection.

Lipooligosaccharides (LOS) are surface exposed, polar, antigenic glycolipids that are present in several mycobacterial species. This study used the opportunistic human pathogens *M. marinum* and *M. kansasii* as a model system to unravel the genes involved in the biosynthesis of LOSs in *Mycobacterium.* Using directed mutagenesis and transposon mutagenesis, mutant strains defective in various parts of the LOS biosynthetic pathway were isolated. Analysis of these strains helped in further delineating the pathway and understanding the role of LOSs in virulence.

A part of this thesis focussed on studying mycolic acid processing and transport using *Mycobacterium smegmatis* as a surrogate system. Mycolic acids are the most distinctive components of the mycobacterial cell wall. While their biosynthesis has been studied in detail, processing and transport across the membrane is not well understood. This study attempted to explore the roles of the two putative type II mycolyltransferases *MSMEG3437* and *MSMEG5851* in mycolic acid processing. Additionally, the role of *M. tuberculosis mmpL11* gene was probed as the *Mtb-mmpL11* had been reported to be involved in virulence. A null

mutant of the *M. smegmatis* homologue, *Ms-mmpL11* (*MSMEG0241*) was generated and analysed for the above study. Deletion mutant strains of the two putative mycolyltransferase II did not show any phenotype, suggesting that their roles are redundant *in vivo*. Although the *Ms-mmpL* gene was found to be non-essential, it was found to be involved in transport of free mycolic acids.

Declaration

The work presented in this thesis was carried out in the School of Biosciences at the University of Birmingham, U.K., B15 2TT during the period November 2008 to November 2011. 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.

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This thesis is dedicated to my Family

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List of Abbreviations

2D-TLC	2-dimensional thin layer chromatography
aa	amino acid
ACP	acyl carrier protein
AG	arabinogalactan
Ag85	antigen 85 complex
AIDS	acquired immuno-deficiency syndrome
Ara*f*	arabinofuranosyl
AraLAM	uncapped LAM
AT	acyl transferase
BC	before Christ
BCG	Bacille Calmette-Guérin
bp	base pair
CFU	colony forming units
CPM	counts per minute
DAG	diacylglycerol
DAT	diacyl trehalose
DCM	dichloromethane
DEAE	diethylaminoethyl
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DOTS	directly observed treatment short course

DPG	dolichol phosphor-glucose
DPM	dolichol phospho-mannose
EMB	ethambutol
ER	enoyl reductase
ES-MS	electrospray-mass spectrometry
ETH	ethionamide
FAME	fatty acid methyl ester
FAS	fatty acid synthase
g	grams
G+C	guanosine and cytosine
Gal*p*	galactopyranose
GlcNAc	N-acetylglucosamine
GPL	glycopeptidolipids
HIV	human immuno-deficiency virus
hr	hour
Hyg	hygromycin
IL	interleukin
INF-γ	interferon gamma
INH	isoniazid
Ins	inositol
Kan	kanamycin
KR	ketoreductase

KS	ketosynthase
L	litre
LAM	lipoarabinomannan
LB	Luria-Bertani
LM	lipomannan
LOS	lipooligosaccharide
mAGP	mycolyl-arabinogalactan-peptidoglycan complex
MAME	mycolic acid methyl-ester
MALDI-TOF	Matrix assisted laser desorption ionisation-time of flight
ManLAM	LAM with mannose caps
MDR	multi-drug resistant
Me	methyl
mg	milligram
min	minutes
MIC	minimum inhibitory concentration
ml	millilitre
MPI	manosyl-phosphatidyl-*myo*-inositol
MS	mass spectrometry
NMR	nuclear magnetic resonance
OD	optical density
PAT	penta-acyl trehalose
PCR	polymerase chain reaction
PDIM	phthiocerol dimycoserosate

PG	peptidoglycan
PGL	phenolic glycolipids
p-HBAD	para-hydroxybenzoic acid derivatives
PI	phosphatidyl-*myo*-inositol
PILAM	LAM with phosphoinositide caps
PIM	phosphatidyl-*myo*-inositol mannoside
PPB	phosphopantetheine binding domain
PZA	pyrazinamide
rcf (g)	relative centrifugal force
Rha*p*	rhamnopyranose
RIF	rifampicin
SL	sulfolipids
STR	streptomycin
TAE	tris-acetate EDTA
TAG	triacyl glycerol
TAT	triacyl trehalose
TB	tuberculosis
TBAH	tetrabutyl ammonium hydroxide
TDM	trehalose dimycolate
TLC	thin layer chromatography
TMM	trehalose monomycolate
TNF-α	tumor necrosis factor-α

TSB	tryptic soy broth
v/v	volume per volume
w/v	weight per volume
WHO	world health organization
Xyl*p*	xylopyranose
XDR-TB	extensive drug-resistant tuberculosis
μCi	microcurie
μg	microgram
μL	microlitre

Published work associated with this thesis

D. Sarkar, M. Sidhu, A. Singh , J. Chen, D. A. Lammas, A. M. van der Sar, G. S. Besra, A. Bhatt. Identification of a glycosyltransferase from *Mycobacterium marinum* involved in addition of a caryophyllose moiety in lipooligosaccharides. Journal of Bacteriology. 2011 May; 193(9):2336-40.Mar 4.

Communicated

A. D. van der Woude A.D., D. Sarkar, A. Bhatt, M. Sparrius, L. Boon, J. Geurtsen, A. M. van der Sar, J. Luirink, E. N. G. Hoban, G. S. Besra and W. Bitter. An unexpected link between lipooligosaccharide biosynthesis and PE_PGRS protein secretion in *Mycobacterium marinum* (Submitted to JBC, under review).

1

General Introduction

1.1 *Mycobacterium* species

Mycobacteria are unicellular, aerobic, non-spore forming bacteria with high G+C content in their DNA. They appear as slightly curved rod shaped cells, 0.2 – 0.6μm in width and 1.0 – 2.0μm in length (Alteri et al., 2007; Holt, 1994). Mycobacteria are a genus of *Actinobacteria*. They are unusual among bacteria in that they have a thick, hydrophobic, lipid rich cell wall. A characteristic feature of mycobacteria is the presence of a wide diversity of unique complex lipids that constitute 60% of the cell wall. This lipid-rich, low permeability matrix contributes to the difficulty in combating mycobacterial diseases by endowing the organism with innate resistance to therapeutic agents and host defences.

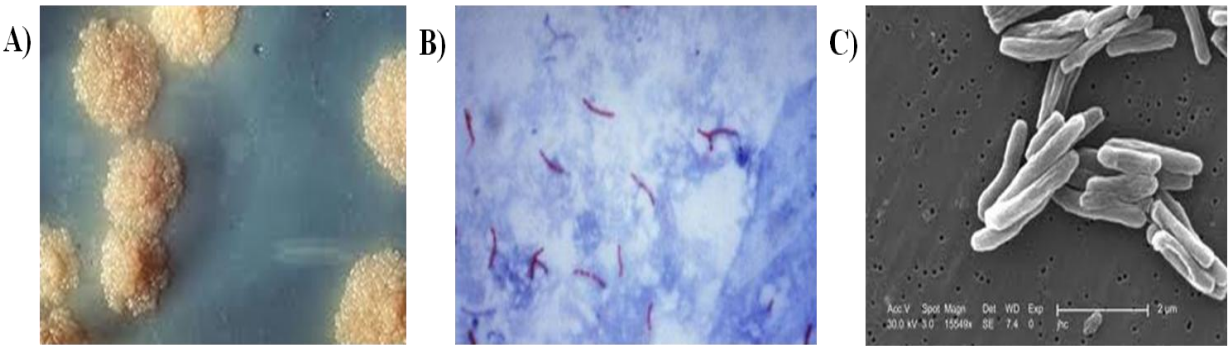

Figure 1.1. A) Colonies of *M. tuberculosis* on Löwenstein-Jensen agar. B) Acid-fast staining of *M. tuberculosis,* C) Scanning electron microscopy of *M. tuberculosis* bacilli, scale bar represents 2μm, Mag 15549X; (adapted from CDC photo publications).

The cell wall core is composed of the mycolyl arabinogalactan – peptidoglycan (mAGP) complex (Besra et al., 1995, Brennan, 2003, McNeil et al., 1990, Dover et al., 2004). Other glycolipids, polysaccharides, lipoglycans intercalate with the mycolic acid layer to form the outer region of the cell wall (Brennan and Nikaido, 1995, Brennan and Crick, 2007). The cell

wall makes a substantial contribution to the persistence of this genus (McNeil and Brennan, 1991, Minnikin et al., 2002). Hence, the biosynthetic pathways of cell wall components are potential targets for new drugs for tuberculosis.

Most species of the genus *Mycobacterium* are closely related to soil bacteria, but some are pathogenic. These include *Mycobacterium tuberculosis* and *Mycobacterium leprae*, the causative agents of tuberculosis and leprosy, respectively. Some are opportunistic pathogens, like *Mycobacterium kansasii*, which causes a disseminated disease in immunocompromised humans, and *Mycobacterium marinum;* a fish pathogen also causes skin infection in immunocompromised humans. Based on their generation time mycobacteria are classified as fast growers and slow growers. *M. tuberculosis* is a slow grower, has a generation time of 16 - 18 hours, while *Mycobacterium smegmatis* is a fast grower, and has a generation time of 3 to 4 hours.

Table 1.1. *Mycobacterium* species, fast and slow growers.

Slow Growing Mycobacteria		**Fast Growing Mycobacteria**	
Mycobacterium tuberculosis	*Mycobacterium leprae*	*Mycobacterium aurum*	*Mycobacterium phlei*
Mycobacterium kansasii	*Mycobacterium africanum*	*Mycobacterium chelonae*	*Mycobacterium porcinum*
Mycobacterium avium	*Mycobacterium marinum*	*Mycobacterium duvalii*	*Mycobacterium pulveris*
Mycobacterium bovis	*Mycobacterium microti*	*Mycobacterium flavescens*	*Mycobacterium rhodesiae*
Mycobacterium malmoense	*Mycobacterium paratubercuosis*	*Mycobacterium fortuitum*	*Mycobacterium shinshuense*
Mycobacterium ulcerans	*Mycobacterium scrofulaceum*	*Mycobacterium gadium*	*Mycobacterium smegmatis*
Mycobacterium gastri	*Mycobacterium xenopi*	*Mycobacterium komossense*	*Mycobacterium vaccae*
Mycobacterium genavense	*Mycobacterium simiae*		
Mycobacterium haemophilum	*Mycobacterium szulgai*		
Mycobacterium intracellulare	*Mycobacterium farcinogenes*		

1.2 Tuberculosis

Diseases caused by mycobacteria claim millions of lives globally, with *M. tuberculosis*, the causative agent of TB being responsible for more deaths each year than any other single pathogen.

TB is an ancient disease and has afflicted mankind for thousands of years. Molecular analysis of human bone samples dating back to 600 BC, support the existence of TB in human populations from those days (Donoghue, 2009). DNA from the members of the *M. tuberculosis* complex has been detected in Egyptian and Peruvian mummies dating back to 5000 BC (Crubezy et al., 1998, Salo et al., 1994). Recent findings show *M. tuberculosis* infection using PCR and lipid biomarkers in a 9000-year-old mummy from the pre-pottery Neolithic settlement period of the Middle East (Hershkovitz et al., 2008).

Ancient literature has mentioned TB by different names. Hippocrates (400 BC) used the term 'Phthisis' to describe the condition associated with fever, cough and loss of appetite. The disease was also known as 'Consumption', 'Kings's evil', 'Scrofula', 'Pott's disease' and 'White Plague'.

The symptoms of TB are chest pain, fever, persistent coughing associated with phlegm, fatigue and loss of appetite and weight. TB was regarded as a deadly and contagious disease; the etiological agent of the disease could not be identified for many years. There was a significant level of understanding about the disease and different measures were adopted to prevent and contain infections. The first major step towards deciphering tuberculosis was the discovery of the tubercle bacilli, the bacteria causing TB, by the German bacteriologist Robert

Koch in 1882 (Koch., 1882). The introduction of 'Sanatoria' in the 19th century was major step towards TB prevention.

1.3 Epidemiology

Tuberculosis remains a major public health concern despite the availability of effective chemotherapy. The World Health Organization (WHO) estimates that one–third of the world population is infected with *M. tuberculosis* which causes nearly 9.4 million new cases and kills 2 million people each year (WHO, 2011) This is largely a result of the persistence of the causative organism itself and also the treatment of a *M. tuberculosis* infection requires many months of therapy with multiple drugs.

The epidemiology of TB varies substantially around the world. The highest are observed in sub-Saharan Africa, India, China, and the islands of Southeast Asia and Micronesia. Intermediate rates of tuberculosis occur in Central and South America, Eastern Europe, and northern Africa, while low rates occur in the United States, Western Europe, Canada, Japan, and Australia. From an epidemiological viewpoint, the vast majority of cases at present occur in the developing world. This poses a serious problem with regard to affordability of antibiotics and logistics involved in drug dispensing.

Human9 Immunodeficiency Virus (HIV) infection remains a great influence in the rising TB trends. This has added to the current epidemic since the probability of TB infection is greatly increased in immunocompromised people. HIV co-infection raised the risk of developing active TB in a population with latent TB, from 5-10% during their lifetime, to the same risk in one year (Cole et al., 1998; Corbett et al., 2003). Hence, the synergy between HIV-1 and TB has lead to increased mortality due to both the diseases.

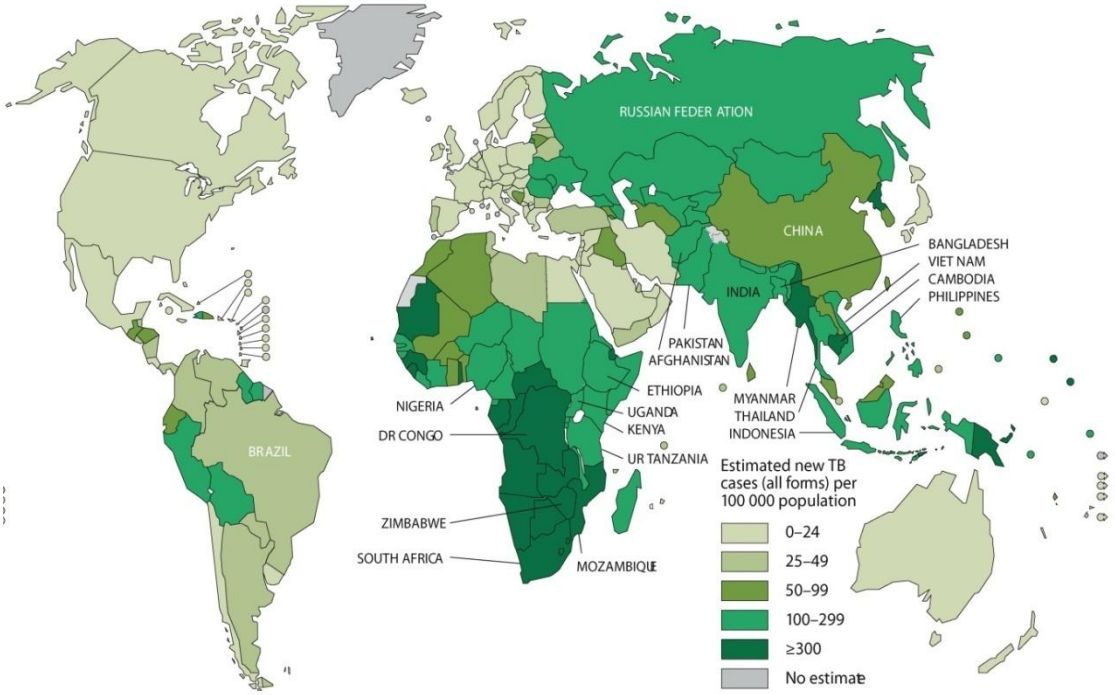

Figure 1.2. Map showing estimated TB incidence rates, by country, 2010. Adapted from WHO TB report 2011.

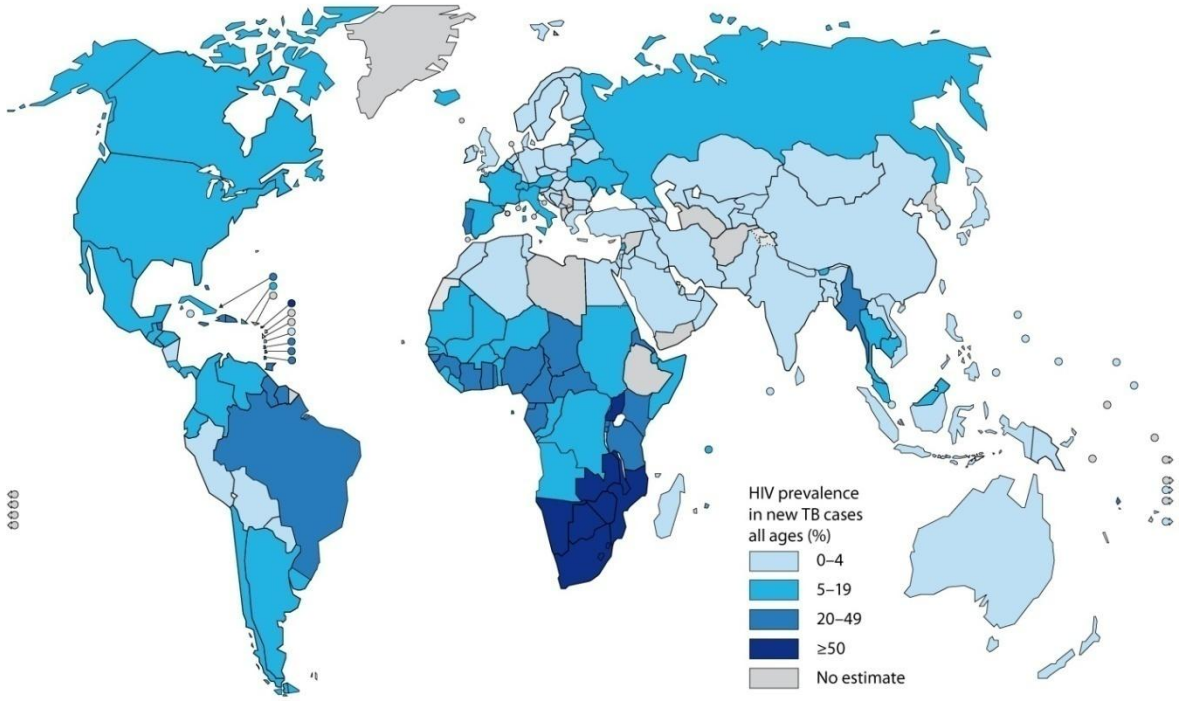

Figure 1.3. Map showing estimated HIV prevalence in new TB cases, by country, 2010 (WHO TB report 2011).

1.4 Immune response to *Mycobacterium tuberculosis* infection

M. tuberculosis infection is transmitted by the airborne route. Infection is initially and primarily of the lungs, alveolar macrophages, infection also occurs in the brain, knee, lymph nodes, other organs and bones. The bacterium is transmitted by the inhalation of aerosol droplets expelled in the cough or sneeze of an infected person (Cole, 2005). Post inhalation of these aerosol droplets containing the bacilli, there are various fates of the inhaled bacteria. The bacilli maybe cleared off by a strong host immune system resulting in no infection establishment. The bacteria can also travel through the narrow passages to the alveoli.

Alveolar macrophages act as the main defence against respiratory pathogens. The tubercle bacilli that reach the alveoli make contact with the macrophages and are ingested via various cell surface receptors (Ernst et al., 1998). A localised pro-inflammatory response is induced by the macrophages and mononuclear cells are recruited from proximal blood vessels to form a granuloma. Granuloma formation is characteristic of *M. tuberculosis* infection. The primary function of the granuloma is to contain and prevent the dissemination of mycobacteria (Russell et al., 1997; Russell, 2007). The granuloma formed can be divided into three layers – a core with the infected macrophages which is surrounded by foamy macrophages. The external layer comprises of lymphocytes which are encircled by extracellular matrix components (Russell, 2007). Infected individuals with granuloma are non-infective. But any changes to their immunity may cause failure of the containment which leads to the rupture of the granuloma and release of infectious bacilli.

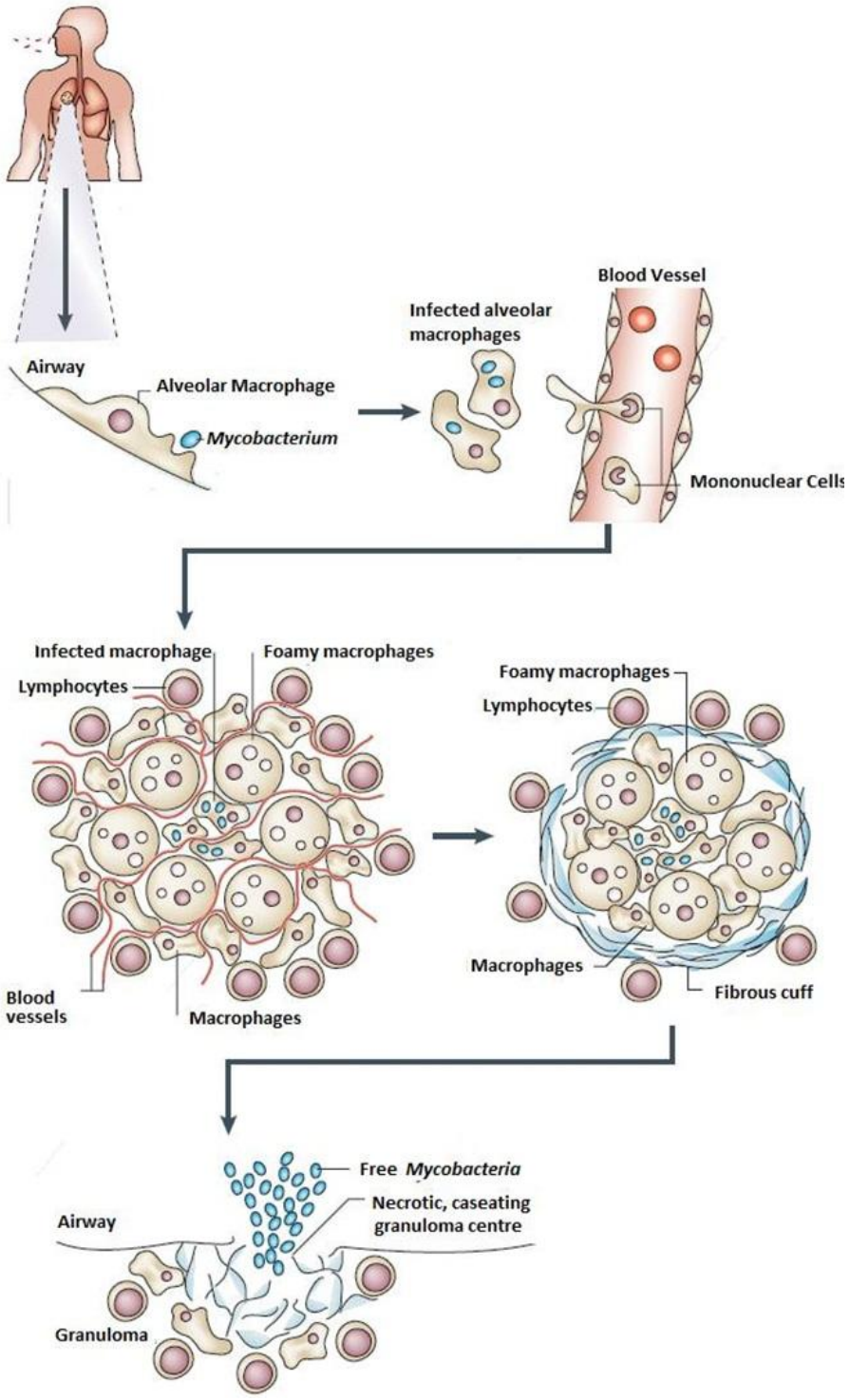

Figure 1.4. Schematic representation of granuloma formation in humans. Adapted from Russell, (2007).

During TB infection, the initial response to *M. tuberculosis* is the binding of the bacilli via the complement receptors (CR1, CR3, and CR4) and mannose receptors of the alveolar macrophages. A glycoprotein Surfactant protein-A on alveolar macrophage surface enhances the binding and uptake of bacteria by up-regulating mannose receptor expression (Beharka et al., 2002). This is followed by phagocytosis of the bacteria. The host cell now commences killing of the phagocytosed bacteria by using reactive nitrogen intermediates or nitrogen oxide (Rich et al., 1997), maturation and fusion of the phagosome to form the phagolysosome,apoptosis, cytokine production (interferon-γ) and chemokine production (interleukins IL-8, IL-10 and tumor necrosis factor-α) (Figure. 1.4). However, it has been shown that *M. tuberculosis* is able to escape these mechanisms by various pathways. *M. tuberculosis* is known to arrest phagosome maturation (Fratti et al., 2003; Vergne et al., 2003) and prevents the phagosome-lysosome fusion by lowering the pH, and the phagocytic vacuole remains at a pH of approximately 6.5 (Vergne et al., 2005). Apart from this, genes such as the *sodC*, a superoxide dismutase (Wu et al., 1998) and cell wall components, such as lipoarabinomannan (LAM) confer resistance to intracellular oxidative stress (Chan et al., 1991; Piddington et al., 2001). *M. tuberculosis* can also maintain its supply of nutrients for intracellular survival by intercepting the host endocytic system (Sturgill-Koszycki et al., 1994).

M. tuberculosis is engulfed by both macrophages and dendritic cells (DCs) and the later represents the main interface between innate and adaptive immunity (Hickman et al., 2002; Orme, 2004). DCs release cytokines that initiate Th1 like activity from T-cells. In order to accomplish this, the DCs have been reported to leave the site of infection and move towards

the lymph nodes, this also aids in distribution of the bacilli. Th1 response is extremely important as it contributes to containment and granuloma formation (Russell, 2007).

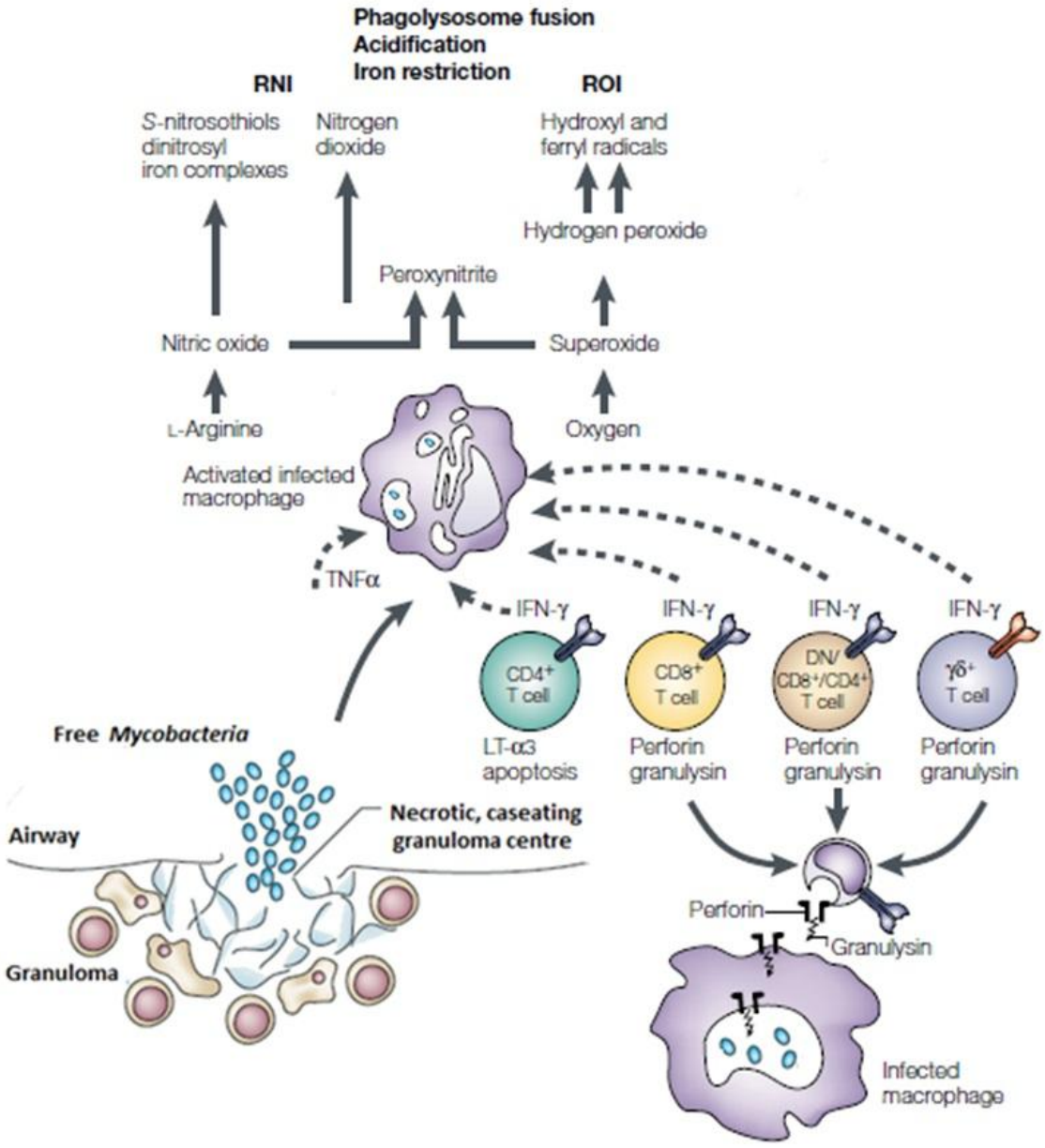

Figure 1.5 Primary immunological events during tuberculosis infection. Adapted from Kaufmann, (2001).

Early literature reports the involvement of a variety of mycobacterial cell-wall products in manipulating the immune response and inducing granuloma formation (Granger et al., 1976; Bekierkunst, 1968; Bekierkunst et al., 1969; White et al., 1964). It has been reported that the virulent lipoglycan LAM and phosphatidylinositolmannosides (PIMs) are released inside infected cells and are presented by CD1b (Zajonc et al., 2006). Death of infected cells releases vesicles containing lipid antigens. These are responsible for cross priming DCs and subsequent antigen presentation by CD1b.

1.5 Treatment

1.5.1 Anti –TB drugs

Initiation of TB chemotherapy was in the 1940s with the discovery of streptomycin (STR) (Jones et al., 1944; Schatz et al., 1944). STR was the main treatment against TB and development of resistance lowered its efficacy. The discovery of streptomycin was followed by the discovery of *para*-amino salicylic acid by Jorgen Lehman (Nagley, 1949; Nagley and Logg, 1949), isoniazid (INH) (Steenken and Wolinsky, 1952a; Steenken and Wolinsky, 1952b), pyrazinamide (PZA) (Mc et al., 1954; Muschenheim et al., 1954; Tompsett et al., 1954), cycloserine (Gold et al., 1955) , ethambutol (EMB) (Forbes et al., 1962) and rifampin (RMP) (Table 1.2). The discovery of INH, established a combined regimen for treatment of TB along with STR and PZA.

INH was the first anti-tuberculosis drug that exhibited high specificity for the tubercle bacilli with very low or no activity against other mycobacterial species and no activity against other bacteria. It was easy to synthesise compared to the other two drugs and considerably

lowered the cost of treatment in the 1950's. INH is a prodrug and is activated by catalase-peroxidase (*KatG*) gene (Khasnobis et al., 2002; Zhang et al., 1992) and inhibits mycolic acid biosynthesis by targeting InhA (enoyl-ACP-reductase) and KasA (β-ketoacylACP synthetase) (Banerjee et al., 1994). Today TB is treated using a combination of drugs mainly to avoid developing drug resistance during single drug usage. The standard treatment for active TB is usually divided into two phases: the initial phase aiming to kill actively growing and semi-dormant bacilli. This employs the use of first line anti-TB drugs; INH, EMB, PZA, and RMP for a period of two months. Streptomycin is generally not used in this phase because of higher toxicity of STR but is used if resistance to INH has been established. This is followed by the continuation phase to eliminate any remaining bacilli and minimise the risk of relapse of infection. INH and RIF are used for four months during this period.

Table 1.2. Commonly used drugs for tuberculosis treatment, adapted from (Zhang, 2005).

Drug	MIC(μg/μl)	Effect on bacterial cell	Target	Genes involved in resistance
Isoniazid	0.01 - 0.2	Bactericidal	Mycolic acid biosynthesis	*KatG*[a] */inhA/ndh*
Ethionamide	0.6 - 2.5	Bacteriostatic	Mycolic acid biosynthesis	*inhA / ethA*[a]
Rifampicin	0.05 - 2.5	Bactericidal	RNA synthesis	*rpoB*
Ethambutol	1.0 - 5.0	Bacteriostatic	Cell wall arabinan biosynthesis	*emb CAB*
Pyrazinamide	50 - 100	Bactericidal / Bacteriostatic	Disrupts membrane transport and energy depletion	*pncA*[a]

[a]katG, ethA and pncA activate the prodrugs INH, ETH and PZA respectively.

According to the WHO, anti-TB drugs are classified on their mode of action and side effects. The first line or essential drugs are INH, STR, PZA, EMB and RMP. The second line or reserved drugs show good efficacy but have relatively higher rates of side effects, toxicity

and unavailability in the developing nations. These are of six classes- aminoglycosides (e.g. kanamycin), polypeptides (e.g. capreomycin), fluoroquinolones (e.g. ciprofloxacin), thioamides (e.g., ethionamide), cycloserines and *p*-aminosalicylic acid). The third line drugs are those which are less effective compared to the above include rifabutin and macrolides (Janin, 2007).

Due to the adverse side effects of these drugs and long treatment times, non-compliance is often observed among patients. Relapse rates of 3-4% have still been reported with this treatment. Non-compliance to this treatment regime is one of the major causes of relapse and also leads to drug resistance. The emergence of multi-drug resistant (MDR) and recently extensively drug resistant (XDR) strains of *M. tuberculosis* pose a serious threat to the TB control programmes all over the world (Gandhi et al., 2006).

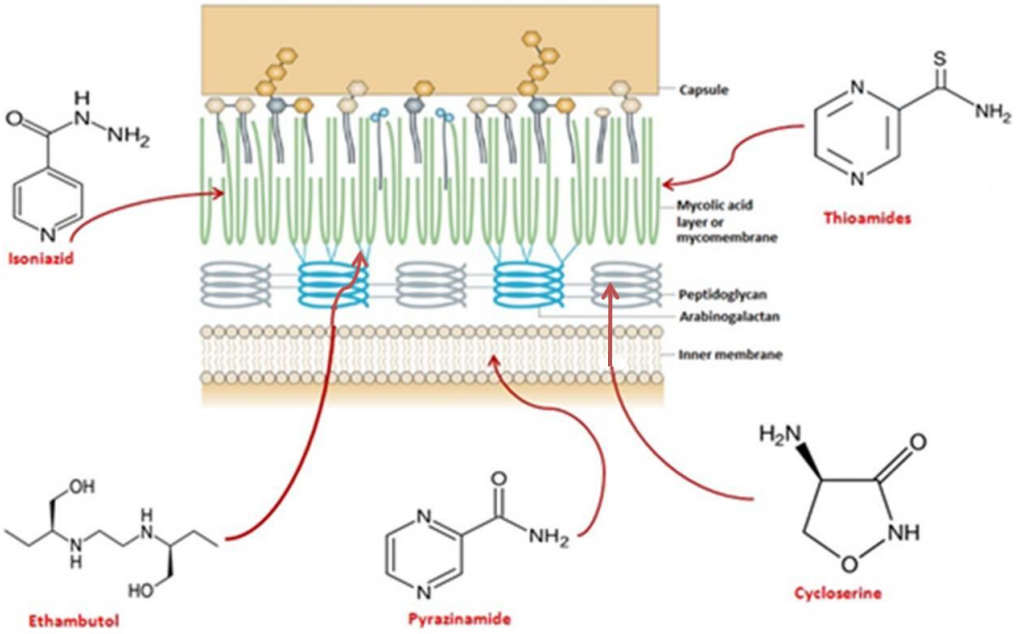

Figure 1.6. Anti-tuberculosis drugs and their target in the cell wall. Adapted from Abdallah et al. (2007).

The past 40 years have not witnessed the discovery of any anti-TB drugs. There is a necessity of development of more efficient drugs that have shorter treatment times when compared with the present treatment regimes to combat the worldwide threat of TB. The unique mycobacterial cell wall remains the most attractive target for chemotherapeutics development. High throughput screenings have identified few potential candidate drugs in recent years. SQ109 (Protopopova et al., 2005), TMC207 (Andries et al., 2005) and a class of benzothiaziones are the newly discovered anti-TB drugs, and SQ109 and TMC207 are in human clinical trials. SQ109 is an N-geranyl-N'-(2-adamantyl)-ethane-1,2-diamine based on the ethylenediamine core as found in EMB. SQ109 is reported to inhibit mycolate export by targeting *mmpl3* (Tahlan et al., 2012), which is involved in transporting trehalose monomycolate. It has been found to be most effective against MDR strains of TB. TMC207 belongs to the diarylquinoline class of compounds and is thought to inhibit the proton pump of the *M. tuberculosis* ATP-synthase (Koul et al., 2007). Its low MIC, long half-life (~173 h in humans) and potent bactericidal activity reduces the time of treatment. TMC207 does not affect any other bacteria apart from mycobacteria, effective against EMB, RIF, PZA, INH and STR resistant strains of TB, and can also be used in conjunction with anti-retroviral drugs and has no cross-resistance with the current anti-TB drugs (Matteelli et al., 2010, Andries et al., 2005). A novel and potent antimycobacterial class of drugs was discovered during the synthesis of sulfur containing antibacterial and antifungal compounds. Known as benzothiazionone (BTZ), these show high specificity for mycobacteria, have a low MIC (1-30ng/ml for members of the *M. tuberculosis* complex) and are comparatively non-toxic

(Makarov et al., 2006). BTZ acts on the decaprenylphosphoryl-β-**D**-ribose 2-epimerase, and blocks production of DPA, a precursor of arabinan biosynthesis (Makarov et al., 2009).

1.5.2 Directly Observed Therapy, Short-course

DOTS stand for "Directly Observed Therapy, Short-course" and is a major strategy of the World Health Organization global TB eradication programme. The number of deaths due to tuberculosis remains alarming inspite of the introduction of DOTS by World Health Organization (WHO) in 1991 which follows the patient from positive testing for the disease through proper medication and monitored outcome (Obermeyer et al., 2008). The WHO also initiated a "Stop TB" campaign in 2006 to increase the uptake of DOTS as well as promoting awareness of the rising MDR and XDR TB cases. Despite being a called short course, DOTS chemotherapy requires a strict therapeutic regimen involving a cocktail of drugs administered over a minimum of six months. The current DOTS treatment involves taking INH, EMB, RIF, PZA and (in some cases) STR daily for the first two months, followed by INH and RIF thrice weekly for the ensuing four months (Cox et al., 2006).

The DOTS strategy focuses on five main points of action. These include government commitment to control TB, diagnosis based on sputum-smear microscopy tests done on patients who actively report TB symptoms, direct observation short-course chemotherapy treatments, a definite supply of drugs, and standardised reporting and recording of cases and treatment outcomes. Treatment with properly implemented DOTS has a success rate exceeding 95% and prevents the emergence of further multi-drug resistant strains of tuberculosis.

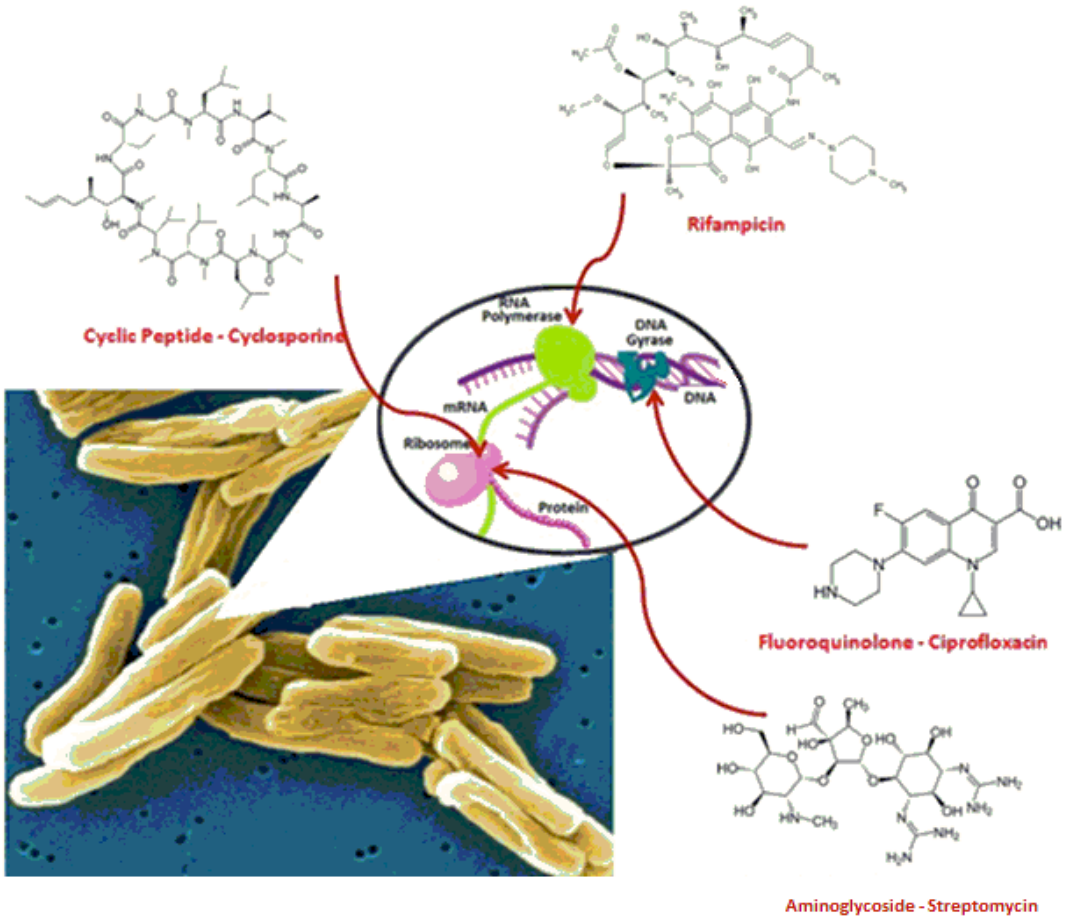

Figure 1.7. Anti-tuberculosis drugs targeting DNA metabolism. Adapted from CDC 2008 photo release and www.health-healths.com.

1.6 Drug resistance

The term ‘drug resistance’ most commonly refers to the resistance acquired by pathogens under selective pressure from chemotherapeutics. The TB bacilli have been reported to develop resistance at a much quicker rate than other human pathogens (Sassetti and Rubin, 2003).

1.6.1 Multi drug resistant TB (MDR-TB)

Multi drug resistant TB (MDR-TB) is resistance picked up by *M. tuberculosis* to at least INH and RIF, the two most effective first line anti-TB drugs. MDR-TB results from either primary infection with resistant bacteria or is developed during the treatments phase. Incomplete treatment and non-compliance during therapy are the main reasons of developing MDR-TB. Drug resistance also arises due to the ability of the *M. tuberculosis* pathogen to mutate various drug targets. This usually happens in cases where the treatment time is prolonged (WHO, 2007; Yew and Leung, 2008). The World Health Organisation (WHO) reported about 440,000 cases of MDR-TB globally in 2008 and deaths caused due to MDR-TB were more than 110,000 every year. Recent MDR-TB cases reported globally ranges from 0 to 28.3%. Amongst new TB cases, 2.9% were MDR-TB, compared with 15.3% amongst previous treated cases ((WHO)) M/XDR TB global surveillance report 2010). Treatment of MDR-TB requires usage of the reserved and more toxic second-line anti-TB drugs. The DOTS-Plus program (an alternative to the DOTS strategy) is used for treatment of reported MDR-TB cases (Farmer et al 2000). The drugs used under this strategy are the most potent second-line reserve drugs – Aminoglycosides and Fluoroquinolones. The usual regimen includes at least 4 to 6 drugs selected on the basis of efficacy and susceptibility testing and treatment history.WHO recommends treatment duration of minimum 18 months post smear negativity, even for HIV-negative patients.

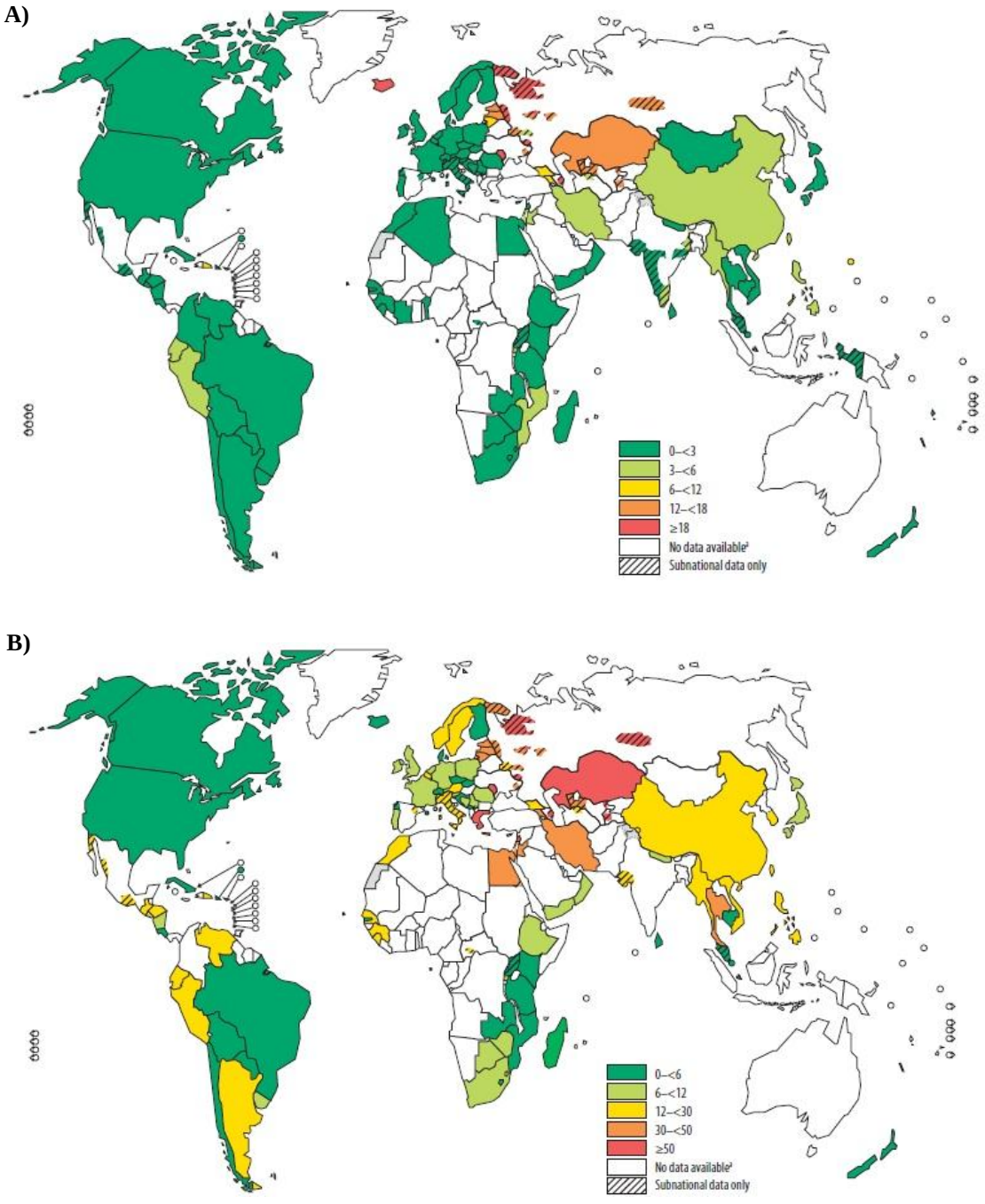

Figure 1.8. Global incidence of MDR TB. A) Map showing the distribution of proportion of MDR-TB among new TB cases, 1994–2009 B) Distribution of proportion of MDR-TB among previously treated TB cases, 1994–2009. Adapted from (WHO, 2010) Multidrug and extensively drug-resistant TB (M/XDR-TB): 2010 global report on surveillance and response.

1.6.2 Extensively drug resistant TB (XDR-TB)

The urgency for new drugs for TB was recently highlighted by the report of XDR-TB strains of TB. XDR strains of *M. tuberculosis* are resistant to INH, RIF and any fluoroquinolones along with resistance to ateast one of the three following injectable second line anti-TB drugs, for instance amikamycin, kanamycin or capreomycin (Raviglione, 2006).

XDR-TB has been reported throughout the world even in HIV-seronegative individuals. Incidence of cases generating from hospital infections have also been reported. The first XDR-TB strains were reportedly found between 1993 to 2004 in the USA, where 4% of the isolated MDR-TB strains during this period were XDR. Representative data from Europe show 19% MDR-TB cases identified in 2000 – 2004 were XDR strains. Asia reported 15% MDR strains as XDR in 2004.

In 53 cases identified in South Africa, between 2005 – 2006, 52 patients died within a median period of 16 days (from sputum smear collection to death)(Gandhi et al., 2006). The epidemic of XDR-TB amongst individuals in South Africa is a major challenge. Of the XDR cases reported, 34% tested HIV positive. Epidemiological data shows that although 64% of the XDR cases have been diagnosed with TB and have been admitted to the hospital, 36% had no history of TB or hospitalisation. This suggests that factors like poor control practices, non-adherence to treatment play a major role in the origination of XDR-TB cases. Infections may also have been acquired at community levels (Migliori et al., 2007).

The United States Centers of Disease Control and Prevention (CDC) has reported the spread of XDR-TB in all continents (CDC, 2006). This form of TB now reported to be present

in 58 countries is almost incurable and represents a serious public health issue globally (WHO report 2011) (Figure.1.9.).

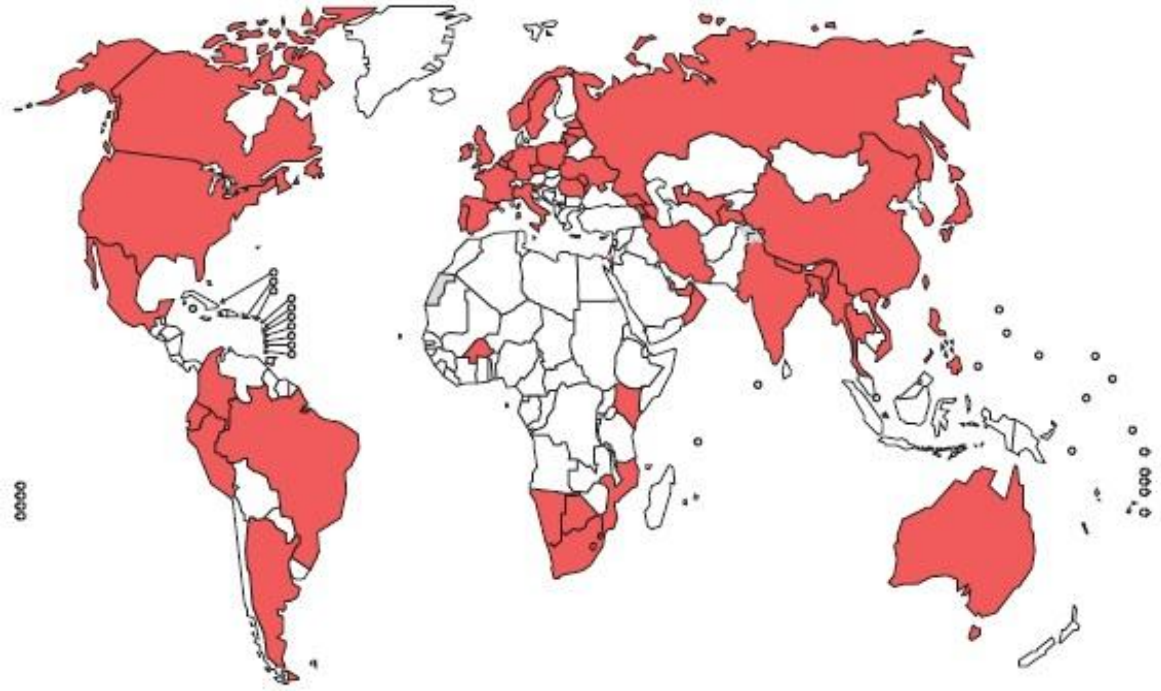

Figure 1.9. Map depicting distribution of countries and territories (in red) reporting at least one case of XDR-TB as of January 2010. Adapted from (WHO, 2010) Multidrug and extensively drug-resistant TB (M/XDR-TB): 2010 global report on surveillance and response.

1.7 The *Mycobacterium tuberculosis* complex (MTBC)

The *M. tuberculosis* complex includes the closely related species – *M. tuberculosis, M. bovis, M. africanum, "M. canettii"* and *M. microti.* Although they differ in epidemiology, these four species have between 85 to 100% similarity in their DNA (Imaeda T. et al., 1988).

M. tuberculosis is the major cause of tuberculosis in humans and infects one third of the world's population annually (WHO). The natural host of *M. tuberculosis* is humans but it has also been reported to infect animals which are in close contact with infected humans.

M. bovis is the causative agent of bovine tuberculosis. *M. bovis* infection is responsible huge agricultural losses globally and public health and animal welfare. It has been reported in various domestic animals e.g., cattle, camels etc.

M. africanum has been isolated from tuberculosis patients in the continent of Africa. Approximately 60% of all isolates from pulmonary tuberculosis patients from some regions in Africa have been identified as *M. africanum* (Haas et al., 1997).

M. microti was identified in 1946 as a pathogen of small mammals like voles, rodents and shrews. Wells in 1946 described an acid-fast bacilli causing tuberculosis like infections in voles. This organism was later identified as *M. microti* (Wells, 1949). Live *M. microti* strains have also been used for vaccine trials in the 1950's and they seemed to be of the same efficacy as the Bacillus Calmette Guerin (BCG) vaccine used then (Hart and Sutherland, 1977). In 1998 first reports of *M. microti* infection in immunocompetent and immunocompromised individuals were obtained from Netherlands and subsequently from Germany, Switzerland, England and Scotland (Kremer et al., 1998) .

M. canettii, identified as *M. tuberculosis*, subspecies Canettii, (now *M. canettii*) was first isolated from a French farmer, by George Canettii in 1969 (van Soolingen et al., 1997).

1.8 Non-tuberculous mycobacteria

Non tuberculous mycobacteria are also known as atypical mycobacteria and some of these cause pulmonary diseases resembling tuberculosis, skin infections and disseminated diseases. These bacteria have been usually classified into four groups, namely –

- Photochromogens – this includes species which develop a pigment after being exposed to light. e.g., *M. kansasii, M. marinum*
- Scotochromogens –these species develop pigmentation in dark. e.g., *M. szulgai, M. scrofulaceum.*
- Non chromogens – this includes a group of opportunistic pathogens like the *Mycobacterium avium complex* (MAC), *M. ulcerans, M. xenopi, M. malmoense, M. terrae, M. haemophilum* and *M. genavense.*
- Rapid growers – this includes some rapidly growing non chromogenic species some of which are pathogenic e.g., *M. chelonae, M. abscessus, M. fortuitum.* Other non disease causing species in this group are *M. smegmatis* and *M. flavescens.*

1.9 Surrogate systems in tuberculosis research -

Deciphering the mechanisms of pathogenesis of *M. tuberculosis* is very central in finding novel drug targets. The two most significant hindrances in *M. tuberculosis* research are -

- Highly infectious nature of *M. tuberculosis* poses risks for researchers necessitates working in a high containment Bio-safety level- 3 (BSL-3).
- *M. tuberculosis* has a long doubling time (~24 hrs generation time) and hence experiments take unusually longer time as compared to working with other fast growing organisms..

This lead to the usage of surrogate models for research on *M. tuberculosis* pathogenesis. Many non-pathogenic mycobacteria and related species are being used as an alternative model to assess *M. tuberculosis*.

1.9.1 *Corynebacterium glutamicum*

C. glutamicum was first discovered in 1950 in Japan. *C. glutamicum* and *M. tuberculosis* belong to the same family *Corynebacterianeae* and has similar cell wall architecture and biosynthetic machinery (Dover et al., 2004). Inspite of sharing similar cell wall ultrastructure, the cell wall of *C. glutamicum* is less complex when compared to *M. tuberculosis* and also gives the advantage over other mycobacterial strains in having a 30 minute generation time and ease of genetic manipulation (Cerdeno-Tarraga et al., 2003).

C. glutamicum has also been reported to tolerate mutations in the homologues of essential *M. tuberculosis* genes, e.g. *Cg-emb* (Seidel et al., 2007). Also *C. glutamicum* is a good model for studying essential genes in the mycolic acid biosynthesis e.g., *pks13* (Gande et al., 2004; Portevin et al., 2004). Hence it has been used as a model organism to study mycobacterial cell wall biosynthesis (Gande et al., 2004; Gibson et al., 2003).

1.9.2 *Mycobacterium smegmatis*

M. smegmatis is most widely used for *M. tuberculosis* research as it is non pathogenic and has a generation time of 4 to 5 hours. This species shares the same cell wall structure of *M. tuberculosis* and other mycobacterial species and genome analysis shows that *M. smegmatis* shares more than 2000 homologues with *M. tuberculosis*. *M. smegmatis* is more tolerant to cell wall alterations as compared to *M. tuberculosis* and some genes essential in *M. tuberculosis*

are non-essential in *M. smegmatis.* This makes *M. smegmatis* an attractive model for tuberculosis research.

On the other hand, there are limitations of using *M. smegmatis* as a model system. Approximately 30% proteins of *M. tuberculosis* do not possess orthologues in *M. smegmatis* and this species is non-pathogenic in nature. Hence even if *M. smegmatis* offers many benefits with working in BSL-1 settings and very short generation time, it still has limitations in screening for an effective anti-tuberculosis drug and potential drug targets (Altaf et al., 2010).

1.9.3 *Mycobacterium bovis* (BCG)

M. bovis is the causative agent of bovine TB (tuberculosis in cattle). The Bacillus Calmette-Guerin (BCG) is an attenuated strain of *M. bovis* and shares a high sequence homology to *M. tuberculosis* and the genes involved in the central metabolic pathway are identical in both the species. The BCG strain takes about 4-5 days to grow and can be used in a BSL-2 facility. Also the BCG strain responds to various molecular /genetic manipulation tools used for metabolic studies, e.g.;– electroporation, transposon mutagenesis etc.

The BCG strain was developed by 230 serial passages of the pathogenic *M. bovis* strain to serve as a vaccine against TB. The disadvantage of this model is that it is an attenuated strain and hence virulence cannot be followed as the strain lacks the RD1 and RD3 (region of difference) regions in the the genome. The RD1 and RD3 are present in the virulent *M. bovis* strain.

1.9.4 *Mycobacterium marinum*

M. marinum, a pathogen of amphibians and an opportunistic pathogen of humans has been recently developed as a model to study *M. tuberculosis* pathogenesis (Clark and Shepard, 1963; Ramakrishnan et al., 1997; Ramakrishnan and Falkow, 1994). *M. marinum* infects macrophages and also causes chronic tuberculosis like disease in pokilothermic hosts like fish and frogs (Rogall et al., 1990). The *M. marinum* genome is 6.6Mb and shares high similarity with the *M.tuberculosis* genome (Stinear et al., 2008; Tonjum et al., 1998). *M. marinum* has a much shorter generation time (~ 4 hrs) compared to *M. tuberculosis,* grows at an optimum temperature of 25°C to 35°C (Clark and Shepard, 1963) and possess similar cell wall lipid profiles as *M. tuberculosis* .

M. marinum can be used to study mechanisms of pathogenesis using its natural host – fish (Swaim et al., 2006) and also in in-vitro macrophage infection models. Zebrafish is a natural host for *M. marinum* and although the fish immune system is not clearly understood, it posesses both innate and adaptive immunity (Traver et al., 2003). Zebrafish infected with *M. marinum* develops granuloma – the hallmark of TB infections and hence suggests that the mechanism of disease formation is conserved between the two species. Host-pathogen interactions can be easily followed in a zebra-fish model using *M. marinum* (Stamm and Brown, 2004; Pozos and Ramakrishnan, 2004). The other advantage of the zebrafish model of infection is transparent embryos, which makes it easy to analyse bacterial migration using fluorescent *M. marinum* strains. *M. marinum* has been shown to enter and replicate within macrophages in a way similar to *M. tuberculosis* (Stamm and Brown, 2004). *M. marinum* has most of the advantages of *M. smegmatis* and is a pathogen, hence can be used for studying

virulence. As it is an opportunistic human pathogen the strain and can be manipulated in BSL-2 settings and do not pose much risk to researchers. This gives *M. marinum* an edge over the non pathogenic *M. smegmatis* for use as a surrogate system for TB research.

1.10 The Mycobacterial cell wall

The cell envelope of mycobacteria differs greatly from other bacteria; it is lipid rich, extremely hydrophobic with very low permeability to drugs (Brennan and Nikaido, 1995). This unique cell wall protects the bacterium from various harsh conditions and confers the property of acid-fastness. Acid-fastness refers to the ability of phenol-based dyes to penetrate the cell wall and resistance to decolourisation by acid-alcohol. Mycobacteria are still detected using the Ziehl-Neelsen staining method which uses the acid-fastness of the mycobacterial cell wall. The cell wall lipids and the arabinogalactan-mycolic acid complex contribute to this ability to retain phenolic dyes (Harada et al., 1976).

Research on the cell envelope of *M. tuberculosis* shows that it consists of the plasma membrane composed of a phospholipid bilayer, similar to the ones found in all free living organisms, the cell wall which is made up of a large complex known as the mycolyl-arabinogalactan-peptidoglycan (mAGP) core and solvent extractable lipids that are intercalated to the mAGP layer and in some cases, a capsule like outermost layer.

M. tuberculosis is reported to form a capsule (Daffé and Etienne, 1999). This capsule forms an interface between the bacterium and its environment (Brennan and Nikaido, 1995). The capsule consists of proteins and polysaccharides with small amounts of lipids and the ratio of proteins to polysaccharides varies from species to species (Daffé and Lanée, 2001). For example, the capsular contents of *M. tuberculosis, M. kansasii, M. gastri* are mainly

polysaccharides, whereas those of *M. smegmatis* and *M. phlei* are mainly proteins. The architecture of the mycobacterial cell wall was first proposed by Minnikin (1982) and subsequently modified by McNeil and Brennan (1991) and Minnikin et al., (2002). This model as representated in Figure. 1.10 shows that the cell wall is a complex arrangement of various macromolecules which can be divided into

- Cell wall composed of a mycolyl-arabinogalactan-peptidoglycan (mAGP) complex.
- Non covalently bound polysaccharides, phospholipids, glycolipids, waxes and proteins.

The cell wall is mainly composed of a large cell-wall core or complex that contains three different covalently linked structures -peptidoglycan, arabinogalactan and mycolic acids and additional lipids attached to outer layer or plasma membrane. The peptidoglycan layer is hydrophobically attached to the plasma membrane (Amar and Vilkas, 1973; McNeil et al., 1991), to which an extended array of polysaccharide is linked (through linker unit) which consist of a highly-ordered galactan attached to a branched arabinan domain which is esterified to a family of long-chain lipids known as mycolic acids. This is known as the mycolyl-arabinogalactan-peptidoglycan (mAGP) complex (Brennan and Besra, 1997).

The covalent linkage of mycolic acids results in a hydrophobic layer of extremely low fluidity. This layer is also referred to as the mycomembrane. The outer part of the membrane contains various lipids, such as phenolic glycolipids (PGL), phthiocerol dimycocerosates (PDIM), cord factor or dimycolyltrehalose (TDM), sulfolipids (SL) and phosphatidylinositol mannosides (PIM). Most of these lipids are specific for mycobacteria. The outer layer, which is generally called the capsule, mainly contains polysaccharides and glucans.

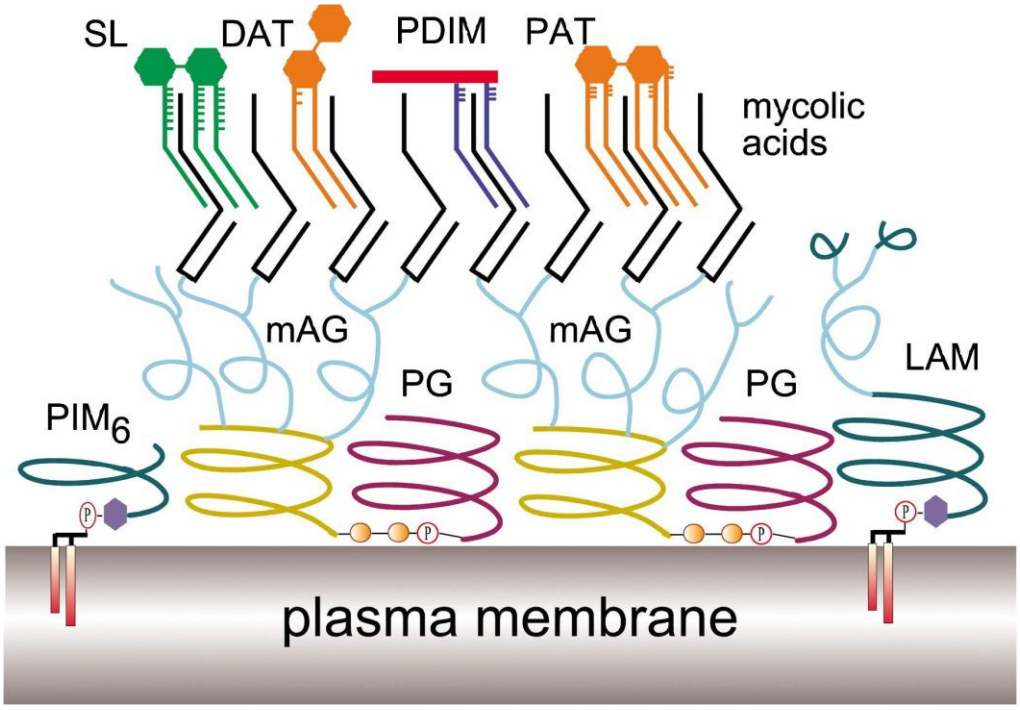

Figure 1.10. Representation of the *M. tuberculosis* cell wall. Adapted from Minnikin et al., (2002). DAT – Diacyl trehalose , PAT – Pentaacyl trehalose, PDIM – Phthiocerol dimycocerosate , LAM – Lipoarabinomamman , mAG –mycolyl arabinogalactan , PG - Peptidoglycan, PIM_6- Phosphatidylinositol hexamannosides.

1.11 Structural components of cell wall skeleton

1.11.1 Peptidoglycan (PG)

The principal structural constituent of the cell wall in mycobacteria is the insoluble cross-linking peptidoglycan (PG) (Alderwick *et al*. 2007). The PG in *M. tuberculosis* is similar to Gram-negative bacteria. It is composed of alternating $\beta(1\rightarrow4)$- N-acetylglucosamine (GlcNAc) and N-glycolyl muramic acid (Mur) residues. Oligopeptide chains of between three and five amino acids, usually *L/D*-alanine, D-glutamate, meso-diaminopimelic acid (DAP) and occasionally glycine, are connected to the muramic acid components. Cross linking occurs between two mDAP residues or mDAP and D-alanine (Zhang et al., 2003). Following the

transglycosylation of the (GlcNAc-β(1→4)-Mur), these linkage formations are catalysed by a transpeptidase (Scheffers and Pinho, 2005). Biosynthesis of the mycobacterial PG has not been investigated in great detail, but is considered to be similar to the biosynthetic process in *Escherichia coli* (van Heijenoort, 2001).

1.11.2 Arabinogalactan (AG)

The arabinogalactan (AG) is one of the major serologically active components of the mycobacterial cell wall and is a branched hetero-polysaccharide. The AG consists of arabinofuranose (Ara*f*) and galactofuranose (Gal*f*) residues. The galactan of AG is made up of a linear chain of 30 β-***D***-Gal*f* residues, alternately linked by β(1→ 5) and β(1→6) linkages. These attach to the muramic acid residues in the PG layer via a unique disaccharide bridge and a phosphodiester bond. The arabinan domain is made up of arabinofuranose (Ara*f*) residues in linear α (1→ 5) linkages. The arabinan chains are also branched to form a fork-like motif (Alderwick et al., 2007; Besra et al., 1995; Daffé et al., 1990). Mycolic acids are esterified to at least two thirds of the terminal Ara*f* .

1.12 Cell wall lipids in Mycobacteria

Over 60 % of the mycobacterial cell wall consists of lipids of which the majority are mycolic acids. It was during the 1930s that the strongly hydrophobic lipid components were first studied and the mycolic acids, which make up approximately half of the lipids, were identified (Minnikin et al., 2002). These are either esterified to the terminal arabinofuranosides or present as solvent-extractable, non covalently bound mycolates, e.g; trehalose di or monomycolates (TDM or TMM). Other cell wall solvent-extractable lipids are pthiocerol

dimycocerosate (DIM/PDIM), sulfolipids (SL), glycopeptidolipids (GPL), phenolic glycolipids, acylglycerols and lipoglycans (LAM/LM/PIMs).

The cell wall lipids differ in terms of modifications and proportions in different mycobacterial species. But they all have a similar organisation in the cell wall. This similarity allows the use of non-pathogenic mycobacteria like *M. smegmatis* for research on the mycobacterial cell wall.

1.13 Cell wall lipids in *Mycobacteria*

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The cell wall lipids differ in terms of modifications and proportions in different mycobacterial species. But they all have a similar organisation in the cell wall. This similarity allows the use of non-pathogenic mycobacteria like *M. smegmatis* for research on the mycobacterial cell wall.

1.13.1 Mycolic acids

Mycolic acids are an essential part of the mAGP domain and also play an important role in the virulence of *M. tuberculosis*; eg TDM (trehalose dimycolate) (Takayama et al., 2005). Mycolic acids are α–alkyl ,β–hydroxy fatty acids (C_{70}-C_{90}) with long alkyl side chain unique α-branched fatty acids found in the cell walls of *Mycobacterium.* They may make up 50% of the dry weight of the mycobacterial cell envelope. There are five known types of mycolic acid in *M. tuberculosis*. All have the same general structure of a α–branch of C_{26} and a longer chain meromycolate of $C_{50\text{-}56}$, but modifications to this give rise to α-mycolates, which contain cyclopropane rings, methoxy-mycolates, which have $–OCH_3$ groups and keto-mycolates, which have keto groups. The latter two are found in both the *cis* and *trans* configuration of their cyclopropane rings.

These conformations and modifications of the mycolic acid structures have a significant effect on their properties. Cyclopropanation of the mycolic acids is reportedly essential for cord formation in mycobacteria and influences virulence (Glickman et al., 2000). The mycobacterial gene *pcaA* is required for cyclopropanation of α-mycolates. A *M. tuberculosis pcaA* mutant strain displayed growth defects in lungs post infection and failed to persist during infection (Glickman et al., 2000). Cyclopropanation modifications have also been shown to affect activation of macrophages in immune responses (Rao et al., 2005; Rao et al., 2006).

A lot of variations are observed in the classes of mycolic acids in various *Mycobacterium* species. For example, *M. tuberculosis* possesses α, methoxy and keto

mycolates while *M. smegmatis* possesses α, α′ and epoxy mycolates. The representative structures of some mycolic acids are shown in Figure 1.11.

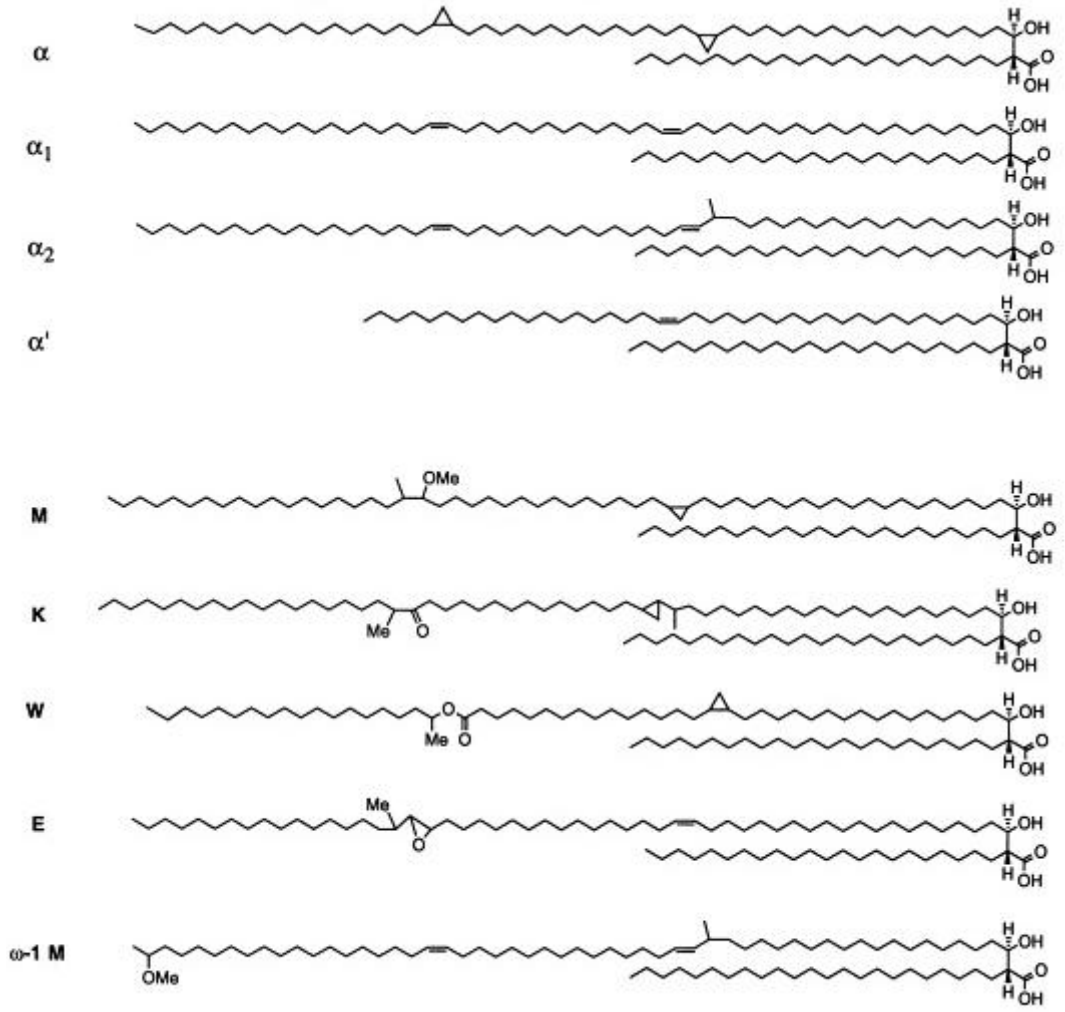


Figure 1.11. Representative structures of different types and classes of mycobacterial mycolic acids, adapted from Barry et al. (1998). α, α1 and α′, lacking the oxygen functions. M – methoxy, K – keto, W – wax ester, E – epoxy, ω-1M - ω-1 methoxy.

Mycolic acids serve as a second permeability barrier. Mycolic acids are also found esterified to the non-reducing terminal of arabinogalactan, forming the mycolyl-arabinogalactan (mAG). They are also found embedded within the outer layer of the cell wall, associated with glucose and trehalose to form glucose monomycolate (GMM), trehalose

monomycolate (TMM), trehalose dimycolate (TDM). Such a distinctive arrangement in the outer layer of *M. tuberculosis* provides limited fluidity to the outer membrane to the cell wall.

Cell wall components like TDM has been reported to have adjuvant activity, antigenicity, innate and adaptive immunity stimulating activity and immunomodifying activity (Dubnau et al., 2000).

1.13.2 Lipoglycans in mycobacterial cell wall

Interspersed within the mAGP framework of the mycobacterial cell are found LAM, LM and various lipoglycans. According to current understanding, lipoglycan biosynthesis in mycobacteria can be simplified as PI $\rightarrow$ PIM $\rightarrow$ LM $\rightarrow$ LAM (Besra and Brennan, 1997), where each step involves formation of an increasingly glycosylated molecule. LAM and its precursor LM and PIMs are related biosynthetically. LM and LAM are attached to the cell wall via the PI anchor (Hunter and Brennan, 1990).

The PI unit is based on a *sn*-glycero-3-phosphate and is acylated. Addition of mannopyranosyl (Man*p*) units to PI generates the PIMs (Ballou and Lee, 1964). The smallest PIM is PIM_1 which is PI glycosylated at the hydroxyl group O-2. Further glycosylation with a Man*p* residue at the O-6 position of the myo-inositol ring of PIM_1 yields PIM_2. Upto five Man*p* units can be added to the O-6 position of the myo-inositol ring to give rise to PIM_6. PIMs are always present in the acylated forms $-Ac_1PIM$ (acyl group at the 3-OH of the *myo*-inositol ring or 6-OH of the Man*p* residue) and Ac_2PIM (acylated at both 3-OH of the *myo*-inositol ring and 6-OH of the Man*p* residue).

LM consists of a linear α-(1→6)-linked mannan backbone (17 -19 Man*p* residues*)* which extends from the mannose (Man*p*) residue linked to the *O*-6 position of inositol in PIM_2

(Chatterjee et al., 1991; Besra et al., 1997) and 7-9 single branched α-(1→2) Man*p* units. Glycosylation of the LM with arabinan forms LAM. LAM consists of three domains – a polysaccharide backbone, the PI-anchor and a capping motif. In LAM, the mannan backbone is further elaborated by the addition of a branched arabinan polymer similar to that found in AG, and mannosylation or capping (Chatterjee et al., 1993; McNeil et al., 1994). The arabinan polymer consists of 60 α-(1→5) - Araf units with branched hexaarabinofuranosides and linear tetraarabinofuranosides.

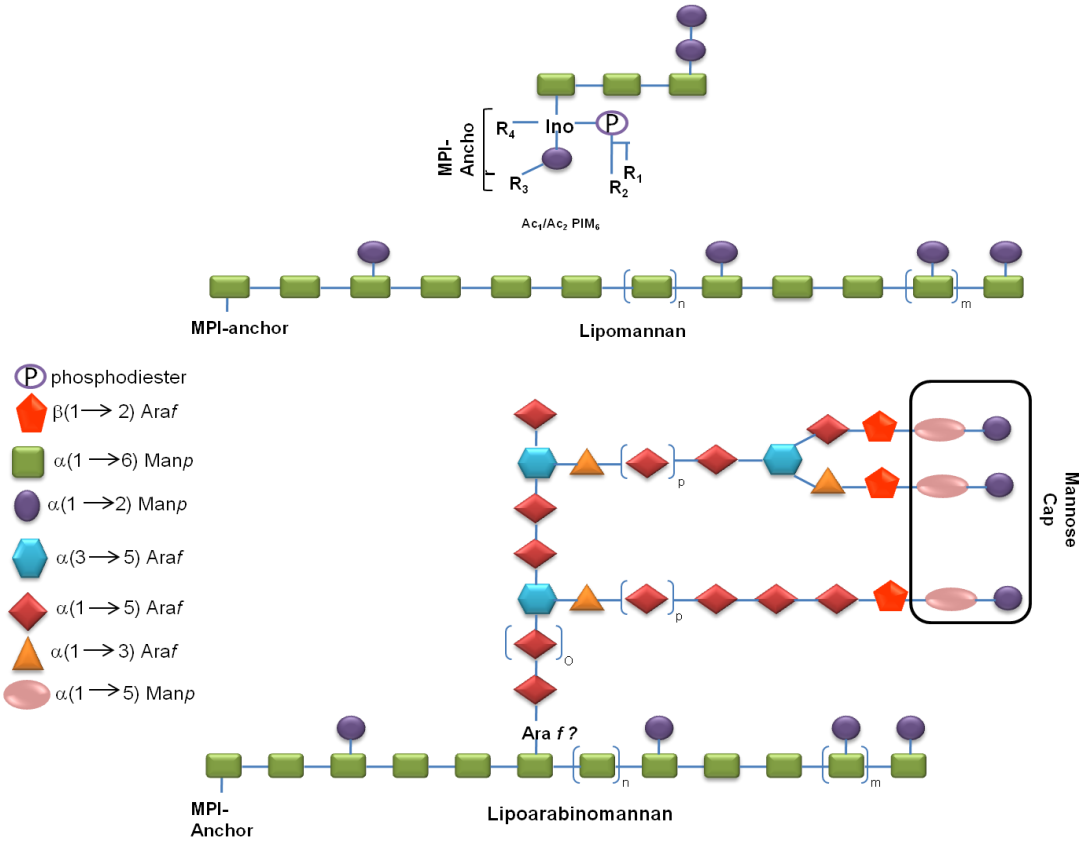


Figure 1.12. The general structure of LAM (Man-LAM) from *M. tuberculosis*, and its relationship with different PI derivatives. R_1 and R_2 indicate the acylation position on the glycerol in the MPI anchor, R_3 – acylation at 3OH of the inositol, and R_4acylations at the 6-OH of the Man*p*. m, n,o,p indicate species specific amounts of glycosylation. ? - unknown linkage between arabinan and mannan. Adapted from (Jankute et al., 2012).

LAM is the major immune modulator leading to immune suppression ,induction of inflammatory cytokines , inhibition of various interferon gamma (IFN-γ) induced activities such as microbicidal and tumoricidal functions of macrophages and neutralization of cytotoxic free radicals (Sibley et al., 1988).

Based on the capping motif, three types of LAM have been identified.

- Mannose-capped LAM (ManLAM) (Nigou et al., 2003) which is found in slow growing pathogenic mycobacterial strains - *M. tuberculosis, M. bovis BCG, M. leprae, M. avium, M. xenopi, M. kansasii* and *M. marinum*.
- Phospho-myo-inositol-capped LAM (PILAM) (Khoo et al., 1995a) which is found in the fast growing, non pathogenic *M. smegmatis* and *M. fortuitum.*
- Non-capped or Ara-LAM (Guerardel et al., 2002) found in *M. chelonae*.

1.14 Solvent extractable lipids in the mycobacterial cell wall

A variety of covalently bound lipids are found interspersed within the hydrophobic environment provided by mycolic acids of the mAGP complex. Some of these lipids are common to all mycobacteria and these include TDMs while others are species / strain specific like PDIM, PGL, SLs, GPLs and LOSs.

1.14.1 Trehalose monomycolate and dimycolate

Trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) is a non-reducing disaccharide of glucose and is found abundantly in bacteria, yeast, fungi, plants, insects and invertebrates and mainly acts as a storage molecule. In mycobacteria, trehalose is either found in the free forms in the cytosol or as acylated forms (esterified to various fatty-acyl groups) in the cell wall. In

mycobacteria, trehalose plays the role of a carrier molecule. Based on these esterifications, acylated trehaloses in mycobacteria can be grouped under three classes:

- Mycolyl trehaloses – trehalose monomycolate and trehalose dimycolate (TMM and TDM).
- Methyl-branched acyl trehaloses – Di-acyl, Tri-acyl and Pent-acyl trehaloses (DATs, TATs, PATs).
- Sulfated acyl-trehaloses – Sulfolipids (SLs).

TDM is also known as 'cord factor' and is the most abundant lipid produced by virulent *M. tuberculosis* (Bloch and Noll, 1953). Removal of the non-covalently bound surface exposed lipids of *M. tuberculosis* cells by petroleum ether showed reduced viability and caused loss of virulence. This petroleum ether extract was known as 'cord-factor' and its composition was found out to be primarily trehalose-6-6'- dimycolate (Noll and Bloch, 1953; Noll et al., 1956). The basic structure comprises of a trehalose sugar esterified to two mycolic acid residues. In TDMs, the trehalose is esterified to mycolates at positions 6 and 6'. TDM is found only extracellularly. This supports the transport of mycolates in the form of TMM (Belisle et al., 1997). TDM constitutes majority of the non-covalently bound surface exposed lipids in mycobacteria and is reported to play an important role in the recruitment of cells for granuloma formation (Hunter et al., 2006), and also modulates expression and production of cytokines and NO (Lima et al., 2001). TMM is the precursor of TDM and is found both intracellularly and extracellularly. It posessess a single mycolate residue esterified to the trehalose sugar (Figure 1.13).

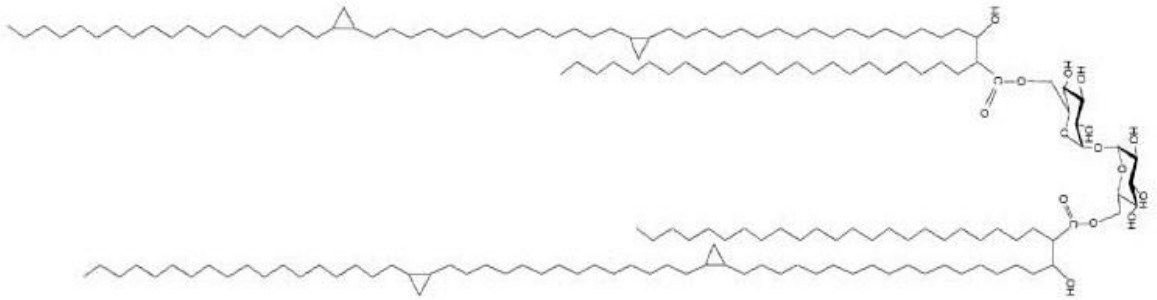


Figure 1.13. Structure of trehalose dimycolate from *M. tuberculosis*. A specific example of the sugar esterified with the α-mycolate is shown.

1.14.2 Methyl-branched acyl trehaloses

Methyl-branched acyl trehaloses carry various methyl branched acyl chains such as mycosanoic acids in diacyl-trehaloses (DATs) and mycolipenic acids in triacyl-trehaloses and pentaacyl-trehaloses (PATs). DATs (Lemassu et al., 1991; Besra et al., 1992), TATs (Munoz et al., 1997) and PATs (Minnikin et al., 1985; Daffé et al., 1988) are exclusively found in the virulent Mycobacterium strains suggesting the importance of these molecules in the virulence of *M. tuberculosis*. These molecules are amphiphillic in nature and hence contribute to the structure of the cell wall by interacting both with the mycolic acid core and the outermost capsule layer (Minnikin et al., 2002). DATs are non-covalently linked to the peptidoglycan in the outer layer of the mycobacterial cell wall (Ortalo-Magne et al., 1996).

While not much is not known about their effects on virulence, both DATs and TATs have been reported to be useful in detection of TB infection. Immunoglobulin G

corresponding to these molecules are detected with very high specificity and sensitivity (Munoz et al., 1997).

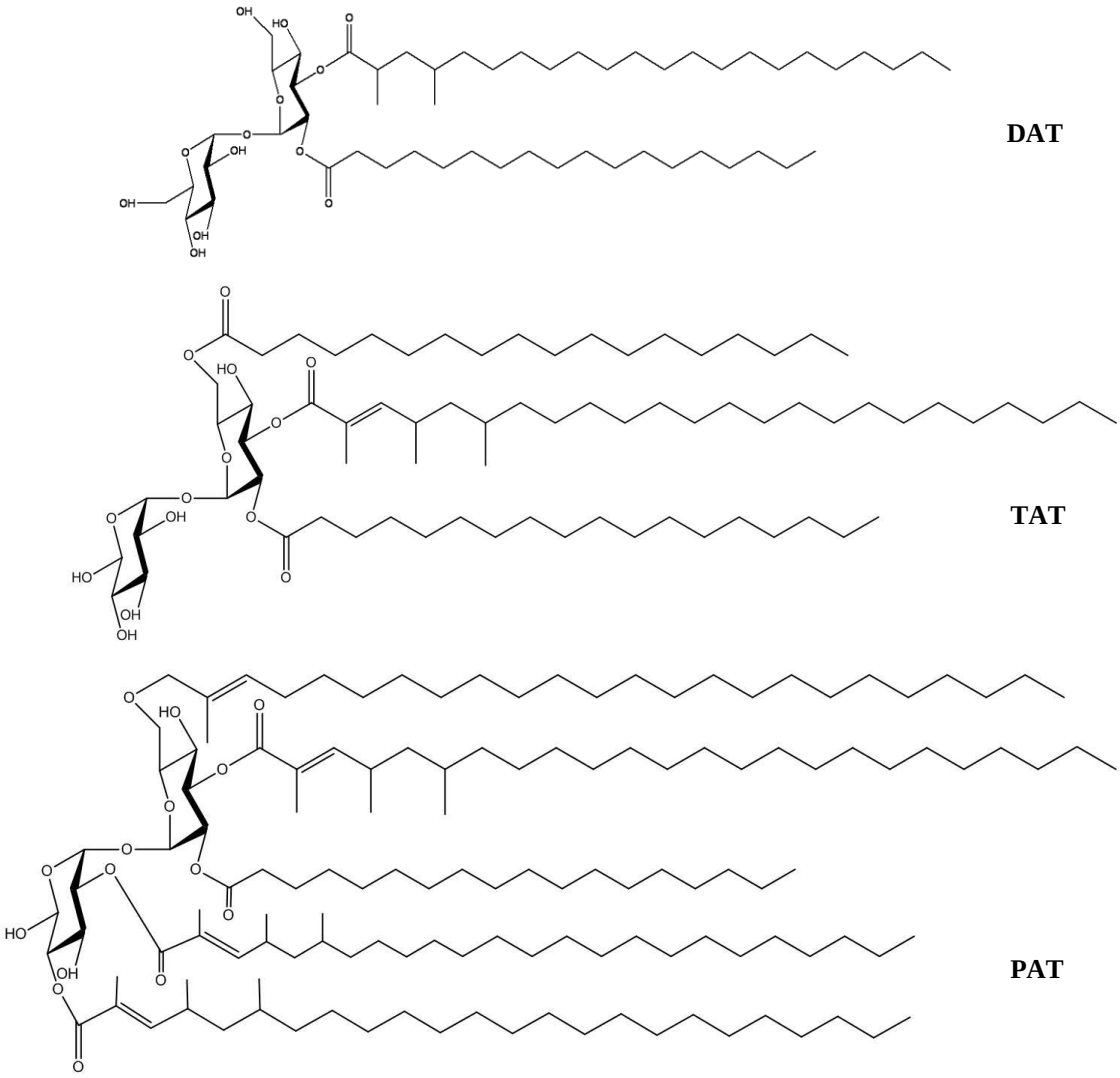


Figure 1.14. Structures of diacyl trehalose (DAT), triacyl trehalose (TAT) and pentaacyl trehalose (PAT) from *M. tuberculosis.*

1.14.3 Phthiocerol dimycocersate (PDIMs) and Phenolic glycolipids (PGL)

Genes involved the biosynthesis of PDIM and PGL play a role in the pathogenesis of the tubercle bacillus (Camacho et al., 2001). PDIMs are composed of a long chain phthiocerol or phthiodiolone esterified with two multimethyl-branched long-chain mycocerosic acids

(Minnikin et al., 2002). Studies by Kolatukuddy and co-workers showed the involvement of PKS genes in biosynthesis of PDIMs in *M. bovis* (Mathur and Kolattukudy, 1992; Azad et al., 1996). Although not much is known about the role of PDIMs in host-pathogen interactions, it has been shown that their presence is essential for successful *M. tuberculosis* infection. Loss of PDIMs was responsible for attenuation of *M. tuberculosis* in mice lungs (Cox et al., 1999). PDIMs have been reported to be involved in the early stages of *M. tuberculosis* infection and protects the bacilli from reactive nitrogen intermediates (Rousseau et al., 2004), arrests acidification of phagosome and is also involved in modification of host cell membranes (Simeone et al., 2007).

Mycobacteria that produce PDIMs, also synthesize the structurally related PGL. In these, the hydroxyl group of the phenol moiety is glycosylated by species/type-specific mono-, tri- or tetrasaccharide units (Daffé et al., 1987). Known as phenolic glycolipids (PGLs), these are found in pathogenic mycobacterial strains such as members of the *M. tuberculosis* complex, especially *M. tuberculosis* strain Canettii, *M. leprae*, *M. kansasii*, *M. marinum*, *M. gastri*, and *M. ulcerans* (Daffé et al., 1987). They are similar in structure to PDIMs, and are comprised of a conserved lipid core with varying carbohydrate residues (Minnikin et al., 1982; Chatterjee et al., 1988). The sugar moiety of PGLs consists of one to four sugar residues, most of which are *O*-methylated deoxysugars.

A precursor of phenolphthiocerols, *p*-hydroxybenzoic acid (*p*-HBA) is found in all strains of *M. tuberculosis*, but only few strains produce PGLs. The gene pks15/1 is involved in the elongation of *p*-HBA derivatives to generate phenolphthiocerols. Based on the sequence of pks15/1, *M. tuberculosis* strains are grouped into different clusters. *"M. canettii"* and strain 210 (belonging to the Beijing strain) carries a six nucleotide insertion in the pks15/1 gene

resulting in a functional Pks15/1 synthase (Brosch et al., 2002; Constant et al., 2002). PGL-1 from *M. leprae* has been used for serodiagnosis of leprosy and TB (Gaylord et al., 1987, Simonney et al., 1995). PGLs have been reported to be involved in immunomodulation during infection by inhibiting release of proinflammatory chemokines (Reed et al., 2004; Prasad et al., 1987), e.g. *M. leprae* PGL-1 inhibits proliferation of T-lymphocytes (Mehra et al., 1984; Fournie et al., 1989)

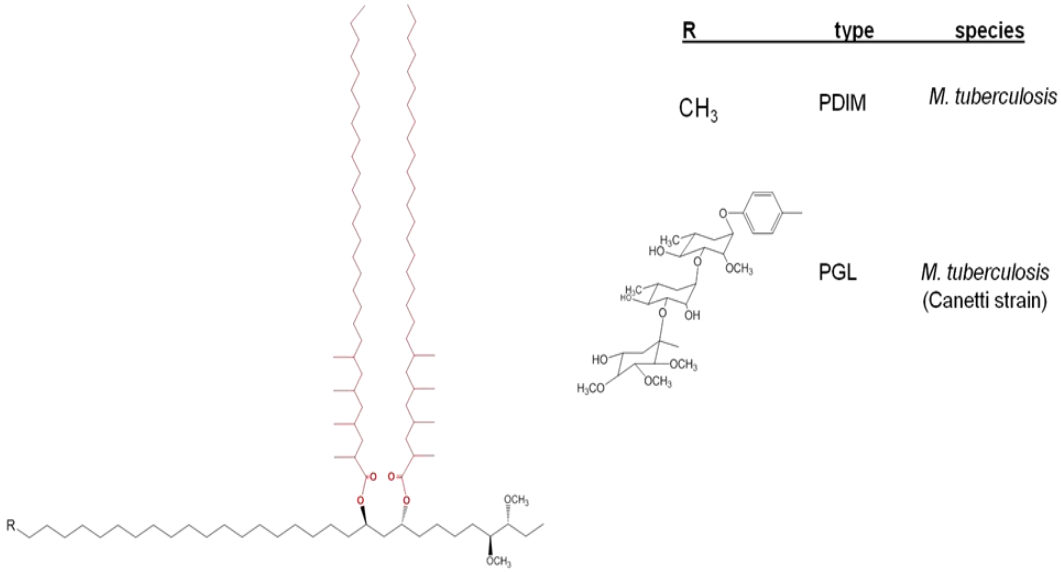


Figure 1.15. Structure of PDIMs and PGLs from *M. tuberculosis*.

1.14.4 Sulfolipids (SL)

Sulfated metabolites are very sparse in prokaryotes. Mycobacteria are one of the three genera in which there is an abundance of sulfolipids. The other two being the plant pathogen *Xanthomonas oryzae* and plant symbiont *Sinorhizobium meliloti* (Schelle and Bertozzi, 2006). Sulfolipids are found in virulent mycobacterial strains (Middlebrook et al., 1959).They are composed of sulfated trehalose esters, acylated with three to four fatty acyl groups which

consist of one short chain saturated fatty acid (e.g. palmitic acid or stearic acid) and different long chain multi-methyl branched fatty acids (e.g., phthioceranic acid and hydroxyphthioceranic acids). The most abundant SL in mycobacterial cell wall is SL-1 which is a 2,3,6,6'-tetraacyl 2' sulfate (Raynaud et al., 1998; Goren et al., 1976). SLs block the release of tumor necrosis factor-alpha (TNF-α) from macrophages stimulated by TDMs. This in turn has an inhibitory influence on granuloma formation. SLs deficiency in mutant strains derived from H37Rv have shown to play a significant effect in persistence and pathogenicity of *M. tuberculosis* in guinea pigs and cultured macrophages (Rousseau et al., 2003b).

SL

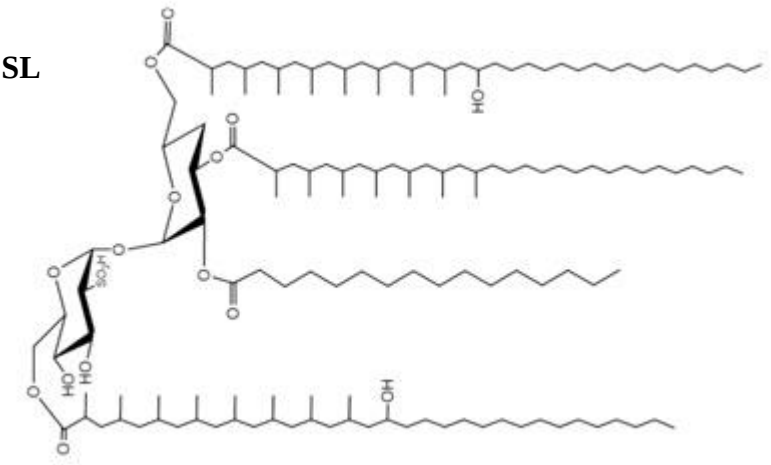


Figure 1.16. Structure of sulfated tetra acyl trehalose (SL) from *M. tuberculosis*.

1.14.5 Oligosaccharide containing lipids

Oligosaccharide containing lipids in mycobacteria are divided into two major classes, the lipooligosaccharides (LOSs), which are acylated by long chain fatty acids, and phenolic glycolipids and glycopeptidolipids (Asselineau and Lanéelle, 1998).

1.14.5.1 Glycopeptidolipids (GPL)

GPLs are non polar mycosides produced by several mycobacterial species such as the non pathogenic, fast growing *M. smegmatis*, mycobacterial pathogens *M. chelonae* and *M. abscessus* (Ripoll et al., 2007), opportunistic human pathogens like members of the *Mycobacterium avium* complex (MAC) that include *M. avium* and *M. intracellulare*, animal pathogens *M. porcinum* and *M. senegalense* (Lopez Marin et al., 1991). GPL's are composed of a common lipopeptide core that is made up of **D**-phenylalanine- **D** -*allo*-threonine- **D** -alanine-**L**-alaninol, with varying glycosylations in pathogenic and non-pathogenic species. Some mycobacterial species such as *M. smegmatis* and *M. avium* display an ability to produce biofilms on PVC surfaces (Recht et al., 2001).This correlates with their ability of sliding motility and GPLs have been identified to play an important role in this process (Martinez et al., 1999). A correlation between virulence and biofilm formation has also been reported. GPLs from *M. smegmatis* have been reported to be involved in inhibition of receptor mediated phagocytosis of *Mycobacterium* by macrophages (Villeneuve et al., 2003). It was observed that clinical strains of *M. avium* were capable of forming biofilms while the strains defective in genes responsible for biofilm formation were retarded in sliding motility when compared to the wild type clinical isolates (Carter et al., 2003; Yamazaki et al., 2006).

1.14.5.2 Lipooligosaccharides (LOS)

LOSs are highly polar, trehalose containing surface immunogens of many environmental mycobacteria. They were first found and characterised from the opportunistic pathogen *M. kansasii* (Hunter et al., 1985), *M. gastri* (Gilleron et al., 1993), *M. szulgai* (Hunter et al., 1988), *M. malmoense* (McNeil et al., 1987), *M. gordonae* (Besra et al., 1993), the fish tank

granuloma causing *M. marinum* (Minnikin et al., 1989), *M. mucogenicum* (Munoz et al., 1998) and the *M. tuberculosis* complex strain, *M. canettii* (Daffé et al., 1991).

LOSs have a poly-acylated trehalose core with further glycosylations. Varying residues of **D**-xylose, 3-*O*-methylrhamnose, fucose, and a N-acyl aminosugar are linked to the core and these sugar compositions are species-specific and responsible for immunogenic properties of LOSs. Depending on the species, the oligosaccharide is linked either on carbon 3, 4 or 6 of the trehalose, and this oligosaccharide is further glycosylated to possess 2 to 6 sugar residues, some of which are new and specific carbohydrates, like the caryophyllose (α-3,6 dideoxy-4-C-(**D**-*altro*-1,3,4,5-tetrahydroxyhexyl)-**D**-*xylo*-hexopyranose) and an N-acyl aminosugar (N-acylated dideoxy galactose) residues.

LOSs have been extensively studied in *M. marinum* and four types of LOSs, LOS-I to LOS-IV have been identified. *M. gastri* is also reported to possess a novel C-4 branched 3,6-dideoxy-α-hexopyranose, and four classes of LOSs were identified from this species (Gilleron et al., 1994). *M. kansasii* has eight different types of these antigenic glycolipids (Hunter et al., 1983; Hunter et al., 1985). The variable sugar residues in this species are xylose, 3-*O*-methyl-rhamnose, fucose and a 4,6-dideoxy-2-*O*-methyl-3-methyl-4-(2'-methoxypropionamido)-α-**L**-mannohexopyranosyl.

Table 1.3 Composition of LOS from different mycobacterial species. Multiple number of identical residues are denoted by 'n'.

M. kansasii	LOS I'	3-*O*-Me-α-L-Rha*p*-(1→3)-α- **D**-Glc*p*-(1↔1)-α-**D**-Glc*p*-β-**D**-Glc*p*-(1→4)
	LOS-I (n=1) LOS II (n=2) LOS III (n = 3)	(β- **D** -Xyl*p*)$_n$-(1→4)- 3-*O*-Me-α-L-Rha*p*-(1→3)-β-**D**-Glc*p*-(1→3)-β- **D** -Glc*p*-(1→4)-α-D-Glc*p*-(1↔1)-α- **D** –Glc*p*
	LOS IV (n=4), LOS- V (n=5), LOS- VI (n=6)	Kan*N*acyl-(1→3)-Fuc*p*-(1→4)-(β-**D**-Xyl*p*)$_n$-3-*O*-Me-α-L-Rha*p*-(1→3)-β- **D** -Glc*p*-(1→3)-β- **D** -Glc*p*-(1→4)-α- **D** -Glc*p*-(1↔1)-α- **D** –Glc*p*
	LOS VII	Kan*N*acyl-(1→3)-Fuc*p*-(1→4)-[(β-L-Xyl*p*)$_6$-(1→4)]6-α-L-3-*O*-Me-α-L-Rha*p*-(1→3)-β- **D** -Glc*p*-(1→3)-β- **D** -Glc*p*-(1→4)-α- D-Glc*p*-(1↔1)-α- **D** –Glc*p*
M. malmoense	LOS II	α- **D** -Man*p*-(1→3)-α- **D** -Man*p*-(1→2)-α-L-Rha*p*-(1→2)-[α-L-3-*O*-Me-Rha*p*-(1→2)]$_2$-α-L-Rha*p*-(1→3)- α- **D** -Glc*p*-(1↔1)-α- **D** –Glc*p*
M. szulgai	LOS I	α-L-2-*O*-Me-Fuc*p*-(1→3)-α-L-Rha*p*-(1→3)-β-L-Rha*p*-(1→3)-β- **D** -Glc*p*-(1→6)-α- **D** -Glc*p*-(1↔1)-α- **D** -2-*O*-Me-Glc*p*
M. tuberculosis "Canettii"	LOS I	N-acyl-4-amino-4,6-dideoxy-Gal*p*-(1→4)-2-*O*-Me-α-L-Fuc*p*-(1→3)-β- **D** -Glc*p*-(1→3)-[2-*O*-Me-α-L-Rha*p*-(1→3)]$_2$-β- **D** -Glc*p*-(1→3)-4-*O*-Me-α-L-Rha*p*-(1→3)-6-*O*-Me-α- **D** -Glc*p*-(1↔1)-α- **D** –Glc*p*
M. gordonae (989)	LOS I	N-acyl-4-amino-4,6-dideoxy-2,3-*O*-Me-α-Gal*p*-(1→3)-2-*O*-Me-4-*O*-Ac-α-L-Fuc*p*-(1→3)-β- **D** -Glc*p*-(1→3)-2-*O*-Me-α-L-Rha*p*-(1→3)-β- **D** -Xyl*p*-(1→2)-α-L-Rha*p*-(1→3)-β- **D** -Glc*p*-(1→3)-α-L-Rha*p*-(1→3)-6-*O*-Me-α- **D** -Glc*p*-(1↔1)-α- **D** –Glc*p*
M. gordonae (990)	LOS I	α-L-Rha*p*-(1→2)-3-*O*-Me-Rha*p*-(1→3)β-**D**-Xyl*p*-(1→2)-α-L-Rha*p*-(1→3)-β-**D**-Glc*p*-(1→3)-β-**D**-Glc*p*-(1→3)-α-L-Rha*p*-(1→3)-6-*O*-Me-α- **D** -Glc*p*-(1↔1)-α-**D**–Glc*p*
M. gastri	LOS I (n = 1) LOS II (n = 2)	[β-L-Xyl*p*-(1→4)]$_n$-3-*O*-Me-Rha*p*-(1→3)-β- **D** -Gal*p*-(1→3)-β- **D** -Glc*p*-(1→4)-α- **D** -Glc*p*-(1↔1)-α- **D** –Glc*p*
	LOS III (n = 6) LOS IV (n = 7)	3,6-dideoy-4-*C*-(1,3-dimethoxy-4,5,6,7-tetrahydroxy-heptyl)-α-*xylo*-Hex*p*-(1→3)-[β-L-Xyl*p*-(1→4)]$_n$-3-*O*-Me-Rha*p*-(1→3)-β- **D** -Gal*p*-(1→3)-β- **D** -Glc*p*-(1→4)-α- **D** -Glc*p*-(1↔1)-α- **D** –Glc*p*
M. marinum	LOS I	3-*O*-Me-Rha*p*-(1→3)-β- **D** -Gal*p*-(1→3)-β- **D** -Glc*p*-(1→4)-α- **D** -Glc*p*-(1↔1)-α- **D** –Glc*p*
	LOS II	[3,6-dideoxy-4-*C*-(**D**-*altro*-1,3,4,5-tetrahydroxyhexyl)-**D**-*xylo*-hexopyranose (1→4)]$_n$- [β-L-Xyl*p*-(1→4)]-3-*O*-Me-Rha*p*-(1→3)-β- **D** -Gal*p*-(1→3)-β- **D** -Glc*p*-(1→4)-α-D-Glc*p*-(1↔1)-α-D-Glc*p*
	LOS III	α-Car (1→4)- [β-L-Xyl*p*-(1→4)]-3-*O*-Me-Rha*p*-(1→3)-β-D-Gal*p*-(1→3)-β- **D** -Glc*p*-(1→4)-α- **D** -Glc*p*-(1↔1)-α- **D** –Glc*p*
	LOS IV	α-4-amino-4,6-dideoxy-Gal*p* (1→c)- α-Car (1→c)- α-Car (1→4)-[β-L-Xyl*p*-(1→4)]-3-*O*-Me-Rha*p*-(1→3)-β-**D**-Gal*p*-(1→3)-β- **D** -Glc*p*-(1→4)-α- **D** -Glc*p*-(1↔1)-α-D-Glc*p*

The Canettii strain of *M. tuberculosis* is characterised by the presence of only two LOSs (Daffé et al., 1991). The trehalose residues were found to be methylated at position 6' and were either 2,3,6- or 3,4,6-tri-*O*-acylated. LOSs from *M. szulgai* contains a 2-*O*-methyl-α-**D**-fucopyranosyl unit and is glycosidically linked to a 2-*O*-methyltrehalose. *M. gordonae* clinical isolates were described to contain novel classes of LOSs. They were reported to possess 6'-*O*-methyl-2,3,4,6-tetra-*O*-acyl-glucose unit and also a novel branched oligosaccharide backbone (Besra et al., 1993).

LOSs are highly antigenic and have been used for serotyping (Munoz et al., 1998). LOSs from *M. marinum* have also been reported to be involved in sliding motility and biofilm formation (Ren et al., 2007). Although the gene cluster involved in LOS biosynthesis in *M. marinum* have been identified (Burguière et al., 2005; Etienne et al., 2009; Ren et al., 2007), the LOS biosynthetic pathway have not yet been deciphered completely. More about LOS biosynthesis from previous research and work reported in this thesis project is discussed in Chapter 2.

1.15 Aims and Objectives

The objectives of this thesis were to study pathways leading to biosynthesis and transport of complex mycobacterial cell wall lipids and their effects on virulence. The study focussed on two lipid metabolites, lipooligosaccharides (LOSs) and mycolic acids.

The broad aim of the studies on LOSs was to identify genes involved in the LOS biosynthesis in *M. marinum* and *M. kansasii* with the long term aim of applying these findings to the *M. tuberculosis* complex strain "*M. canettii*". *M. marinum* was used predominantly in these studies as a model organism. A combination of transposon mutagenesis and directed

knockouts were used to achieve these goals and the results are discussed in chapters 2, 3,4 and 5. Additionally, the effects of alteration in LOS profile on virulence were studied using zebrafish and macrophage models of infection.

The studies on mycolic acids focussed on deciphering transport and processing pathways and make use of *M. smegmatis* as a surrogate. These results are described in Chapter 6.

2

Identification of a caryophyllose transferase involved in lipooligosaccharide biosynthesis in *Mycobacterium marinum*

2.1 Introduction

As discussed in the previous chapter, solvent extractable glycolipids are predominant in mycobacteria and can vary between different mycobacterial species, and within strains of the same species. While some 'free' lipids like TDM are common to all mycobacteria, others are only species and/or strain specific such as sulfolipids, glycopeptidolipids and phenolic glycolipids. Included in the latter class are the highly polar lipooligosaccharides (LOSs) which are produced by a number of mycobacteria, including the opportunistic pathogen *M. kansasii*, the fish tank granuloma-causing *M. marinum*, and the *M. tuberculosis* complex strain, *"M. canettii"* (Daffé et al., 1987).

Given the absence of LOSs in *M. tuberculosis* strains, the exact role of LOSs in virulence is unclear. However, LOS-deficient *M. kansasii* strains were unable to establish infection in a mouse model and mutants of *M. marinum* with altered LOSs patterns were found to be defective in macrophage entry (Belisle and Brennan, 1989). Furthermore, purified *M. marinum* LOSs were found to inhibit TNF-α secretion by LPS-stimulated macrophages (Rombouts et al., 2009). Collectively, these data suggested a significant role for LOSs in virulence of pathogenic mycobacteria.

Earlier experiments on biochemical and genetic analysis of LOS biosynthesis were done on *M. marinum*. *M. marinum* produces four subclasses of LOSs; LOS-I, LOS-II, LOS-III and LOS-IV. Each of these subclasses contains a common glycan core consisting of four glucose residues and one methylated rhamnose (Burguière et al., 2005). The structures are shown in Figure 2.1.

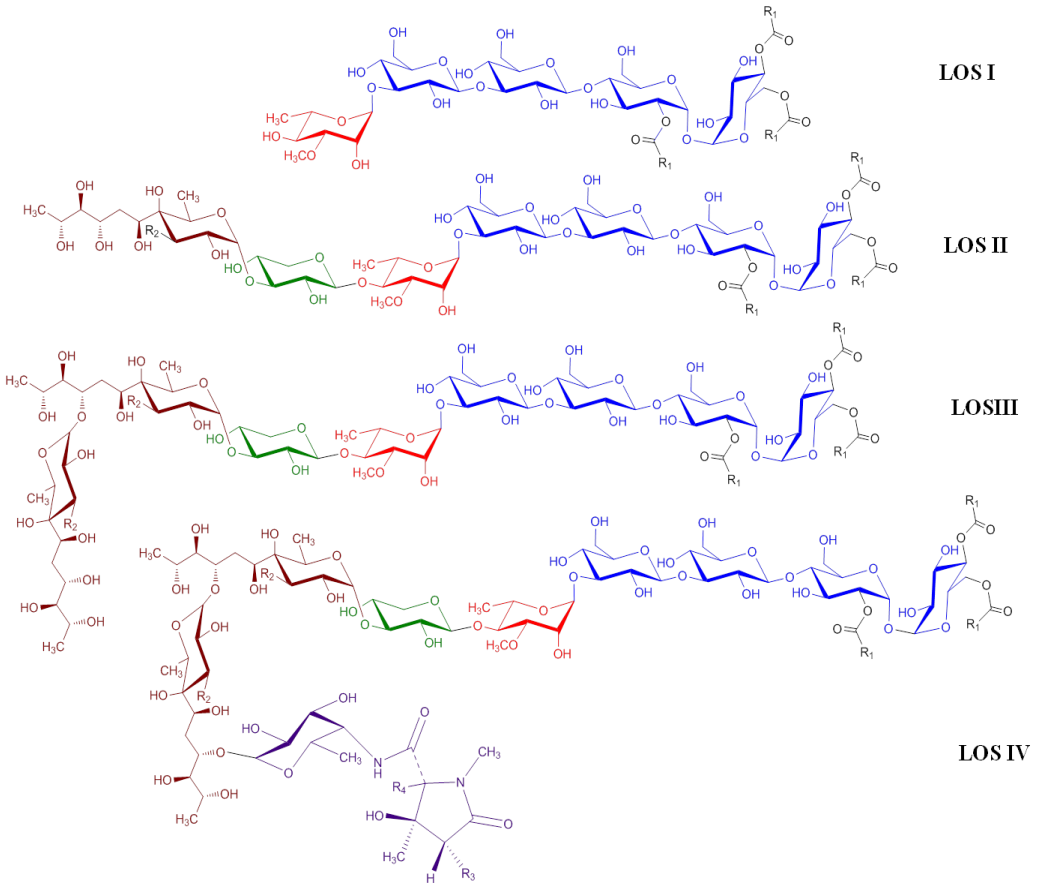


Figure 2.1. Structures of LOS-I to LOS-IV from *M. marinum.* The tetraglucose core (in blue) with the methylated rhamnose (red) in LOSI , with a xylose (red) and caryophyllose (brown) in LOSII, an additional caryophyllose in LOSIII , and LOSIV with the terminal N-acylated 4,6, dideoxygalactose (purple) . 'R$_1$' in the core stands for either 2,4,-dimethylhexadecanoate, or 2,4-dimethyl-2-pentadecenoate; and R$_2$ is either -H or –OH, R$_3$ is either –H or -OCH$_3$ and R$_4$ is –H or –COOH.

A **D**-Xyl*p* residue is found in LOS-II, LOS-III and LOS-IV in addition to the glycan core. LOS-II contains a further caryophyllose sugar that was initially referred to as sugar 'X'(Burguière et al., 2005), and now has been characterized to be a 3,6-dideoxy-4-*C*-(**D**-*altro*-1,3,4,5–tetrahydroxyhexyl)-**D**-*xylo*-hexopyranose (Rombouts et al., 2009), while LOS-III and LOS-IV contain two caryophyllose residues, with LOS-IV containing an extra, sugar that has been characterised as an N-acylated-α-amino-4,6-dideoxy galactose (Rombouts et al., 2010). The glycan

core is also acylated, predominantly with a dimethylated $C_{18:0}$ fatty acid, and to a lesser extent with a range of non-methylated and dimethylated C_{15}-C_{20} fatty acids (Burguière et al., 2005). By isolating *M. marinum* transposon mutants with altered colony morphology, Ren et al., (2007) identified the genetic locus for LOS biosynthesis.

Surprisingly, *M. tuberculosis* H37Rv which is not known to produce LOSs, contains a similar locus which is however missing a number of genes that are present in the equivalent locus in *M. marinum*. *MMAR2313 (losA*), encoding a glycosyl transferase was the first mycobacterial gene demonstrated to play a role in LOS biosynthesis. A *losA* transposon mutant was reported to be defective in synthesising LOS-IV. This suggested that LosA was involved in the transfer of the N-acylated-α-amino-4, 6-dideoxy galactose to LOS-III resulting in the formation of LOS-IV (Burguière *et al* 2005). The only other LOS biosynthesis genes identified by mutational analysis in *M. marinum* include *MMAR2309* (encoding a UDP-glucose dehydrogenase) and *MMAR2332* (encoding a carboxylase); disruption of either gene led to the accumulation of intermediates. Further progress in delineating LOS biosynthesis pathways in *M. marinum* has recently been made by comparative study on a *M. marinum* strain Mma7 that fails to produce LOS-IV and instead accumulates large quantities of LOS-III (Rombouts *et al*., 2009). *M. marinum* Mma7 contained a chromosomal deletion that extended from the 3´-end of *losA* to *MMAR2318*, suggesting that genes in this region were likely involved in the biosynthesis of the terminal N-acylated-α-amino-4,6-dideoxy galactose that is found in LOS-IV. Based on the sugar rich

composition of LOSs, numerous glycosyltransferases (GTFs) potentially play a substantial role in LOS biosynthesis pathways.

However, at the start of the studies described in this chapter, *losA* remained the only GTF-encoding gene shown to be involved LOS biosynthesis in *M. marinum*. The gene cluster involved in LOS biosynthesis in *M. marinum* has five GTFs as shown in Table 2.1.

Table 2.1. Predicted glycosyltransferases in the LOS biosynthesis gene cluster in *M. marinum*.

Gene	Product / type	Conserved domains
MMAR 2311	Glycosyltransferase / type II	S-adenosyl methionine dependant transferase / DPM 1 synthase
MMAR 2313 (*losA*)	Glycosyltransferase / type II	Bacterial DPM 1 synthase
MMAR 2333 (*wcaA*)	Glycosyltransferase / type II	Bacterial DPM1 synthase
MMAR 2349 (*wbbl2*)	Glycosyltransferase / type II	Rhamnosyltransferase domain
MMAR 2351	Glycosyltransferase / type II	DPG synthase

The protein encoded by *MMAR2311* is a type II GTF and contains a methyltransferase domain at the N-terminus and a type II DPM1 like domain at the C-terminus. These GTFs are known to transfer sugars to various substrates like dolichol-phosphate. *MMAR2313 (losA)* is involved in the transfer of a dideoxy-galactopyranose residue to LOS-III to form LOS-IV. Burguiére et al., (2005) reported that inactivation of *losA* results in the inability of the mutant strain to produce LOS-IV. *MMAR2349 (wbbl2)* posesses a rhamnosyltransferase domain and belongs to type 2 GTFs. *MMAR2351* is a type 2 GTF with a dolichol

phosphate mannose - dolichol phosphate glucosyltransferase (DPM-DPG) synthase like domain. The effects of disruption of *MMAR2351* are described in Chapter 4 of this thesis. A transposon mutant disrupted in *MMAR2332* was found to be defective in LOS biosynthesis (Ren et al.,2007). Given the proximity of *MMAR2333* to genes involved in LOS biosynthesis it was likely that the *MMAR2333*-encoded GTF was involved in the transfer of one or more sugar residues found in *M. marinum* LOSs. In this study we report the identification and characterisation of a null mutant of *M. marinum MMAR2333* generated by specialised transduction, a highly efficient, phage-based, knockout methodology that has proven successful in generating targeted mutants in other mycobacteria. The mutant strain was also assessed for changes in virulence by infecting cultured bone marrow-derived murine macrophages, and zebrafish larvae.

2.2 Materials and Methods

2.2.1 In silico analysis of MMAR2333 (WcaA)

The *MMAR2333 (wcaA)* gene sequence was obtained from http://genolist.pasteur.fr/MarinoList. Multiple sequence alignments of WcaA from different mycobacterial species were rendered using CLUSTAL W (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and ESPript (http://espript.ibcp.fr/ESPript/ESPript/). Expasy server (http://expasy.ch/) and Mobyle portal (http://mobyle.pasteur.fr/cgi-bin/portal.py?#forms::toppred) were used to predict the transmembrane domains.

2.2.2 Plasmids, DNA manipulations and bacterial growth conditions

Plasmids, bacterial strains and phages used in this study are listed in Table 2.1. *Escherichia coli* strains were routinely cultured in Luria-Bertani (LB) broth at 37ºC. *M. smegmatis* strain mc^2155 was used for generation and propagation of mycobacteriophages and was routinely grown at 37°C in Tryptic Soy Broth with 0.05% Tween-80. All *M. marinum* strains were grown either in 7H9 broth supplemented with 10% OADC (oleic acid/albumin/dextrose/catalase, BD) or in 7H10 broth (composition based on Middlebrook's 7H10 agar components) supplemented with 10 % OADC, at 30°C, with 0.05% Tween-80. *M. marinum* 1218R (ATCC 927) was used as the wild type (parental) strain for generation of the *MMAR2333* null mutant. For plate growth, 7H10 agar plates were incubated at either 30°C, or when required, at 37°C (for selecting transductants; see below). For experiments involving the usage of phages, Tween 80 was not used in media. Mycobacteriophages were routinely propagated on soft agar lawns of *M. smegmatis* mc^2155 on basal Middlebrook's 7H9 agar with 0.2% glycerol. Phage high titres were generated using protocols described by Larsen et al., (2007).

Antibiotics were added as required: Hygromycin B (Roche) 150µg/ml for *E. coli*, 100µg/ml for *M. smegmatis*, 75µg/ml for *M. marinum*. Kanamycin sulfate (Sigma) 50µg/ml for *E. coli* and 25µg/ml for *M. marinum*, Apramycin 25µg/ml for *M. marinum.*

Table 2.2. Bacterial strains, plasmids and phages used in this study

Plasmids, phages and strains	Description	Reference
Plasmids		
p0004S	Cosmid vector containing Hyg-SacB cassette	(Larsen et al., 2007)
p∆*MMAR2333*	Derivative of p0004S obtained by cloning the right and left PCR flanks of MMAR2333	This work
pMV261	Kan^R , *E. coli*-mycobacterial shuttle vector (ColE1 *oriM* P*hsp60*)	Stover *et al.*, 1991
pMV261-*MMAR2333*	pMV261 containing a copy of the gene *MMAR2333*	This work
pMSP12-dsRed-Apr	*E. coli*-mycobacterial shuttle plasmid, containing dsRed and Apr^R	J. Chen Ph.D. thesis, University of Birmingham 2010
Phages		
phAE159	Conditionally replicating shuttle phasmid derived from lytic mycobacteriophage TM4.	(Larsen et al., 2007)
ph∆*MMAR2333*	Derivative of phAE159 obtained by cloning *p∆MMAR2333* into its PacI site	This work
Bacterial strains		
E.coli TOP 10	F– *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen
HB101	*E. coli* K-12 *F__*(*gpt-proA*)62 leuB1 glnV44 ara-14 galK2 lacY1 hsdS20 rpsL20 xyl-5 mtl-1 recA13	Stratagene
M.smegmatis mc^2155	Wild type strain, E*pt* mutant of *M. smegmatis* strain mc^26	Snapper et al., 1990
M. marinum 1218R	Wild type strain of *M. marinum*	ATCC927
Δ*MMAR 2333*	*M. marinum* strain with replacement of *MMAR2333* with *hyg*	This work
Δ*MMAR2333*-C	Δ*MMAR2333 c*omplemented with pMV261-*MMAR2333*.	This work

2.2.3 Construction of knockout phage for deletion of *MMAR2333*

Approximately 1 kb sequences of the upstream and downstream regions of *MMAR2333* were PCR-amplified from *M. marinum* 1218R genomic DNA using the primer pairs listed in Table 2.3. The PCR products were purified and the

primer incorporated *Van*91I sites were digested with *Van*91I, following which the digested PCR fragments were cloned into *Van*91I-digested p0004S to generate the allelic exchange plasmid pΔ*MMAR2333*. Plasmids obtained by miniprep (Qiagen miniprep kit) were digested with *Van*91I and *Pac*I and sequenced to confirm presence of the left and right flanks. One positive plasmid was packaged into the temperature sensitive phage, phAE159. *Pac*I digested pΔ*MMAR2333* was ligated to *Pac*I digested phAE159 DNA. The ligation mix was then packaged into empty λ-phage heads and transduced into *E. coli* HB101. Cells containing phasmid DNA were selected for on LB agar containing Hygromycin at 37°C. Packaging of pΔ*MMAR2333* into phAE159 was confirmed by PacI digestion.The phasmid DNA was subsequently used to generate the phage phΔ*MMAR2333* using protocols described by (Larsen et al., 2007).

Table 2.3. Primers used for generation of knockout construct in *M. marinum*

Primer	Sequence 5' -3'	Description	Size
MMAR2333_LL	TTTTTTTTCCATAAATTGGGTCTGCACCGGCTACAAGAG	left side flank sequence of MMAR2333	960 bp
MMAR2333_LR	TTTTTTTTCCATTTCTTGGGTCCTGGGTGGGCTGAAGTA		
MMAR2333_RL	TTTTTTTTCCATAGATTGGCGTGGGTCGGCTTCAAATCG	Right side flank sequence of MMAR2333	902 bp
MMAR2333_RR	TTTTTTTTCCATCTTTTGGCTTCAGACGGACCTGTGGGT		

The positive phasmids were transformed by electroporation into *M. smegmatis* at 1800V and recovered at 30°C for ~4 hours in TSB. The recovered cells were then harvested and resuspended in 200µl of MP buffer. This was mixed with 200µl of freshly growing *M. smegmatis* and 5ml molten soft agar (50°C) and poured on 7H9 basal agar plates and incubated at 30°C for 2 -3 days and allowed

to form plaques. The plates were soaked in minimum amount of MP buffer for 5-6 hours and the solution containing phages was filtered and stored at 4°C. This generated the recombinant phage - ph∆*MMAR2333* designed to replace the gene *MMAR2333* with *hyg*.

2.2.4 Generation of a *MMAR2333* null mutant

Specialised transduction of *M. marinum* 1218R was performed as described previously for other mycobacteria (Bardarov et al., 2002). *M. marinum* cultures were grown in 50ml of 7H9+10% OADC (0.05% Tween 80) to an OD_{600} of about 0.8 and harvested by centrifugation at 4500xg for 10 minutes. The cell pellet was washed twice with 50ml of MP buffer. Finally the pellet was resuspended in 2ml of MP buffer and 2ml of high titre (10^{-10} pfu /ml) phage lysate was mixed with the cells. A control was set up where 0.5 ml of resuspended cells were mixed with 0.5ml of MP buffer. The mix was incubated overnight at 37°C followed by harvesting and recovery with 10ml 7H9+10% OADC with Tween-80 overnight at 37°C. This was plated onto 7H10+10% OADC-agar plates with hygromycin B and plates were incubated at 37°C for 2 weeks. Hygromycin resistant colonies obtained after transduction of *M. marinum* 1218R (wild type strain) were inoculated in 10ml 7H9+10% OADC -Tween 80 with hygromycin B for genomic DNA extraction and further characterisation. Allelic exchange of *MMAR2333* with a hygromycin resistance cassette in hygromycin resistant transductants was confirmed Southern blot. One such transductant was selected for subsequent experiments.

Restriction enzymes were selected based on the sequence of the knockout plasmids. For Δ*MMAR2333* the restriction enzyme used was *Van*91I. The wildtype genomic DNA was also digested with Van91I. Following digestion, the genomic DNA fragments were separated by gel electrophoresis. In the mutant strains the gene is replaced by a *hyg-SacB* gene, thus after digestion the expected sizes were 7123bp, 8316bp and 10,373 bp whereas in the wildtype the fragments sizes would be 3823 bp and 6971bp. PCR products of the left and right flanks of the gene were used as probes. The procedure was performed as described as suggested by manufacturer's guidelines in (DIG High Prime DNA Labelling and Detection Starter Kit II; cat no – 11585614910, Roche). This kit uses digoxigenin, a steroid to label DNA probes by random priming. The hybridized probes are then detected by anti-digoxigenin-AP (Fab fragments), and subsequently visualised by chemiluminescence.

2.2.5 Generation of complemented strain of Δ*MMAR2333*

The *MMAR2333* ORF was PCR amplified from *M. marinum* 1218R genomic DNA using the primers listed in Table 2.4. Using the primer incorporated *Bam*HI and *Hin*dIII restriction sites, the PCR product was cloned into the *E. coli-Mycobacterium* shuttle vector pMV261 (containing the kanamycin resistance cassette *aph*) (Stover et al., 1991) and verified by sequencing. The resultant plasmid pMV261-*MMAR2333* was introduced by electroporation into *M. marinum* Δ*MMAR2333* and transformants were selected on 7H10-agar with kanamycin. One such transformant was named Δ*MMAR2333*-C and was used for further characterisation.

Table 2.4. Primers used for generation of complemented strains

Name	Sequence (5' → 3')	Product	Size
MMAR2333_F	GCAGGATCCGGTGAGCGTGGGAGTGGGTG *	MMAR2333 gene sequence	957 bp
MMAR2333_ R	GCGAAGCTTTCACATGCCCACCTTTCGAG #		

*Underlined sequence shows *Bam*HI restriction site, # Underlined sequence shows *Hind*III restriction site

2.2.6 Analysis of growth patterns

Growth of the wild type *M. marinum* 1218R, mutant Δ*MMAR2333* and complemented strain Δ*MMAR2333*-C was monitored at O.D. 600nm over a period of 0 to 60 hours at intervals of 12, 24, 36 ,48 60 hours. All the strains were grown in 7H10 broth +10% OADC with 0.05% Tween 80 and appropriate antibiotics at 30° C. The starting O.D. for all the cultures was 0.1.

Mid-log cultures of the bacterial strains were streaked on 7H10 +10% OADC agar plates. The plates were incubated for 5 - 8 days at 30°C and isolated single colonies were observed under a binocular microscope.

2.2.7 Extraction and analysis of *M. marinum* lipids

For labelling lipids with [^{14}C], *M. marinum* strains were grown to mid-logarithmic phase in 10ml of Middlebrook's 7H10 broth at 30 degrees in a shaking incubator, following which 50 µCi of [1,2-^{14}C] acetate (57 mCi/mmol, GE Healthcare, Amersham Bioscience) was added to the culture and the incubation was continued for another for 24 h. The labelled bacterial cells were harvested, washed, and freeze-dried, and polar and apolar lipids were extracted and analysed by 2D-TLC

(Dobson et al., 1985). [^{14}C]-labelled lipids were visualised by autoradiography by exposing a Kodak BioMax MR film to the TLC plates for 3 days.

2.2.8 Purification of LOS-U

For purification of LOS-U, 400 mg of polar lipids, extracted from 40 g dried cells, were applied to a DEAE cellulose column. The column was eluted with 500ml $CHCl_3$:CH_3OH (2:1 v/v) and 10 ml fractions were monitored for elution of LOS-U by TLC ($CHCl_3$:CH_3OH:H_2O; 60:30:6;v/v/v) and spraying the plates with α-naphthol/sulfuric acid followed by charring revealed the sugar containing lipids. LOS-U containing fractions were pooled, concentrated and further purified by preparative TLC on a 10 x 20cm plastic backed silica gel TLC plates (Merck) run in $CHCl_3$:CH_3OH:H_2O (60:30:6, v/v/v). The plates were sprayed with 0.01% 1,6-di-phenyl-1,3,5-hexatriene in petroleum ether /acetone (9:1,v/v) and the glycolipids were visualised under UV light (wavelength 366nm), and the area was marked with a pencil. The plates were then developed in toluene to remove the 1,6-di-phenyl-1,3,5-hexatriene and after drying, the marked area was scraped from the TLC plates, extracted with $CHCl_3$:CH_3OH (2:1, v/v) and Per-*O*-methylation of LOS-U was performed (described in Chapter 8). MALDI-MS and ES were performed by Dr. P.R. Ashton and M r N.G. May (School of Chemistry) and described in Chapter 8.

2.2.9 Extraction of bone marrow derived macrophages (from Balb/c mice) and infection by *M. marinum*

Bone marrow derived macrophages (BMDM) were obtained as described in Chapter 8. 24 hours before infection, the BMDM were activated using

recombinant mouse INF-γ (Invitrogen) at a concentration of 1000units/ml. The concentration of BMDM used for this study was 1.0 X 10^6 cells / ml.

Late log phase bacteria (*M. marinum 1218R*, Δ*MMAR2333*, Δ*MMAR2333*-C) were washed with PBS and resuspended in infection medium (DMEM with 10% FBS) to 10^7cfu/ml. Murine bone marrow macrophages treated with INF-γ at 1000U/ml (Invitrogen) was infected with *M. marinum* strains at an MOI of 10 and incubated at 37^0C under 5% (v/v) CO_2 for 3-4 hours. The infection medium was removed and the cells were washed twice with PBS and once with complete medium (DMEM with 10% FBS and 50μg/ml gentamicin). Cells were incubated in 1ml complete medium at 37^0C under 10% (v/v) CO_2 overnight. On Day 1, the supernatant was removed and cells were washed once with PBS. Fresh complete medium without gentamicin was added. Cells were incubated at 37^0C under 10% (v/v) CO_2 for 1 hour. This supernatant was collected as Day 1 sample for future cytokines study and cells were lysed with 1ml PBS with 0.1% TritonX-100. Samples were collected on Day 3 and Day 5. Cell lysates as well as infection medium were 10 fold serial diluted and plated out on 7H10 agar with OADC. The plates were incubated at 30^0C for 1 week before counting colonies. Infection with each strain was triplicated. TNF-α levels in the infection supernatant was estimated according to manufacturer's instructions using Quantikine^R Mouse TNF-α Immunoassay Kit (MTA00B), RnD Systems.

2.2.10 Generation of a ds-Red *M. marinum* strain for visualizing infection in zebrafish embryos.

The plasmid pMSP12-ds-Red-Apr was obtained as a gift from J. Chen and A. Bhatt, University of Birmingham, for this study. The plasmid was electroporated into the *M. marinum* 1218R (wild type), mutant strain Δ*MMAR2333* and Δ*MMAR2333*-C (complemented strain) to generate red-fluorescent labelled bacteria. The electroporated strains were selected on apramycin (30μg/ml). The positive clones were grown in 7H9 the culture visualized by fluorescence microscopy. Images were captured using Photometric Sensys CCD camera and Smart Capture 2 software (Digital scientific Ltd UK), courtesy Dr. Sue Armstrong, University of Birmingham. The red-fluorescence bacteria were used for infecting zebrafish embryo. The zebrafish infection studies were performed by our collaborators Dr. A. Van der Woude, VUMC, Amsterdam, The Netherlands.

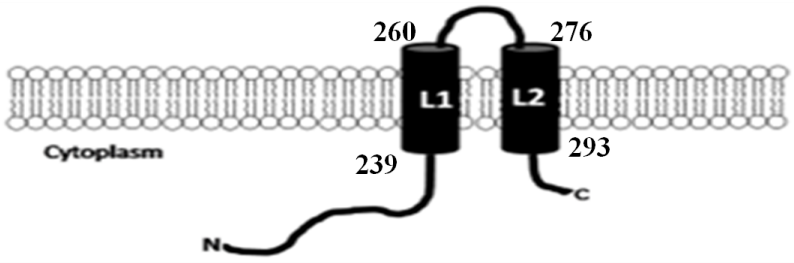
2.3 Results

2.3.1 *In silico* analysis of MMAR2333

The gene *MMAR2333* encodes a putative glycosyltransferase (GTF) and is present in a locus containing genes shown to be involved in LOS biosynthesis. It is annotated as WcaA in the *M. marinum* M genome due to similarity in domains with a GTF in enteric bacteria *E. coli* K-12 involved in the biosynthesis of the exopolysaccharide colanic acid (Stevenson et al., 1996). The protein is predicted to contain two transmembrane domains located near the C-terminus (Figure.2.2), which suggests that the enzyme is membrane associated. This is an attribute of many mycobacterial GTFs that associated with biosynthesis of cell wall components.

Additionally, MMAR2333 contains a characteristic domain found in eukaryotic dolichol-phophate mannose (DPM) synthases. Eukaryotic DPM synthases are members of the GTF-2 super family that catalyse the transfer of nucleotide sugars to dolichol phosphate. In bacteria, homologues of DPM synthases use polyprenol phosphate, rather than dolichol phosphate. Example of such an enzyme from *M. tuberculosis* is Ppm1, which catalyses the transfer of Mannose (Man) from GDP-Man for subsequent use as substrate for biosynthesis of LAM (Gurcha et al., 2002). The MMAR2333 protein sequence contains the characteristic residues found in GTF type II, including a conserved DXD motif (Figure.2.2). Interestingly, the best matches obtained from a BLAST search using the MMAR2333 amino acid sequence as the query were putative GTFs from cyanobacteria, rather than than GTFs from other LOS-producing mycobacteria (Figure.2.3). Given the proximity of *MMAR2333* to genes involved in LOS

biosynthesis, it is likely that the *MMAR2333*-encodes a GTF that is involved in the generation of a lipid-bound sugar substrate, which can be subsequently utilised for the addition of one or more sugar residues found in *M. marinum* LOSs. To test this, we generated a null mutant of *MMAR2333* using specialised transduction, a highly efficient mycobacterial knockout method, not previously reported to be used in *M. marinum*. Phage delivery methods have earlier been used to generate transposon mutants in *M. marinum* (Alexander et al., 2004); this is the first report of the use of phages for delivering allelic exchange substrate for targeted gene knockouts in this species.



MSVGVGAGTKKKIAVIAPAYNESECVEELARQLAAVFDSEPAYDFEAIIVENGSTDDTMEKLLAIN 63
AADPRFKILQLARNFRMDGGLTAGLNVVDADAVVLMTADLQDPPDFIPDMIRAWEQGYENVY 122
GVVTERGGVGPIRRMNSQLFYWLAGRLTDDRITSNASDFRLVDRKVYEAVRLMDERNRFVRGL 182
FSWVGFKSIGLPMKRAPRFAGESKAYTFKVMDLAGKGILAHSYVPLRLITLTGFLLSAVAAIAVFV 257
LAVRFVFYGVPFPGFGSLVSLMLVGFGVITLLLGVVGEYLALIYEEVKQRPNFVVTRKVGM 318

Figure 2.2. Predicted topology of MMAR2333. The predicted transmembrane domains are denoted by L1 and L2. N, N terminus; C, C terminus. Amino acid residues in blue belongs to L1, and in red to L2. The residue numbers are shown next to the transmembrane helices.

Figure 2.3. Alignment of the MMAR2333 amino acid sequence with that from putative *Synechococcus* sp.glycosyltransferase (CYB1435) and the *Mycobacterium kansasii* homologue MKAN1150. Characteristic sugar binding residues are indicated below the alignment. The sequences spanning the transmembrane domains of MMAR2333 are indicated by bars above the sequence. Dots indicate gaps.Numbers indicate the amino acid co-ordinates of MMAR2333. Black boxes with white letters indicate identical amino acid sequences at the aligned position for all three proteins. Gray boxes indicate similar or identical residues for two of the three proteins at the aligned position; the bold sequence letters in these boxes indicate identical or similar residues at the aligned position.

2.3.2 Effect of deletion of *MMAR2333* on colony morphology

Colony morphology changes often relate to changes in cell wall lipid composition (Chen et al., 2006; Alexander et al., 2004). Thus, alteration in LOSs would be expected to alter colony morphology in the mutant strain. *M. marinum* 1218R, Δ*MMAR2333* and Δ*MMAR2333*-C strains were grown on Middlebrook 7H10 agar supplemented with 10% OADC in the presence of 0.05% Tween-80. The Δ*MMAR2333* mutant showed altered colony morphology as compared to the wild type strain (Figure 2.4) suggesting potential alterations in the cell wall. The Δ*MMAR2333* mutant had a rough colony. Colony morphology reverted to smooth type upon complementation with the wild type gene *MMAR2333* (Δ*MMAR2333*-C).

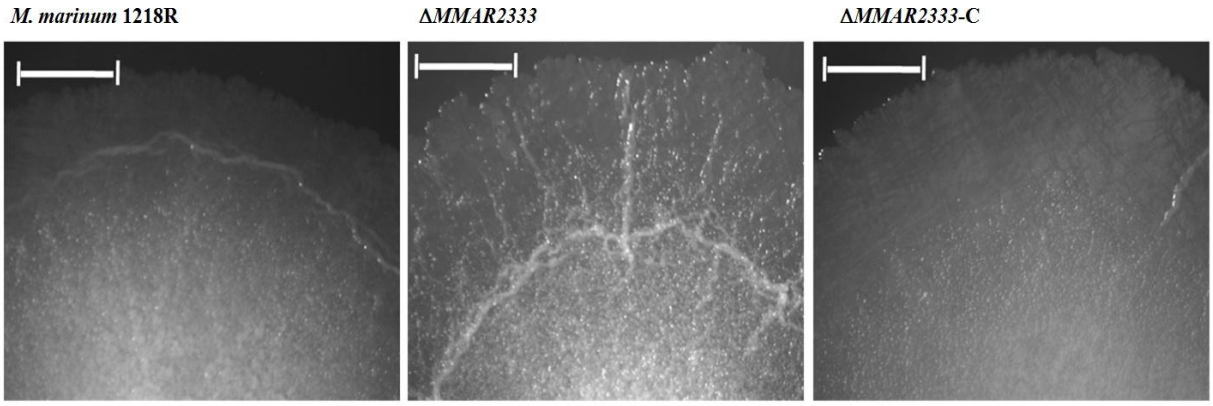


Figure 2.4. Colonies of *M. marinum* strains 1218R (wild type), Δ*MMAR2333* and Δ*MMAR233*-C grown on 7H10 agar supplemented with 10% OADC, after incubation at 30°C for 6-7 days. Scale bar represents 1mm.

2.3.3 Effect of *MMAR2333* deletion on growth characteristics in liquid media

Growth of wild type *M. marinum* 1218R (WT), Δ*MMAR2333* and Δ*MMAR2333*-C was compared in 7H10 broth supplemented with 10% OADC in the presence of 0.05% Tween-80. Growth of *M. marinum* 1218R was 1.31 after 60 hours at A_{600}, but the Δ*MMAR2333* mutant

strain reached to an O.D of 0.79 (Figure 2.5). However, introduction of plasmid borne wild type copy of the gene *MMAR2333* to generate a complemented strain Δ*MMAR2333*-C appears to restore the growth rates to wild type phenotype. The complemented strain reached an O.D. of 1.24 after 60 hours.

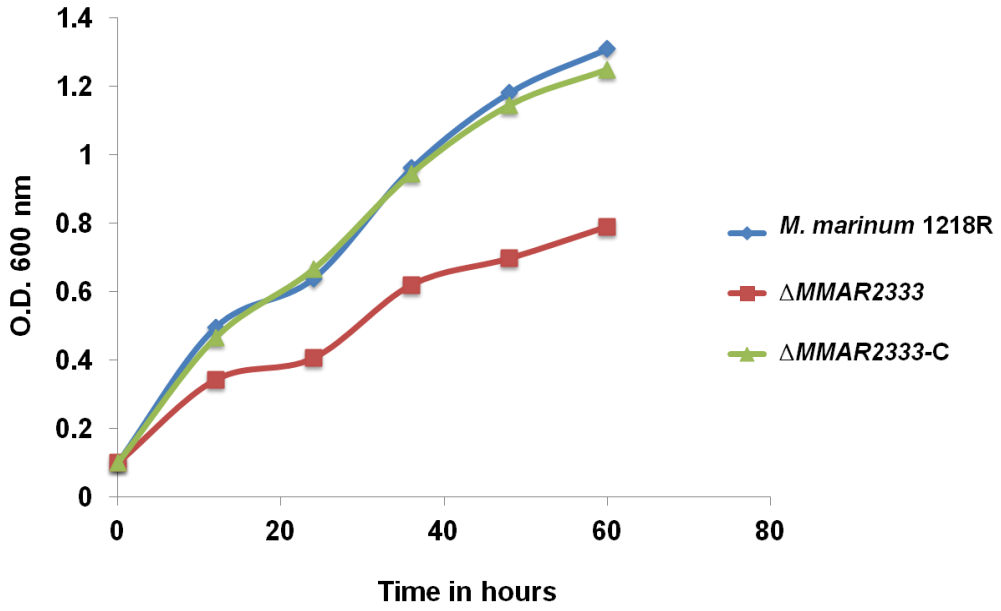


Figure 2.5. Consequences of deletion of *MMAR2333* on the growth of *M. marinum*. Growth curve of *M. marinum* 1218R, Δ*MMAR2333* and Δ*MMAR2333*-C strains in 7H10 broth medium supplemented with 10% OADC and Tween-80 at 12hours intervals up to 60 hours.

2.3.4 Analysis of total lipids from the Δ*MMAR2333* mutant

To assess the effects of loss of *MMAR2333* function on cell wall lipid composition, cultures of wild type, mutant and complemented strains were pulsed with [^{14}C]-acetate to label lipids. Labelled polar and apolar lipids were extracted and analysed by 2D-TLC using the five solvent systems as described by Dobson et al. (1985). Solvent system E is designed to separate LOSs and phospholipids. In order to visualize differences in the lipid profiles of the wild type and Δ*MMAR2333*, 2D-TLCs were developed in solvent system E. The Δ*MMAR2333* mutant

showed presence of LOS-I, while LOS-II, LOS-III and LOS-IV were missing and instead the strain accumulated, a [^{14}C]- labelled species that migrated to a position between that of LOS-I and LOS-II (Figure 2.6). Staining of TLC plates with α-naphthol / sulfuric acid revealed that this accumulated species was a glycolipid, and as its appearance was accompanied by the disappearance of LOS-II, III and IV. It was quite likely that the accumulated glycolipid was a LOS intermediate and we thus termed this unidentified lipid LOS-U (LOS-unknown, later confirmed to be LOS-II').

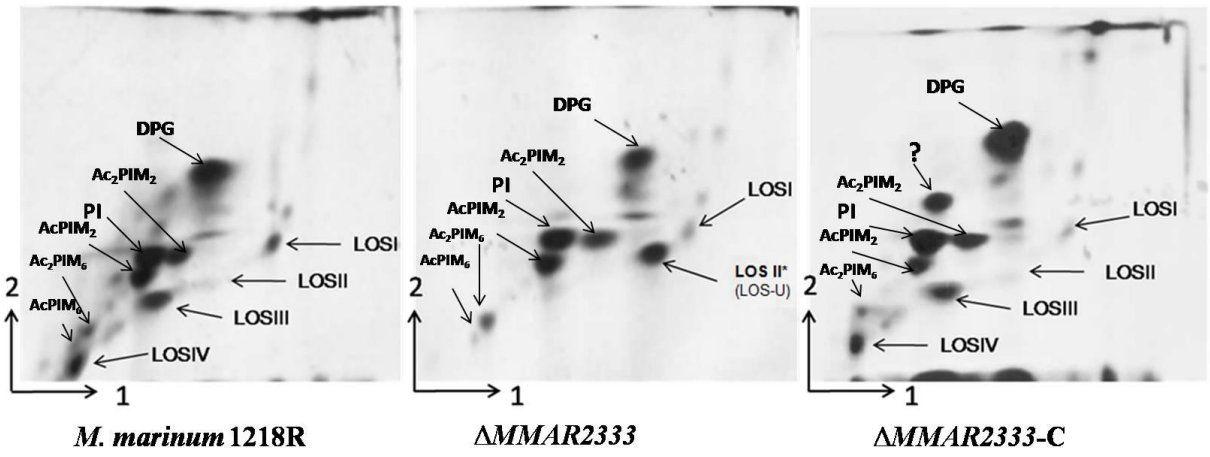


Figure 2.6. 2D TLC autoradiograph of ^{14}C labelled polar lipids from *M. marinum* 1218R, Δ*MMAR2333*, Δ*MMAR2333*-C grown in Middlebrook 7H10 broth. Direction 1- chloroform: methanol: water, 60:40:6 (v/v/v), direction 2- chloroform: acetic acid: methanol: water, 40:25:3:6 (v/v/v/v). Δ*MMAR2333* shows accumulation of a LOS species, named LOS-U (LOS-II*) and absence of higher LOSs. $AcPIM_2$ and Ac_2PIM_2, mono and di-acyl phosphatidyl-inositol dimannosides; $AcPIM_6$ and Ac_2PIM_6, mono and di-acyl phosphatidyl-inositol hexamannosides; LOS I-IV, lipoologosaccharides; PI, phosphatidylinositol; DPG, diphosphatidylglycerol; PE. Phosphatidylethanolamine; P, unknown phospholipids.

2.3.5 Characterisation of LOS-U

A LOS biosynthesis intermediate, LOS-II*, isolated from *M. marinum* MRS1178 (a transposon mutant of *MMAR2332*) was also reported to migrate to a position intermediate

between LOS-I and LOS-II on 2D-TLC plates (Ren et al., 2007). LOS-II* is a precursor of LOS-II and contains **D**-Xyl*p* attached to the glycan core, but the caryophyllose found in LOS-II is missing in LOS-II*. When ^{14}C-labelled lipids from the strains Δ*MMAR2332* and Δ*MMAR2333* were mixed and separated on the same 2D-TLC plate, LOS-II* and LOS-U migrated to same position, appearing as a single spot (Figure 2.7).

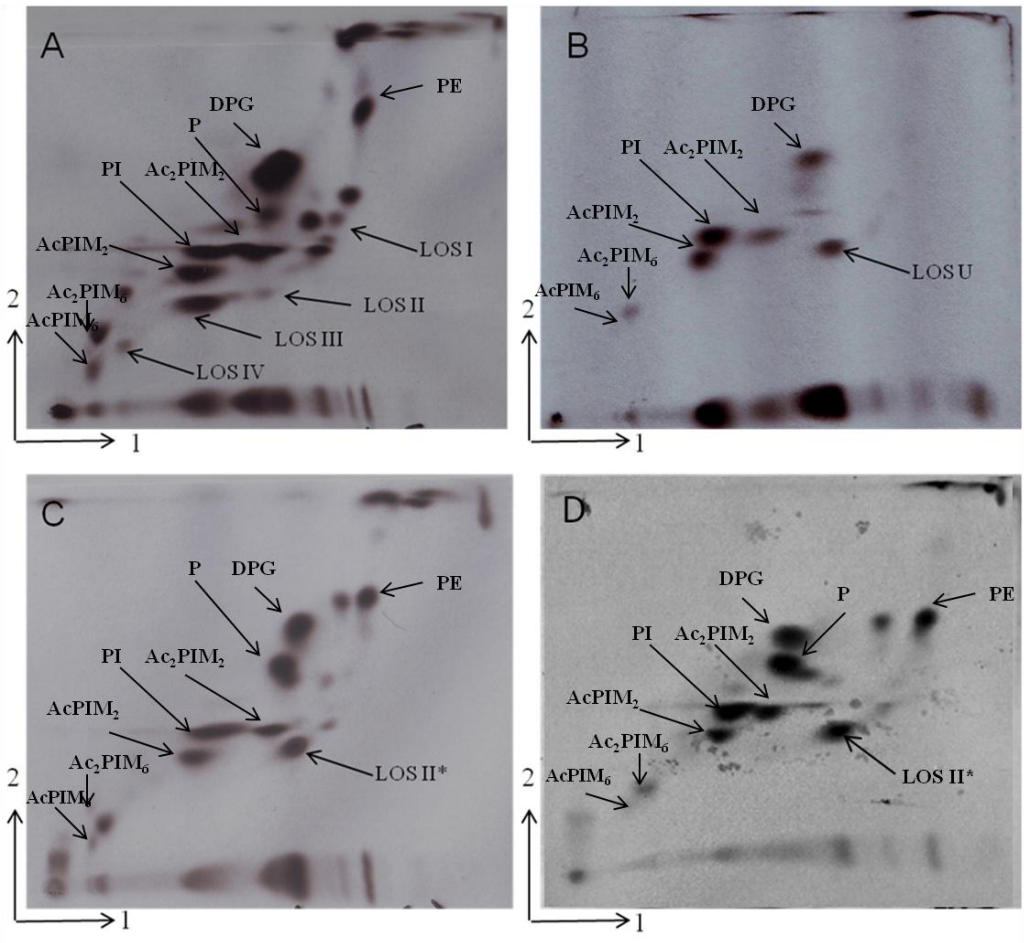


Figure 2.7 Two dimensional TLC autoradiograph of ^{14}C labelled *M. marinum* lipids. Polar lipids from (A) *M. marinum* 1218R, (B) Δ*MMAR2333*, (C) MRS1178 (*MMAR2332*::ϕMycoMar) and (D) a mix of polar lipids from Δ*MMAR2333* and MRS1178 (*MMAR2332*::ϕMycoMar) grown in Middlebrook 7H10 broth. Lipids were analysed by 2D TLC using solvent systems chloroform: methanol: water, 60:40:6 (v/v/v) in the first direction, and chloroform: acetic acid: methanol: water, 40:25:3:6 (v/v/v/v) in the second direction.

This suggests that LOS-U and LOS-II* were likely the same glycolipid species. In order to ascertain this by determining the chemical nature of LOS-U, we first purified LOS-U using a combination of column chromatography and preparative TLC, and per-*O*-methylated LOS-U was analysed by MALDI-MS.

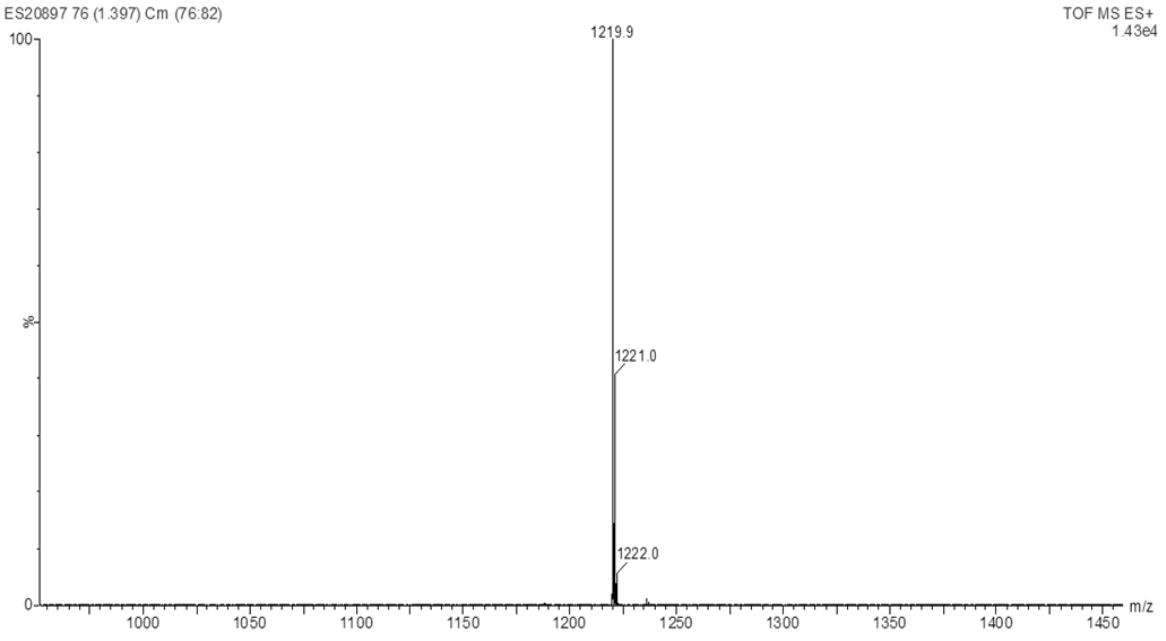


Figure 2.8. Mass spectrometric analysis of per-*O*-methylated LOS-II* isolated from Δ*MMAR 2333*. The accumulating LOS-U afforded a signal at 1219.9 *m/z* which corresponds to the previously isolated intermediate LOS-II*.

A prominent signal was obtained at *m/z* 1219.4 [M + Na] + (Figure. 2.8), which was identical to that obtained for LOS-II*. These results indicate that LOS-U, the intermediate isolated from Δ*MMAR2333*, was the precursor of LOS-II, LOS-II* and similar to the Δ*MMAR2332* transposon mutant, the Δ*MMAR2333* deletion mutant also accumulated LOS-II*. In other words, the addition of the first caryophyllose residue to the **D**-Xyl*p*-glycan core

did not take place in the Δ*MMAR2333* null mutant. Given the similarity of *MMAR2333* to DPM-like glycosyltransferases, these findings suggests the involvement of *MMAR2333* in the cytoplasmic transfer of nucleotide bound caryophyllose residue (or its precursor) to a polyprenol phosphate or other lipid-based carrier. This can be subsequently used as a sugar donor by another glycosyltransferase to extend LOS-II* to LOS-II (Sarkar et al., 2011).

Additionally the identical LOS-pattern of the *MMAR2332-Tn* mutant and Δ*MMAR2333* strain makes it likely that *MMAR2332*, which encodes a protein homologous to a putative thiamine pyrophosphate-containing carboxylase, is involved in the biosynthesis of the unique caryophyllose sugar.

2.3.6 Intracellular survival of *MMAR2333* null mutant in bone marrow derived macrophages

Inability of LOS-IV deficient *M. marinum* mutants in entering murine macrophages have been reported previously (Burguière et al., 2005). The mutant strain Δ*MMAR2333* accumulated an intermediate LOS-II* and was deficient in production of the higher LOSs, LOS-II, LOS-III and LOS-IV. In order to assess the role of the *MMAR2333* deficient strain in virulence an intracellular survival assay was performed with murine bone marrow derived macrophages. Macrophages were thus infected with Δ*MMAR2333* to determine the ability to enter and survive within the macrophages. Infection experiments were done using a multiplicity of infection (MOI) of 10. The survival of intracellular bacteria will be followed over a period of 1, 3 and 5 days. The number of intracellular bacteria (colony forming units) was enumerated

by lysing the macrophages and plating on 7H10 agar. The infection experiments were done in triplicates and were repeated thrice.

Transposon-mediated disruption of *MMAR2332*, also led to the accumulation of LOSII* and the ability of the mutant strain to survive inside cultured macrophages was not altered. Similarly in this case, no difference was observed in the ability of the mutant strain Δ*MMAR2333* to enter and survive inside the bone marrow derived macrophages (Figure 2.9).

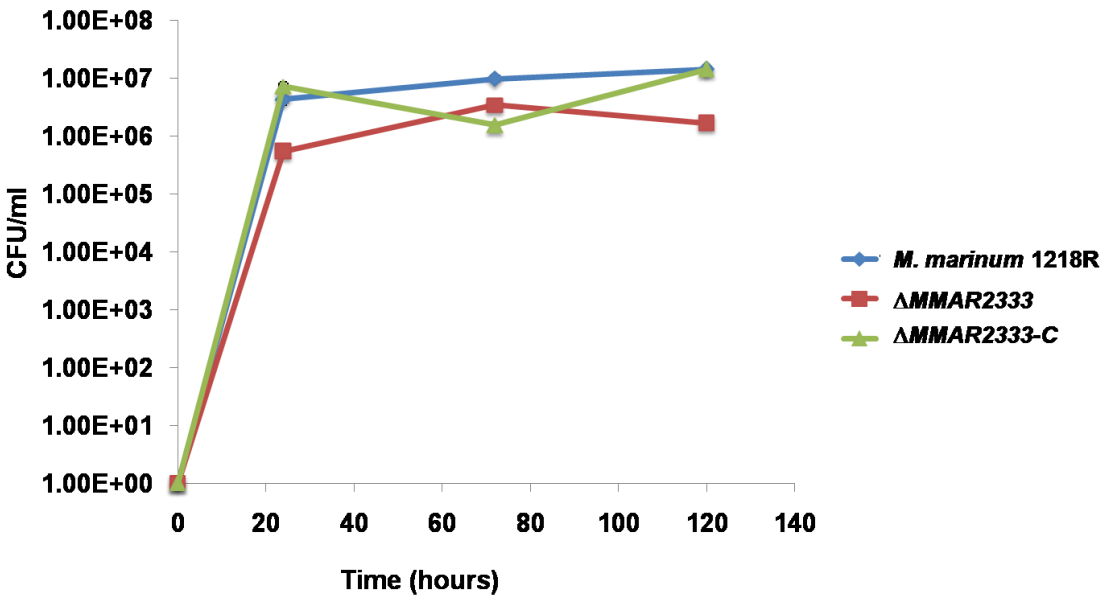


Figure 2.9. Survival of *M. marinum* strains in murine (Balb/c) bone marrow derived macrophages. BMDM cells were lysed and plated on 7H10 agar plates and the colony forming units /ml were enumerated.

Previous studies have shown that the loss of a glycolipid may not necessarily influence bacterial loads in infected cells but may alter cytokine signalling thereby influencing the

inflammatory response (Rao et al., 2005; Glickman et al., 2000). *M. tuberculosis* infection induces production of the proinflammatory cytokine TNF-α in macrophages and dendritic cells (Orme, 2004). TNF- α levels released by infected bone marrow derived macrophages were measured using a mouse TNF-α kit. Cell supernatant analysis for TNF-α release in infection experiment with the strain Δ*MMAR2333* appear elevated as compared to levels of TNF-α released by the wild type and complemented strains (Figure 2.10). TNF-α release is an indication of infection establishment, but in this case, the whole bacterium was used to infect BMDMs. Further studies with LOS deficient strains will provide more insight into the role of LOSs in virulence and details are discussed in Chapters 3 and 4 of this thesis.

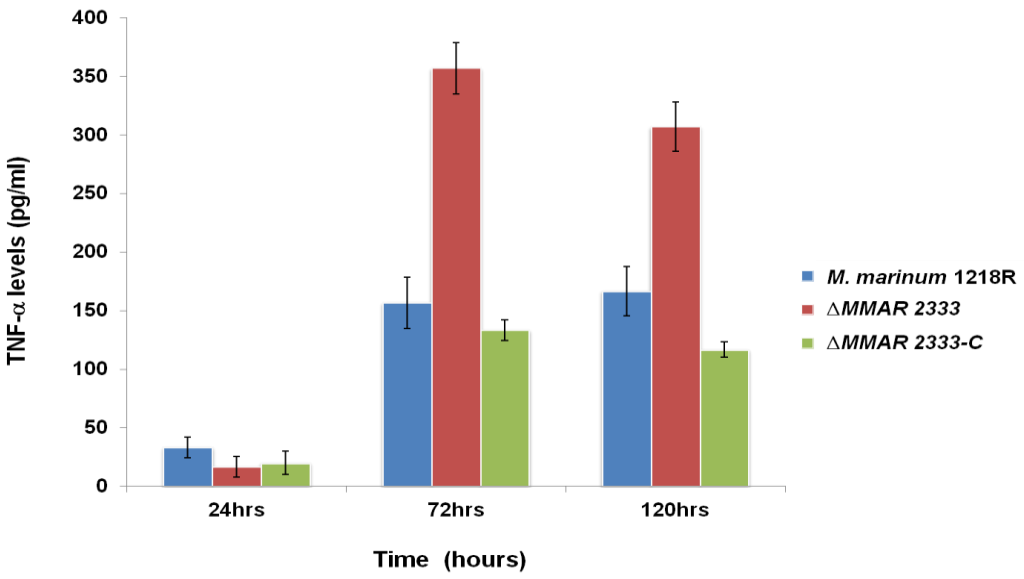


Figure 2.10. TNF- α production by murine (Balb/c) bone marrow derived macrophages infected with different *M. marinum* strains.

The natural host of *M. marinum* is the fish and hence to determine changes in virulence patterns, a whole animal model - the zebra fish-infection model was used. The *M. marinum*

strains were electroporated with ds-red plasmid (gift from J. Chen / A. Bhatt, University of Birmingham) with apramycin resistance and the ds-red positive clones were used for infecting zebra fish embryos. Infection experiments were repeated twice in triplicates by Dr. A. Van der Woude and colleagues (data shown was generated at VUMC, Netherlands).

2.3.7 Generation of ds-red strains of *M. marinum*

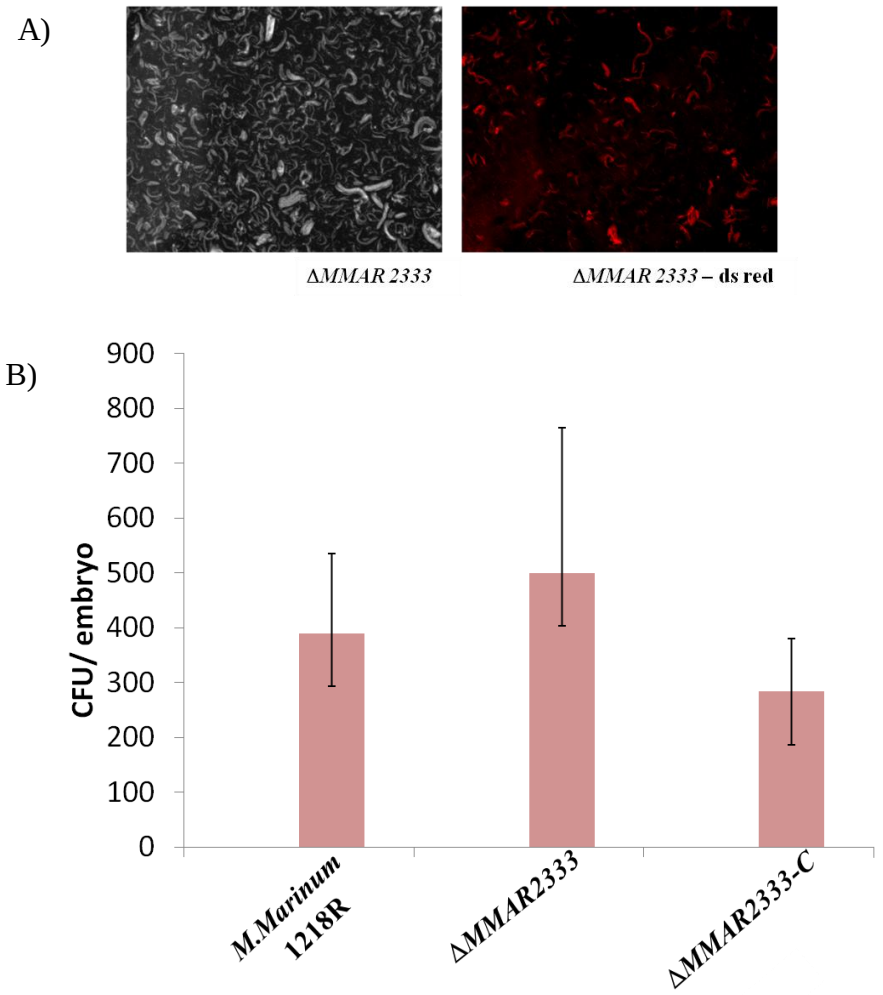


Figure 2.11 Survival of ds-Red *M. marinum* strains in zebrafish embryo. A) Δ*MMAR2333* with and without ds-red, viewed under a microscope under 40X zoom. B) Counts of intracellular bacteria (cfu) per embryo post infection with *M. marinum* strains. Data generated by Dr. Aniek van der Woude, VUMC, The Netherlands.

The plasmid pMSP12-ds-Red-Apr was electroporated into the *M. marinum* strains to generate red-fluroscence labelled bacteria. The apramycin resistant colonies were grown in 10% OADC supplemented 7H9 broth and visualized by fluroscence microscopy (Figure 2.11A). The red-fluorescence *M. marinum* 1218R, Δ*MMAR2333* and *MMAR2333*-C were used for infecting zebrafish embryo. Our collaborators Dr. A. Van der Woude, VUMC, Netherlands, did the zebrafish embryo infection. The survival of the *M. marinum* strains in terms of CFU / embryo is shown in Figure 2.11B.

No difference was observed in bacterial loads in zebrafish embryos infected with the wild type compared to those infected with the mutant strains suggesting that loss of LOS-II, LOS-III and LOS-IV and accumulation of LOS-II* in the mutant strain Δ*MMAR2333* did not affect the survival of the mutant in the above models of infection.

2.4 Discussion

M. marinum causes tuberculosis like disease in poikilothermic animals and is an opportunistic pathogen of humans (Ramakrishnan, 2004). *M. marinum* has a comparatively faster generation time and can be handled without BSL-3 facilities. These factors make *M. marinum* an attractive model to study mycobacterial pathogenesis (Cosma et al., 2003).

Mycobacterial cell wall associated lipids play a critical role in the virulence of pathogenic mycobacteria (Daffé and Draper, 1998). Recent studies have shown that approximately one third of the LOS gene cluster is conserved between *M. tuberculosis* and *M. marinum,* the former having the least number of genes. These lipooligosaccharides (LOSs) have been reported to play a role in sliding motility, biofilm formation and macrophage

infections (Ren et al., 2007). The basic structure of LOSs show that the core structure is formed of four glucose units with a methylated rhamnose. The higher LOSs contains complex sugars along with this basic structure and a number of sugar transferring enzymes are involved in their synthesis. In a previous study, many glycosyltransferases have been identified in the LOS biosynthesis gene cluster in *M. marinum* (Burguière et al., 2005) .

In this study, a glycosyltransferase involved in the LOS biosynthesis cluster in *M. marinum* have been identified. The gene *MMAR2333* encodes a glycosyltransferase and lies in the cluster identified to be involved in LOS biosynthesis (Burguière et al., 2005). This study also demonstrated the utility of Specialised Transduction to generate null mutants of *M. marinum.* While phage delivery methods have been used to generate transposon mutants in *M. marinum*, this is the first report of the use of phages for delivering allelic exchange substrates for targeted gene knockouts in this species.

Inactivation of *MMAR2333* led to an alteration in colony morphology. The mutant strain exhibited a rough colony as compared to the smooth type of the wild type strain. The smooth colony morphology reverted upon complementation with the wild type gene. The mutant strain was defective in the biosynthesis of three of the four subclasses of LOSs found in *M. marinum*, and accumulated a precursor of LOS-II, termed LOS-II*. The wild type phenotype was restored on complementation with a wild type gene indicating that the observed phenotypes in the mutant were solely due to loss of *MMAR2333* function

The polar glycolipid (LOS-II*) identified in our work corresponds to β-Xyl*p*-(1→4)-α-3-O-Me-Rha*p*-(1→ 3)-β-Glc*p*-(1→ 3)-β-Glc*p*-(1→ 4)-α-Glc*p*-(1→ 1)-α-Glc*p*. This was different from LOS-I in having a terminal Xyl*p* residue and also differed from LOS-II in

lacking the caryophyllose unit, described as 'X' in earlier work (Burguière et al., 2005). The LOS-II* corresponds to a previously described polar lipid accumulated by the MRS1178 mutant. The strain MRS1178 is disruptied in the gene *MMAR2332*, the protein product of which is a carboxylase belonging to a family of TPP-requiring enzymes. Acetolactate synthase belongs to this family of enzyme, and these are known to be involved in branched amino acid synthesis. Ren et al., (2007), however showed that *MMAR2332* was not involved in amino acid synthesis and was probably involved in the synthesis or transfer of the then unknown sugar moiety in LOS-II. The unknown sugar has now been reported to be a 3, 6-dideoxy-4-C-(**D**-*altro*-1, 3, 4, 5-tetrahydroxyhexyl) **D**-*xylo*-hexopyranose, which is termed Caryophyllose. This unit is a rather unusual 4-C- polysaccharide with a non-glycosidic linkage. These types of sugars have only been reported in a few gram-negative bacteria, and a similar sugar has been reported only in *Pseudomonas caryophylli* (Adinolfi et al., 1995).

The resemblance of MMAR2333 to bacterial DPM-like synthases suggested that the GTF was not directly involved in the transfer of caryophyllose to LOS-II*, but it was more likely to catalyse the transfer of mucleotide-bound caryophyllose to a lipid (polyprenol) carrier. Alternatively, MMAR2333 could catalyse the formation of a polyprenol-bound precursor of caryophyllose, which is subsequently modified to caryophyllose. *In vitro* confirmation of the above functions could not be accomplished due to the unavailability of nucleotide-bound caryophyllose substrates. In addition to *MMAR2333,* 2 other ORFs *MMAR2311* and *MMAR2313* (*losA*) also encode DPM-like GTFs. The presence of this class of glycosyltransferases in LOS biosynthetis cluster suggests that some of these enzymes maybe involved in the generation of lipid bound sugar substrates which are 'flipped' to the extracytoplasmic side of the membrane. These lipid bound sugar substrates can then be used

by extracytoplasmic glycosyltransferases to extend the oligosaccharide moiety of LOSs.

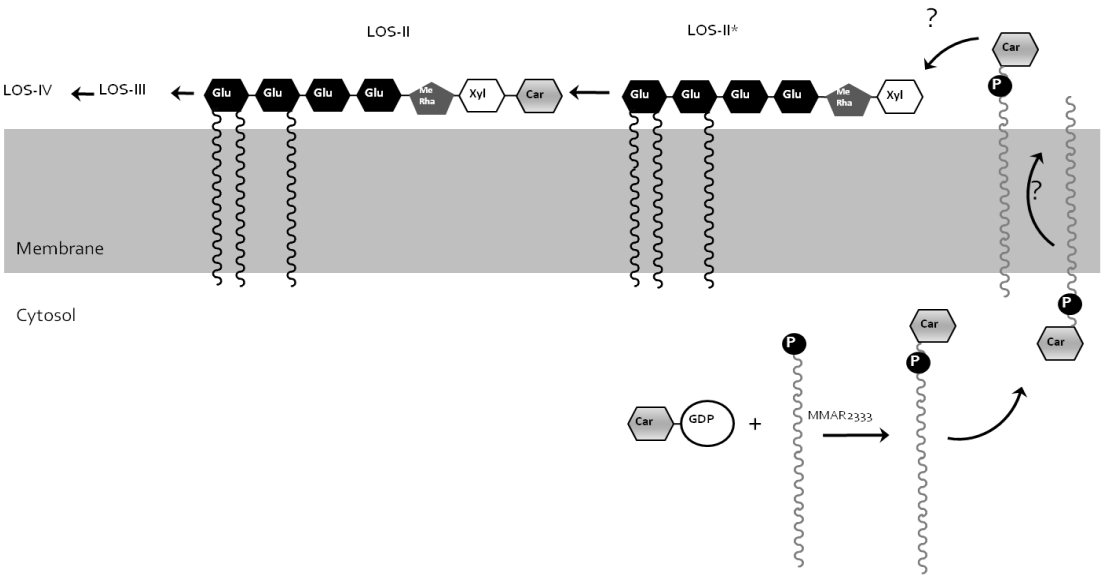


Figure 2.12 Proposed pathway of LOS biosynthesis in *M. marinum* showing the involvement of MMAR2333 in transferring a nucleotide sugar (caryophyllose) to a polyprenol unit. The polyprenoid bound caryophyllose is then used by other enzymes to finally add the caryophyllose to the LOS-II*.

Indeed, the LosA mutant is devoid of LOS-IV suggesting that LosA could likely be involved in the generation of a lipid-bound N-acyl 4,6 dideoxygalactose which is subsequently transferred by an extracellular glycosyltransferase to LOS-III. This affords a model for LOS biosynthesis (Figure 2.12), wherein the acylated hexasaccharide LOS-II* is synthesised intracellularly and transported across the membrane. This process could be initiated by *MMAR2342*, which encodes a transmembrane protein belonging to a group of larger mycobacterial proteins termed MmpLs, which are involved in the transport of mycobacterial glycolipids or their intermediates (Camacho et al., 2001; Converse et al., 2003; Domenech et al., 2004; Sonden et al., 2005). LOS-II* would then be extended by specific GTFs on the

extracytoplasmic side that use lipid-bound sugars (caryophyllose or the N-acyl 4,6 dideoxygalactose) as sugar donors to yield LOS-II, LOS-III and LOS-IV. In an alternative model, LOS-I could be transported by the MmpL protein to the extracytoplasmic side with the addition of xylose as the initial sugar, a process that would require the generation of a lipid-bound xylose substrate.

MMAR2311, the third putative DPM-like glycosyltransferase in the LOS cluster, is a potential candidate for this function. In summary, later stages of LOS biosynthesis may involve a distinct set of glycosytransferases catalysing the formation lipid bound sugar donors, and another set that extends the LOSs. The generation of mutants of *MMAR2311* and other putative glycosyltransferase genes in the LOS cluster will shed more light on the biosynthesis of these carbohydrate-rich mycobacterial glycolipids.

3

Role of a polyketide synthase gene (*pks5*) in lipooligosaccharide biosynthesis in *Mycobacterium marinum*

3.1 Introduction

Cell wall lipids play an important immunomodulatory role during infection by pathogenic mycobacteria. Lipooligosaccharides (LOSs) are one such class of polar lipid antigens found in some mycobacterial species. LOSs were first reported in *M. kansasii* (Hunter et al., 1983) an opportunistic pathogen, the fish pathogen *M. marinum* (Burguière et al., 2005) , *M. smegmatis* (Saadat and Ballou, 1983), *"M. canettii"* of the *Mycobacterium tuberculosis complex* (Daffé et al., 1991) and six other mycobacteria *M. gastri* (Gilleron et al., 1993; Gilleron and Puzo, 1995) , *M. gordonae* (Besra et al., 1993), *M. malmoense* (McNeil et al., 1987), *M. szulgai* (Hunter et al., 1988), *M. mucogenicum* (Munoz et al., 1998), and *M. butyricum* (Khoo et al., 1995b). LOSs are key factors contributing to biofilm formation, sliding motility, infection and infection of murine macrophages by *M. marinum* (Burguière et al., 2005; Ren et al., 2007). As discussed in the previous chapter, they possess an acylated trehalose core with various glycosylations. The trehalose in the core structure of LOSs is acylated by polymethyl branched fatty acids. These long chain methyl branches are a unique feature of mycobacterial cell wall lipids, some of which are involved in virulence e.g., phthiocerol dimycocerosate (DIM) (Cox et al., 1999; Camacho et al., 1999). The methyl branched component of DIM is synthesised by a specialised fatty acid synthase termed mycocersic acid synthase (*mas*) (Minnikin et al., 2002; Rainwater and Kolattukudy, 1983; Rainwater and Kolattukudy, 1985). MAS resembles a type-I fatty acid synthase, i.e. it is a single polypeptide carrying multiple domains required for the reductive cycles of fatty acid biosynthesis: an acyl transferase (AT) domain for loading of the starter unit; acyl carrier proteins (ACP) which

holds the growing chain; keto-acyl synthases (KS) which catalyse chain extension; keto-reductase (KR) and dehydratase (DH) domains (reduction to an alcohol and generation of unsaturated thiol ester, respectively); enoyl reductase (ER) for catalysing the final reduction step and a releasing thioesterase (TE). MAS is a multifunctional enzyme and has the domains KS-AT-DH-ER-KR-ACP (Mathur and Kolattukudy, 1992), and the KS and AT domains selectively utilises methyl-malonyl-CoA over malonyl-CoA for elongation (Fernandes and Kolattukudy, 1997).

The mycobacterial genome encodes many polyketide synthase genes including 7 MAS-like (*msl*) genes (Sirakova et al., 2001). Initially identified as Pks's, they are fatty acid synthases involved in biosynthesis of long chain or methyl-branched fatty acids. Pks's are involved in the synthesis of various glycolipids of *M. tuberculosis*. Pks2 was shown to be responsible for synthesis of the hepta and octa-methyl hydroxyphthioceranic acid which constitutes the major acyl chains of sulfolipids (Sirakova et al., 2001). Mycolipenic acids, which are a constituent of the penta-acyl trehalose (PATs) of *M. tuberculosis* are synthesised by *pks3/4* (Dubey et al., 2002) and pks12 was shown to be involved in mycoketide synthesis in *M. tuberculosis* (Matsunaga et al., 2004). A polyketide synthase gene was also shown to be involved in the synthesis of the lipopeptide core of GPLs in *M. smegmatis* (Sonden et al., 2005). A recent study also reported that the disruption of the *M. smegmatis* gene *MSMEG4727*, the *pks5* orthologue of *M. tuberculosis* resulted in a strain deficient in LOS and polymethyl-branched fatty acid production (Etienne et al., 2009).

Mycobacterial LOSs possess upto four methyl branched fatty acids and also species specific linear fatty acids. The LOSs from *M. kansasii* have three 2, 4-dimethyl tetradecanoic acid chains (Hunter et al., 1983) while LOSs from *M. marinum* are acylated by two different polymethyl-branched fatty acids, namely – 2,4-dimethylhexadecanoate and 2,4-dimethyl-2-pentadecenoate (Rombouts et al., 2011). This is reflected in the presence two separate *pks* genes and two polyketides associated acyl transferases (PapA3 and PapA4) in the LOS biosynthesis locus in *M. marinum*. Due to their similarity with the *M. tuberculosis* Pks5 the PKSs in *M. marinum* are annotated as *pks5* (*MMAR2340*) and *pks5_1* (*MMAR2344)* but are probably not functionally related to *M. tuberculosis pks5*. Additionally, acyltransferases encoded by polyketide associated proteins sharing high similarity to the PapA2 gene from *M. tuberculosis*, are annotated as *papA3* (*MMAR2355*) and *MMAR2343* was shown to be involved in acylation of the LOSs and termed *papA4* (Rombouts et al., 2011).

The studies outlined in this chapter were aimed at deciphering the role of *MMAR2340* and *MMAR2344* in the biosynthesis of the acyl chains found in *M. marinum* LOSs. I planned to do this by generating a null mutant of the *p*ks5 gene (*MMAR2340*) and of the *pks5_1* (*MMAR2344*) gene by specialised transduction. I was able to generate only the *MMAR2340* mutant using this method and analysis of the polar lipid profiles of the mutant strain are presented in this chapter. Also, the mutant was tested for changes in virulence using cultured murine bone marrow macrophages.

3.2 Materials and Methods

3.2.1 *In silico* analysis of MMAR2340 (Pks5)

The *MMAR2340 (pks5)* gene sequence was obtained from the Marinolist website (http://genolist.pasteur.fr/MarinoList). Multiple sequence files were produced using CLUSTAL W (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and rendered using ESPript (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi). Domain prediction was performed using PFAM (http://pfam.sanger.ac.uk/) and PKS-DB (http://www.nii.res.in/pksdb.html) web sites.

3.2.2 Plasmids, DNA manipulations and bacterial growth conditions

Plasmids, bacterial strains and phages used in this study are listed in Table 3.1. *E. coli* strains were routinely cultured in Luria-Bertani (LB) broth at 37ºC. *M. smegmatis* strain mc^2155 was used for generation and propagation of mycobacteriophages and was routinely grown at 37°C in Tryptic Soy Broth with 0.05% Tween-80. All *M. marinum* strains were grown either in 7H9 broth supplemented with 10% OADC (oleic acid/albumin/dextrose/catalase, BD) or in 7H10 broth (composition based on Middlebrook 7H10 agar components) supplemented with 10 % OADC, at 30°C, with 0.05% Tween-80. *M. marinum* 1218R (ATCC 927) was used as the wild type (parental) strain for generation of the *MMAR2340* null mutant. For plate growth, 7H10 agar plates were incubated at either 30°C, or when required, at 37°C (for selecting transductants; see below). For experiments involving the usage of phages, Tween 80 was not used in media. Mycobacteriophages were routinely propagated on Middlebrook's 7H9 with 0.2% glycerol. Phage high titres were generated using protocols described by (Larsen et

al., 2007). Antibiotics were added as required: Hygromycin B (Roche) 150µg/ml for *E. coli*, 100µg/ml for *M. smegmatis*, 75µg/ml for *M. marinum*. Kanamycin sulfate (Sigma) -50µg/ml for *E. coli* and 25µg/ml for *M. marinum*.

Table 3.1. Bacterial strains, plasmids and phages used in this study

Plasmids, phages and strains	Description	Reference
Plasmids		
p0004S	Cosmid containing Hyg-SacB casette	(Larsen et al., 2007)
p∆*MMAR2340*	Derivative of p0004S obtained by cloning the right and left PCR flanks of MMAR2340	This work
Phages		
phAE159	Conditionally replicating shuttle phasmid derived from lytic mycobacteriophage TM4	(Larsen et al., 2007)
ph∆*MMAR2340*	Derivative of phAE159 obtained by cloning *p∆MMAR2340* into its PacI site	This work
Bacterial strains		
E.coli TOP 10	F– *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen
HB101	*E. coli* K-12 *F__*(*gpt-proA*)62 leuB1 glnV44 ara-14 galK2 lacY1 hsdS20 rpsL20 xyl-5 mtl-1 recA13	Stratagene
M. smegmatis mc^2155	Wild type strain, E*pt* mutant of *M. smegmatis* strain mc^26	Snapper et al.,1990
Δ*MMAR 2340*	*M. marinum* strain disrupted in gene *MMAR2340* was replaced by *hyg*	This work

3.2.3 Construction of a knockout phage for deletion of *MMAR2340*

Approximately 1 kb sequences of the upstream and downstream regions of *MMAR2340* were PCR-amplified from *M. marinum* 1218R genomic DNA using the primer pairs listed in Table 3.2. The PCR products were purified and the primer incorporated *Van*91I sites were digested with *Van*91I. The digested PCR fragments were then ligated into *Van*91I-digested p0004S to generate the allelic exchange plasmid p∆*MMAR2340*. Plasmids obtained by miniprep (Qiagen miniprep kit) were digested with *Van*91I and *Pac*I and sequenced to confirm presence of the left and right flanks. One positive plasmid was selected for packaging into phAE159. The *Pac*I digested knockout plasmids p∆*MMAR2340* was then ligated with *Pac*I digested phAE159 DNA. The ligation mix was packaged in the temperature sensitive mycobacteriophage phAE159 heads, transduced into *E. coli* HB101, and selected on LB agar with hygromycin at 37°C. This yielded phasmid DNA, which was subsequently used to generate the phage ph∆*MMAR2340*.

Table 3.2. Primers used for generation of knockout construct in *M. marinum*

Primer	Sequence 5' -3'	Description	Size
MMAR2340_LL	TTTTTTTTCCATTTCTTGGGGTGATGCCGAAGAACT	left side flank sequence of MMAR2340	960 bp
MMAR2340_LR	TTTTTTTTCCATAAATTGGCTTCCCACTCGTGCTCA		
MMAR2340_RL	TTTTTTTTCCATAGATTGGGGGTCCAGCAAATTCCG	Right side flank sequence of MMAR2340	902 bp
MMAR2340_RR	TTTTTTTTCCATCTTTTGGCCAGCGTCCTCAAACAT		

Following protocols detailed in Larsen et al., (2007), the cosmids recovered from *E. coli* HB101 were confirmed by digestion with *Pac*I digestion. The

positive cosmids were transformed by electroporation into *M. smegmatis* at 1800V and recovered at 30°C for ~4 hours in TSB. The recovered cells were then harvested and resuspended in 200µl of MP buffer. This was mixed with 200µl of freshly growing *M. smegmatis* and 5ml molten soft agar (50°C) and poured on 7H9 basal agar plates and incubated at 30°C for 2 -3 days and allowed to form plaques. The plates were soaked in minimum amount of MP buffer for 5-6 hours and the solution containing phages was filtered and stored at 4°C. This generated the recombinant phage - ph∆*MMAR2340* designed to replace the gene *MMAR2340* with *hyg*.

3.2.4 Generation of a *MMAR2340* null mutant

Specialised transduction of *M. marinum* 1218R was performed as described previously for other mycobacteria (Bardarov et al., 2002) and as detailed in Chapter 8. A culture of *M. marinum* grown to an OD_{600} of 0.8 was harvested and the cell pellet was washed twice with 50ml of MP buffer. Finally the pellet was resuspended in 2ml of MP buffer and then mixed with 2ml of high titre (10^{-10} pfu /ml) phage lysate. Cells in MP buffer with no phage added were used as a control. The mix was incubated overnight at 37°C followed by harvesting and recovery with 10ml 7H9+10% OADC with Tween-80 overnight at 37°C. This was plated onto 7H10+10% OADC-agar plates with hygromycin B and plates were incubated at 37°C for 2 weeks. Hygromycin resistant colonies obtained after transduction of *M. marinum* 1218R (wild type strain) were inoculated in 10ml 7H9+10% OADC - Tween 80 with hygromycin B for genomic DNA extraction and further characterization. Allelic exchange of *MMAR2340* with a hygromycin resistance

cassette in hygromycin resistant transductants was confirmed Southern blot. One such transductant was chosen for subsequent experiments.

Restriction enzymes were selected based on the sequence of the knockout plasmids. The restriction enzyme used was *Hind*III. Following digestion, the *g*DNA fragments were separated by gel electrophoresis. In the mutant strains the gene is replaced by a *hyg-SacB* gene; thus after digestion the expected sizes for a mutant were 6.5Kb and 7.7Kb, while those for wild type genomic DNA were 16.4Kb. PCR products of the left and right flanks of *MMAR2340* were used as probes.

The procedure was performed as suggested by manufacturer guidelines in (DIG High Prime DNA Labelling and Detection Starter Kit II cat no – 11585614910, Roche). This kit uses digoxigenin, a steroid to label DNA probes by random priming. The hybridized probes are then detected uisng anti-digoxigenin-AP (Fab fragments), and subsequently visualised by chemiluminescence.

3.2.5 Extraction and analysis of *M. marinum* lipids

For labelling lipids with [^{14}C], *M. marinum* strains were grown to mid-logarithmic phase in 10ml of Middlebrook 7H10 broth at 30°C in a shaking incubator, following which 50 μCi of [1,2-^{14}C] acetate (57 mCi/mmol, GE Healthcare, Amersham Bioscience) was added to the culture and the incubation was continued for another for 24 h. The labelled bacterial cells were harvested, washed, and freeze-dried, and polar and apolar lipids were extracted and analysed by 2D-TLC according to the procedures of Dobson et al. (1985). [^{14}C]-labelled lipids were

visualised by autoradiography by exposing a Kodak BioMax MR film to the TLC plates for 3-5 days.

3.3 Results

3.3.1 *In silico* analysis of MMAR2340

The LOS biosynthetic gene cluster in *M.marinum* was identified in the region between *MMAR2307* to *MMAR2344*. The genes *MMAR2340* and *MMAR2344* encode polyketide synthase Pks5 and Pks5_1, respectively. The other genes in this locus are believed to encode enzymes responsible for the transfer of the Pks-products. These include genes encoding a fatty acyl-AMP ligase (MMAR2341/fadD25), a mycobacterial membrane protein (MMAR2342 mmpL12) and a polyketide synthase associated protein (MMAR2343/papA4).

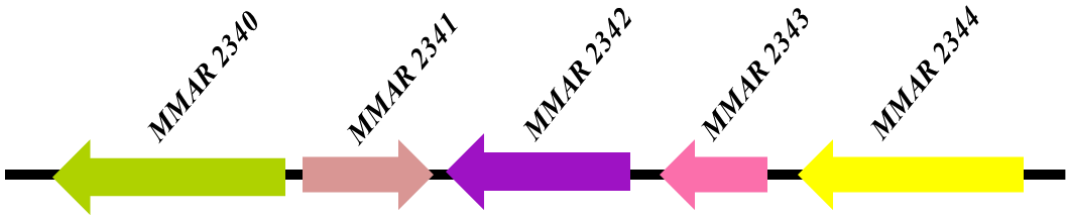


Figure 3.1. Map of *pks5* (*MMAR2340*) and neighbouring genes.

The 2090 amino acid protein encoded by *MMAR2340* comprises of a ketoacyl synthase domain with catalytic sites at the N-terminal and C-terminal, acyltransferase domain, Gro-ES like alcohol dehydrogenase domain, zinc-binding dehydrogenase domains, ketoreductase domain and a phosphopantheteine attachment site towards the C-terminus (Figure 3.2 A). The second *pks* gene,

MMAR2344 encodes a 2084 amino acid and has similar domain architecture as pks5 (Figure 3.2 B).

Predictions regarding the nature of the fatty acids produced by a Pks can be made based on the characteristic malonyl or methyl malonyl accepting motifs in the acyltransferase (AT) domains of the *pks* genes (http://linux1.nii.res.in/ ~pksdb/DBASE/page.html).

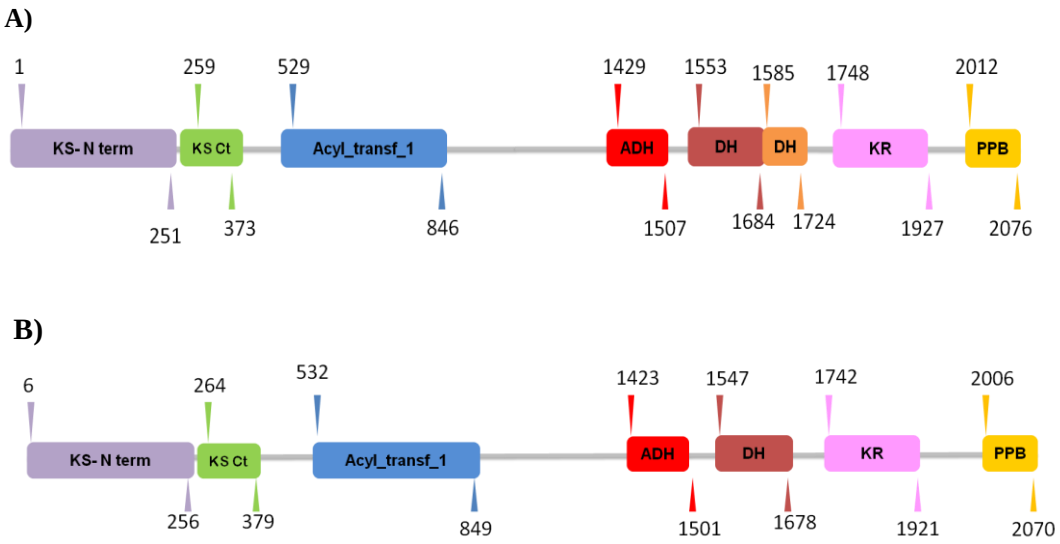


Figure 3.2. The Pks5 and Pks5_1 domain organisation of *M. marinum*. (A) Domain organisation of *M. marinum* Pks5 (B) Domain organisation of *M. marinum* Pks5_1, predicted using Pksdb and Pfam server. KS-Nterm/ Ct, keto acyl synthase domains , ADH-alcohol dehydrogenase like domain, DH-zinc binding dehydrogenase domain, KR, ketoreductase domain and PPB- phosphopantetheine binding domain.

MMAR2340 shares 76% identities with MKAN1200 (*M. kansasii* Pks5), 74% identity with "*M. canettii*" Pks5, *M. tuberculosis* Rv1527 and *M. bovis*. MMAR2340 also shares 63% identity with MSMEG4727/Pks5, a MAS-like Pks responsible for synthesis of methyl branched fatty acyl chains in LOSs from *M. smegmatis*.

Figure 3.3. Alignment of the MMAR2340 amino acid sequence with MMAR2344 (Pks5_1) and Pks5 homologues from *M. kansasii* MKAN1200, *M. canetti* Pks5, *M. tuberculosis* Rv1527c and *M.* smegmatis MSMEG4727. Numbers indicate the amino acid co-ordinates of MMAR2340. Dots indicate gaps. Red boxes with white letters indicate identical amino acid sequences at the aligned position for all three proteins. White boxes indicate similar or identical residues for two of the three proteins at the aligned position; the bold sequence letters in these boxes indicate identical or similar residues at the aligned position.

3.3.2 Analysis of total lipids from the Δ*MMAR2340* mutant

One knockout strain confirmed by Southern blot was selected for analysis of cell wall lipids. To assess the effects of loss of *MMAR2340* function on cell wall lipid composition, cultures of wild type, mutant and complemented strains were pulsed with [^{14}C]-acetate to label lipids. Labelled polar and apolar lipids were extracted and analysed by 2D-TLC using the five solvent systems described by Dobson et al. (1985). Differences in the lipid profiles of the wild type and Δ*MMAR2340* were visible only in TLCs run in solvent system E that separates phospholipids and LOSs. The mutant strain Δ*MMAR2340* was completely defective in LOS production, as none of the four *M. marinum* LOS lipids were visible (Figure 3.4). A transposon mutant of *MMAR2340* generated the *M. marinum* E11strain, described in Chapter 4 showed an identical similar phenotype. These results indicate that *pks5* played a role in the biosynthesis of LOS and that deletion of *pks5* results in the loss of all four LOS subtypes.

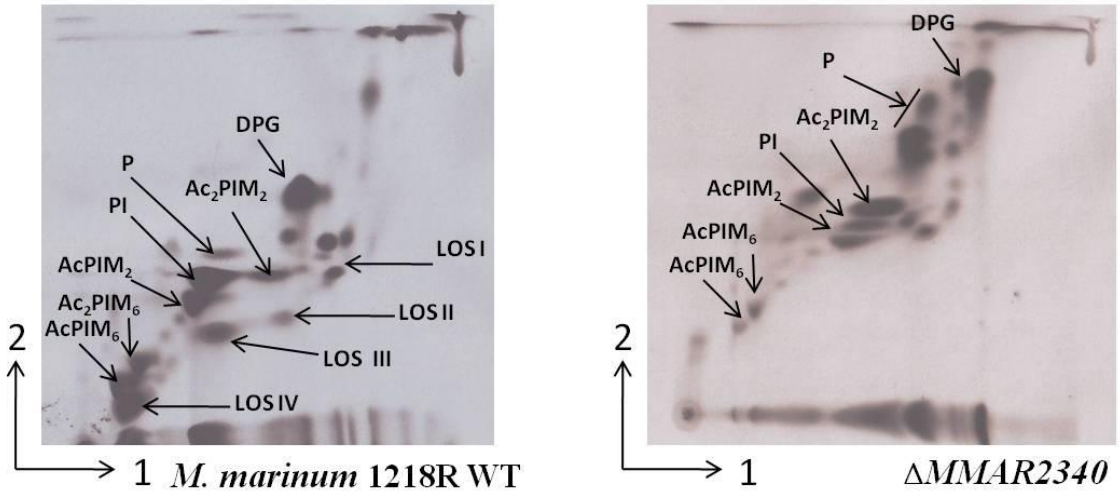


Figure 3.4. Autoradiograph of a 2D-TLC showing labelled polar lipids from *M. marinum* 1218R (wild type) and Δ*MMAR2340* grown in Middlebrook 7H10 broth. Direction 1- chloroform: methanol: water, 60:40:6 (v/v/v), direction 2- chloroform: acetic acid: methanol: water, 40:25:3:6 (v/v/v/v). Δ*MMAR2340* is devoid of all the four LOS species. $AcPIM_2$ and Ac_2PIM_2, mono and di-acyl phosphatidyl-inositol dimannosides; $AcPIM_6$ and Ac_2PIM_6, mono and di-acyl phosphatidyl-inositol hexamannosides; LOS I-IV, lipoologosaccharides; PI, phosphatidylinositol; DPG, diphosphatidylglycerol; PE. Phosphatidylethanolamine; P, unknown phospholipids.

3.3.3 Intracellular survival of the *MMAR2340* mutant in bone marrow derived macrophages

Studies using *M. kansasii* to infect murine models have reported that LOS producing strains, which had glossy colony morphology, were readily cleared from mice models of infection while the LOS negative rough appearing strains were able to survive and produce a systemic infection in mice. It was reported that LOS-IV deficient *M. marinum* mutants were unable to enter murine macrophages (Burguière et al., 2005). In the previous chapter a mutant strain Δ*MMAR2333* which produced LOS-I and accumulated an intermediate LOS-II* was found to be able to enter and survive in bone marrow macrophages with no apparent effect on survival and intracellular growth of the bacterium.

In the case of Δ*MMAR2340,* we had a mutant strain that could be used to assess the effects of complete loss of all the LOSs. In order to assess the role of a LOS deficient strain in virulence, an intracellular survival assay was performed with murine bone marrow derived macrophages. Macrophages were thus infected with Δ*MMAR2340* to determine the ability to survive in the macrophages. Infection experiments were done using a multiplicity of infection (MOI) of 10 and survival of intracellular bacteria was followed over a period of 1, 3 and 5 days. The number of intracellular bacteria (colony forming units) was enumerated by lysing the macrophages and plating on 7H10 agar plates (Figure 3.5).There was no change in the ability of the mutant strain mutant Δ*MMAR2340* in surviving inside the macrophages, indicating the absence of LOSs did not affect intracellular survival of the bacteria.

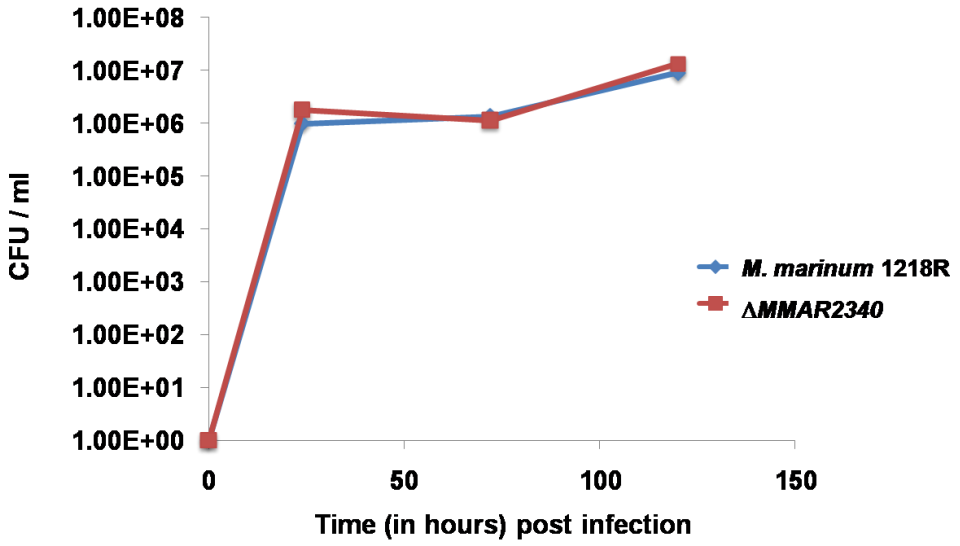


Figure 3.5. Survival of *M. marinum* strains in murine (Balb/c) bone marrow derived macrophages. Counts of intracellular bacteria after lysing infected BMDM are expressed in colony forming units per ml.

The loss of a glycolipid may not necessarily affect bacterial loads in infected cells but might alter cytokine signalling thus influencing the inflammatory response (Rao et al., 2005; Glickman et al., 2000). *M. tuberculosis* infection induces production of the proinflammatory cytokine TNF-α in macrophages and dendritic cells (Orme, 2004). TNF-α levels released by infected bone marrow deived macrophages were measured using a mouse TNF-α kit. Cell supernatant analysis for TNF-α release in infection experiment with the strain Δ*MMAR2340* revealed slightly lower levels in the mutant strain as compared to the wild type and complemented strains. Post infection at 72 hours, the wildtype strain infected BMDM TNF-α levels were recorded at 95 pg /ml while it was 126.67 pg/ml in case of the mutant strain. Post infection at 120 hours the values recorded was 76.7 pg/ml in case of the wildtype and 112.22 pg/ml for Δ*MMAR2340.*

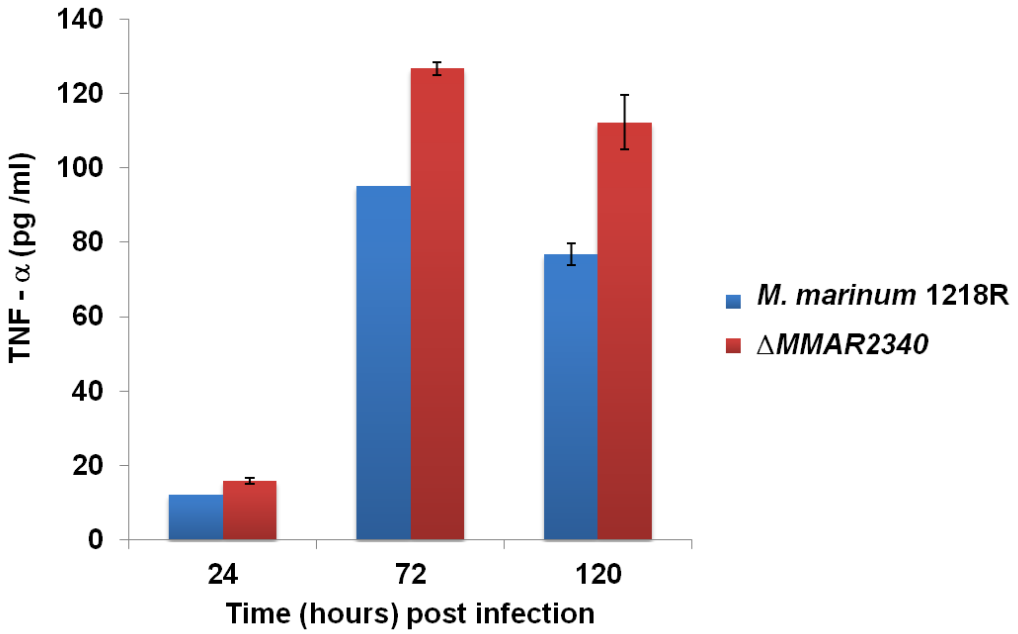


Figure 3.6. TNF- α production by murine (Balb/c) bone marrow derived macrophages infected with different *M. marinum* strains.

3.4 Discussion

Cell wall lipids of pathogenic mycobacteria play a crucial role in virulence (Daffé and Draper, 1998). LOSs play an important role in sliding motility, biofilm formation and virulence (Burguière et al., 2005). *M. marinum* is characterised by the presence four classes of LOSs. The core structure comprises of four units of glucose and a methylated rhamnose. Addition of xylose and complex sugars like caryophyllose and N-acyl 4, 6-dideoxygalactose makes up the higher LOSs. In addition to this, the glucose core is acylated by the presence of two methyl branched fatty acyl chains (Rombouts et al., 2011).

A MAS-like *pks5* gene *MSMEG4727* was reported to be essential in generation of the acyl chains in LOSs from *M. smegmatis.* Although, *M. tuberculosis* does not produce LOSs, the genes *losA* (*Rv1500*) and *pks5* (*Rv1527c*) are present in *M. tuberculosis.*

Interestingly, a *pks5* mutant of H37Rv was reported to be affected in virulence in mice (Rousseau et al., 2003a) but no missing metabolites were detected. The MTBC strain *M. canettii* produces LOSs and shares 74% identity to the pks5 from *M. marinum*. LOSs have been suggested to act as a mask for virulence or avirulence factors in mycobacteria (Daffé et al., 1991). In this study, a polyketide synthase involved in the LOS biosynthesis cluster in *M. marinum* has been identified. The mutant strain Δ*MMAR2340* was defective in the biosynthesis of all the four subclasses of LOSs found in *M. marinum* and was not altered in survival and replication in cultured bone marrow macrophages. But infection with this strain elicited a strong release of TNF-α which is a hallmark of infection.

The gene *MMAR2340* encodes a MAS like PKS enzyme –Pks5 and lies in the cluster identified to be involved in LOS biosynthesis (Burguière et al., 2005). The genome of *M. marinum* also reveals the presence of another polyketide synthase 5 gene, pks5_1 (MMAR2344). The occurrence of two *pks5* genes could relate to the unique structure of *M. marinum* LOSs which posess two methyl branched fatty acyl chains, 2,4-dimethylhexadecanoate and 2,4-dimethyl-2-pentadecenoate. Based on this hypothesis, it is expected that deletion of either the *pks5* or *pks5_1* gene would give rise to a partially acylated intermediate of the lipooligosaccharide. On the contrary, we observed that deletion of *MMAR2340* results in complete abolition of LOS production in the mutant strain. A similar phenotype was displayed by a transposon mutant of *M. marinum* E11 strain, *MMAR2340::aph* (detailed in chapter 4 in this thesis) which was deficient in producing all the four LOS subtypes. Given the similarity of the MMAR2340/pks5 with MSMEG4727/pks5 and the LOS negative phenotype of Δ*MMAR2340*, we predict that *MMAR2340* (*pks5*) is involved in the synthesis of one of the two acyl chains - 2, 4-dimethylhexadecanoate and 2,4-dimethyl-2-pentadecenoate. The acyl chains are further

transferred to the tetraglucose core of LOS by one of the acyl transferases (PapA3 or PapA4). It is possible that acyl chain generated by Pks5 is the first to be attached to the hexose ring; this then serves as an intermediate or substrate for addition of the second acyl chain, which is probably synthesized by Pks5_1. Thus a loss of pks5 would be expected not just in the loss of a specific fatty acyl chain on the LOSs, but a complete loss of the glycolipids due to unavailability of a partially acylated intermediate that serves as a substrate for subsequent enzymes in the LOS biosynthesis pathway. A deletion mutant of *MMAR2344* (*pks5_1*) could clarify this and shed light on the type of acyl chains synthesized by the individual LOS-cluster associated *pks* genes. Unfortunately repeated attempts at generating a knockout strain of *MMAR2344* by specialised transduction were unsuccessful. Further studies on the effect of deletion of putative transporter *MMAR2342* (*mmpL12*) and *MMAR2344* (*Pks5_1*) will provide further insights into LOS biosynthesis.

4

Identification of genes involved in the *Mycobacterium marinum* lipooligosaccharide biosynthesis using transposon mutagenesis.

4.1 Introduction

Lipooligosaccharides (LOS's) are antigenic glycolipids present in the outer membrane of many mycobacteria. Although this glycolipid is not present in *Mycobacterium tuberculosis*, they have been reported in *Mycobacterium marinum* , *Mycobacterium kansasii* (Hunter et al., 1985; Hunter et al., 1983), *Mycobacterium smegmatis* , and "*Mycobacterium canettii"* of the *M. tuberculosis* complex (Daffé et al., 1987). Unlike most mycobacterial glycolipids, LOS's vary in length and composition between different species. *M. marinum* produces under laboratory conditions four different LOS structures with increasing length, in which different unusual sugar moieties are added to a tetra glucose core which is acylated by two 2,4 dimethyl hexadecanoate and 2,4 dimethyl 2- pentadecenoate. The gene cluster involved in LOS biosynthesis of *M. marinum* has been proposed to comprise *MMAR2302* through *MMAR2341*. In a study using transposon mutants, Ren et al. (2007), showed that a disruption in *MMAR2309* leads to production of only LOS-I. The protein encoded by *MMAR2309* belongs to the UDP-glucose/GDP-mannose dehydrogenase family and possess conserved NAD-binding domains. Based on the sequence analysis and lipid profile, it was suggested that MMAR2309 was responsible for the biosynthesis of **D**-xylose. The same study also reported that a disruption in *MMAR2332* resulted in a mutant strain that produces only LOS-I and LOS-II*, an intermediate between LOS-I and LOS-II, which contains Xyl*p* but lacks caryophyllose. *MMAR2332* encodes a protein which is similar to various thiamine pyrophosphate (TPP) requiring enzymes, like acetolactate synthetase. It is predicted that MMAR2332 is involved in the biosynthesis of the caryophyllose sugar residue and hence a disruption in

this gene resulted in LOS-II*. The *losA* gene (*MMAR2313*) was shown to have a role in LOS-IV production (Burguière et al., 2005). So far, only three selected genes and a genomic region of four genes have been demonstrated to be involved in the late steps of LOS biosynthesis in *M. marinum*.

The previous chapter detailed the generation of targeted mutants in the LOS biosynthetic cluster in *M. marinum*. The work discussed in this chapter describes a parallel approach that makes use of a library of transposon mutants to isolate mutant strains defective in LOS biosynthesis. We made use of two resources to isolate LOS-deficient mutants. The first was a mariner transposon library generated in this study to isolate LOS deficient mutants based on altered colony morphology. The second made use of a transposon library generated in the laboratory of our collaborators. In a unrelated screen for PE-PGRS mutants, Dr. A. Van der Woude and Prof W. Bitter (VUMC, Netherlands) noticed that a number of mutants defective in the secretion of the Esx-5 substrate EsxN had a transposon insertions in genes located near known LOS biosynthesis genes (the LOS cluster). The work described in this chapter reflects the lipid analysis done by me as a part of this collaboration. In total, we have identified ten *M. marinum* genes with a role in different stages of LOS biosynthesis.

4.2 Materials and Methods

4.2.1 Bacterial strains and culture conditions

Wild-type *M. marinum* strains 1218R, M^{USA} and E11, and corresponding mutants were routinely grown at 30°C in Middlebrook 7H9 (Difco), 7H10 liquid medium and on Middlebrook 7H10 plates (Difco) supplemented with 10% Middlebrook OADC (BD, Biosciences) and 0.05% Tween 80.

4.2.2 Plasmids, DNA manipulations and bacterial growth conditions

Plasmids, bacterial strains and phages used in this study are listed in Table 4.1. *Escherichia coli* strains were routinely cultured in Luria-Bertani (LB) broth at 37ºC. *Mycobacterium smegmatis* strain mc^2155 (wild type) was used for generation and propagation of mycobacteriophages and was routinely grown at 37°C either in Middlebrook 7H9 Tween 80 or Tryptic Soy Broth (TSB) / agar supplemented with 0.05%. All *M. marinum* strains were grown either in 7H9 broth supplemented with 10% OADC (oleic acid/albumin/dextrose/catalase, BD) or in 7H10 broth (composition based on Middlebrook 7H10 agar components) supplemented with 10 % OADC, at 30°C. For plate growth, 7H10 agar plates were incubated at either 30°C, or when required, at 37°C (for selecting transductants; see below). For experiments involving the usage of phages, Tween 80 was not used in media. Mycobacteriophages were routinely propagated on Middlebrook's 7H9 with 0.2% glycerol. Phage high titres were generated using protocols described by Larsen et al (2007). Antibiotics were added as required : hygromycin B (Roche) 150µg/ml for *E. coli*, 100µg/ml for *M. smegmatis*,

50µg/ml for *M. marinum*; kanamycin sulfate (Sigma) - 50µg/ml for *E.c oli* and 25µg/ml for *M. marinum*; chloramphenicol 30µg/ml for *M. marinum.*

4.2.3 Generation of transposon mutants

Transposon mutagenesis was performed on *M. marinum* 1218R using the mycobacterial Transposon delivery phage phAE181 to produce a transposon library with an objective of generating disruption mutants in the LOS cluster, primarily to isolate mutants in glycosyltransferases. *M. marinum* cultures were grown in 50ml of 7H9+OADC with 0.05% Tween 80 to an optical density (OD_{600nm}) of 0.8. The cells were harvested by centrifugation at 4500g for 10 minutes and washed twice with 50 ml MP buffer. The cell pellet was then gently resuspended in 2 ml MP buffer and mixed with 1 ml of high titre phage lysate (10^{-8} to 10^{-10} pfu/ml). Separately, 500 µl cells were mixed with 500 µl of MP buffer to serve as control. The cell – phage mix was incubated at 37°C overnight (static) , the cells were then harvested by centrifugation and resuspended in 10 ml 7H9 broth+OADC with 0.05% Tween 80. *M. marinum* cells were recovered by overnight incubation at 37°C. Cells were again harvested and resuspended in 1 ml fresh media and plated on 7H10+OADC agar plates (100 µl per plate) with 50µg/ml hygromycin B. Plates were incubated at 37°C for 2 to 3 weeks.

Additionally we had access to a set of transposon mutants of *M. marinum* E11 and M^{USA} strains as a part of an ongoing collaboration with Dr. Aniek Van der Woude and Prof. Wilbert Bitter of VUMC, Amsterdam, The Netherlands.

Table 4.1. Bacterial strains, plasmids and phages used in this study

Plasmids, phages and strains	Description	Reference
Plasmids		
pVV16	Hyg^R/Kan^R , *E.coli*-mycobacterial shuttle vector (ColE1 *oriM* P*hsp60*)	Gift from Dr. V. Vissa, Colorado State University
Phages		
phAE181	Conditionally replicating phage TM4 derivative carrying *Tn-5371*	(Kriakov et al., 2003)
Bacterial strains		
E. coli TOP 10	F– *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen
M. smegmatis mc^2155	Wild type strain, E*pt* mutant of *M.smegmatis* strain mc^26	Snapper et al., 1990
M. marinum 1218R	Wild type strain used for generation of transposon mutants	ATCC927
M. marinum E11	Wild type strain used for generation of transposon mutants	(van der Sar et al., 2004)
M. marinum M^{USA}	Wild type strain used for generation of transposon mutants	(Talaat et al., 1998)

4.2.4 Isolation and sequencing of *Tn* insertion sites

Transductants obtained were selected based on their colony morphology. Selected mutants were grown in 10ml of 7H9+OADC with 0.05% Tween 80 +50μg/ml hygromycin B, and genomic DNA was extracted. The gDNA was digested with *Bss*HII (NEB) and ligated with T4 ligase (NEB). The ligation mix was used to transform *E. coli* cc118λpir competent cells. To select self-ligated genomic DNA

fragments containing the transposon, the transformation mix was plated on LB-plates with 150μg/ml hygromycin B and incubated overnight at 37°C. Colonies obtained were inoculated in LB-broth with hygromycin to obtain plasmid DNA. Plasmid DNA isolated from the hygromycin resistant transformants was digested with *Bss*HII and checked on gel. The plasmids were then sequenced using the primers HOPS1 (5' GCTTACAATTTAGGTGGCACT 3') and KMN1 (5' AGTGCCACCTAAATTGTAAGC 3') to get the sequence of the left and right flanks of the transposon insertion sites.

4.2.5 Generation of complemented strains of E11 *MMAR2327::aph* and E11 *MMAR2336::aph.*

The *MMAR2327* and *MMAR2336* genes were PCR amplified from *M. marinum* E11 genomic DNA using the primers listed in Table 4.2. Using the primer incorporated *Nde*I and *Pst*I restriction sites, the PCR products were cloned into the *E. coli-Mycobacterium* shuttle vector pVV16 (containing hygromycin and kanamycin resistance cassette) and verified by sequencing. The resultant plasmids pVV16-*MMAR2327* and pVV16-*MMAR2336* were introduced by electroporation (Snapper et al., 1990) into *M. marinum* E11 *MMAR2327::aph* and *M. marinum* E11 *MMAR2336::aph* to generate the complemented strains E11*MMAR2327::aph-C* and E11*MMAR2336::aph.*

Table 4.2. Primers used for generation of complemented strains

Name	Sequence (5' → 3')	Product	Size
MMAR2327_F	GATCGATCCATATGAGTCGACCTG TGGAGGTT	MMAR2327 gene sequence	1569 bp
MMAR2327_R	GATCGATCCTGCAGTCATCCAGGC TTTCGAATG		
MMAR2336_ F	GATCGATCCATATGCATTACCTGA TTACTGG	MMAR2336 gene sequence	1017 bp
MMAR2336_R	GATCGATCCTGCAGCTACCGCAGT TGCCTAGCCTC		

*Underlined sequence shows *Nde*I restriction site, # Underlined sequence shows *Pst*I restriction site.

Table 4.3 Table of complemented strains generated in this study

Complemented strains	Description	Reference
E11*MMAR2327::aph-C*	*MMAR2327::aph* complemented with wild type copy of the gene *MMAR2327* in p VV16.	This work
E11*MMAR2336::aph-C*	*MMAR2336::aph* complemented with wild type copy of the gene *MMAR2336* in pVV16.	This work

4.2.6 Lipid extraction and analysis

For labelling lipids with ^{14}C-acetate, *M. marinum* strains were grown to mid-logarithmic phase in 10ml of Middlebrook 7H10 broth supplemented with 10% OADC, 0.05% Tween 80 with appropriate antibiotics where required in a shaking incubator at 30 degrees, following which 1 mCi/ml [1,2-^{14}C] acetate (57 mCi/mmol, GE Healthcare, Amersham Bioscience) was added to the culture and the incubation was continued for another for 24 h. The labelled bacterial cells were harvested, washed, and freeze-dried, and polar and apolar lipids were extracted and analysed by 2D-TLC according to the procedures described by

Dobson et al. (1985), detailed in Chapter 8. ^{14}C-labelled lipids were visualised by autoradiography by exposing a Kodak BioMax MR film to the TLC plates for 3-5 days.

4.2.7 Large scale extraction of accumulating LOS's from mutant strains

For purification of accumulating LOSs, 400 mg of polar lipids, extracted from 40 g dried cells, were applied to a DEAE cellulose column. The column was eluted with 500ml $CHCl_3$:CH_3OH (2:1 v/v) and 10 ml fractions were monitored by separation of LOSs by 1D-TLC ($CHCl_3$:CH_3OH:H_2O; 60:30:6) and spraying the plates with alpha-naphthol/sulfuric acid followed by charring. Fractions containing LOSs were pooled, concentrated and further purified by preparative TLC on a 10 x 20cm plastic backed silica gel TLC plates (Merck) run in $CHCl_3$:CH_3OH:H_2O (60:30:6, v/v/v). The plates were sprayed with 0.01% 1,6-di-phenyl-1,3,5-hexatriene in petroleum ether /acetone (9:1,v/v) and the glycolipids were visualised under UV light, and the area was marked with a pencil. The plates were then run in toluene to remove the di-phenyl hexatriene and after drying, the marked area was scraped from the TLC plates, extracted with $CHCl_3$:CH_3OH (2:1, v/v), per methylated and subjected to mass spectroscopy analyses (described in Chapter 8).

4.2.8 Extraction of bone marrow derived macrophages (from Balb/c mice) and infection by *M. marinum*

Bone marrow derived macrophages (BMDM) were obtained as described in Chapter 8. 24 hours before infection, the BMDM were activated using

recombinant mouse INF-γ (Invitrogen) at a concentration of 1000units/ml. The concentration of BMDM used for this study was 0.5 X 10^6 cells / ml. Late log phase bacteria (*M. marinum* E11WT, *MMAR2327::aph, MMAR2327::aph-C*, *MMAR2336::aph, MMAR2336::aph-C,* and *MMAR2340::aph*) were washed with PBS and resuspended in infection medium (DMEM with 10% FBS) to 10^7cfu/ml. Murine BMDM was infected with *M. marinum* strain at an MOI of 10 and incubated at 37^0C under 10% (v/v) CO_2 for 3-4 hours. Infection protocol was followed as described in chapter 6. Infection with each strain was triplicated and TNF-α levels in the infection supernatant was estimated using Quantikine^R Mouse TNF-α Immunoassay Kit (MTA00B), RnD Systems.Cell lysates were 10 fold serial diluted and plated out on 7H10 agar with OADC. The plates were incubated at 30^0C for 1 week before counting colonies.

4.3 Results

4.3.1 Selection of transposon mutants in this study

In the transposon screen using colony morphology change, we were able to isolate a strain disrupted in the gene *MMAR2351*, a glycosyltransferase in the *M. marinum* LOS gene cluster. The mutant strain was first isolated based on colony morphology (Figure 4.1).

Additionally we had access to a set of transposon mutants of *M.marinum* E11 and M^{USA} strains as a part of an ongoing collaboration with Dr. Aniek Van der Woude and Prof. Wilbert Bitter of VUMC, The Netherlands. The following table (Table 4.4) lists all the mutant strains discussed in this study.

Table 4.4 Table of all LOS *Tn*-insertions with putative genes that are disrupted.

Mutant strain	Gene product	Parental strain
MMAR2307::aph	hypothetical transmembrane protein	*M. marinum* M^{USA}
MMAR2319::aph	conserved hypothetical transmembrane protein	*M. marinum* E11
MMAR2320::aph	sugartransaminase (WecE)	*M. marinum* E11
MMAR2327::aph	conserved hypothetical transmembrane protein	*M. marinum* E11
MMAR2336::aph	GalE6 epimerase	*M. marinum* E11
MMAR2340::aph	Pks5	*M. marinum* E11
MMAR2341::aph	FadD25	*M. marinum* E11
MMAR2351::hyg	glycosyltransferase	*M. marinum* 1218R
MMAR2353::aph	UDP-glycosyltransferase	*M. marinum* E11
MMAR2355::aph	PapA3	*M. marinum* E11
MMAR2356::aph	isoleucine t-RNA synthetase	*M. marinum* E11
MMAR5170::aph	WhiB4	*M. marinum* E11

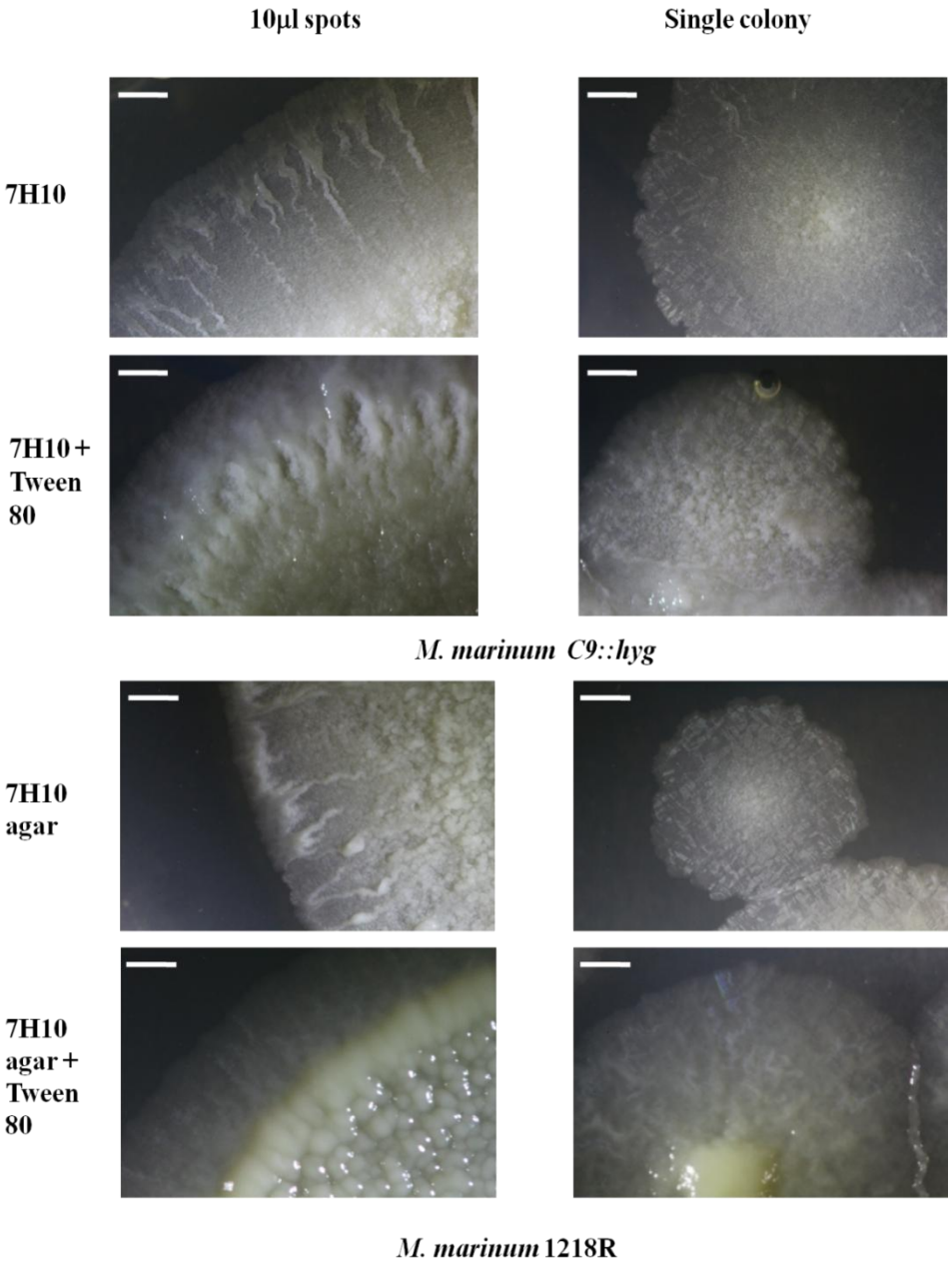


Figure 4.1 Colony morphology of *M. marinum* wild type and *Tn*-mutant strain. A 10µl spot of a mid-log phase culture and single colony on 7H10 agar and 7H10 agar containing 0.05% Tween 80 plates. Scale bar represents 1mm.

4.3.2 2D TLC analysis of mutant strains within the LOS gene cluster

M. marinum produces four different classes of LOS's, designated LOS-I to LOS-IV. The biosynthesis of these LOS variants is proposed to be sequential. The structure of LOS-I is 3-*O*-Me-Rha*p*-(1-3)-Glc*p*-(1-3)-Glc*p*-(1-4)-Glc*p*-(1-1)-Glc*p* (Burguière et al., 2005). The addition of xylose together with one or two molecules of the highly unusual sugar caryophyllose (Rombouts et al., 2009) produces LOS-II and LOS-III, respectively. The molecule added to produce LOS-IV has been partially characterized and seems to be a heterogenic group of mainly one acidic form of a *N*-acylated 4-amino-4,6-dideoxy-Gal*p* residue (Rombouts et al., 2010). The biosynthetic pathway for LOS and the genes involved remains largely unknown. In Chapter 2 a mutant strain of *MMAR2333* was detailed which was unable to produce LOS-II to LOS-IV but accumulated a LOS species- LOS-II*, which lacked the unique caryophyllose sugar. To determine the effects of genes disruption on LOS biosynthesis in the newly identified mutant strains the polar lipid profiles of these mutants were examined by two-dimensional thin-layer chromatography (2D-TLC).

4.3.2.1 Mutant strains defective in LOS-IV production

The mutant strain *MMAR2320::aph,* disrupted in *MMAR2320,* showed a distinctive 2D-TLC pattern with a specific accumulation of a LOS species that migrated to a position similar to LOS-III (Figure 4.2). *MMAR2320* a homologue of *E. coli* WecE, has been shown to be a sugar aminotransferase (Hwang et al., 2004). LOS-IV has a unique aminosugar and given the loss of LOS-IV in the mutant strain it seemed likely that MMAR2320 was involved in the biosynthesis

of the aminosugar residue. Complementation of the *MMAR2320::aph* mutant with a copy of MMAR2320 either on the shuttle vector pSMT3 or the integrative vector pUC-int-cat restored wild type LOS patterns in the strain (Figure 4.2).

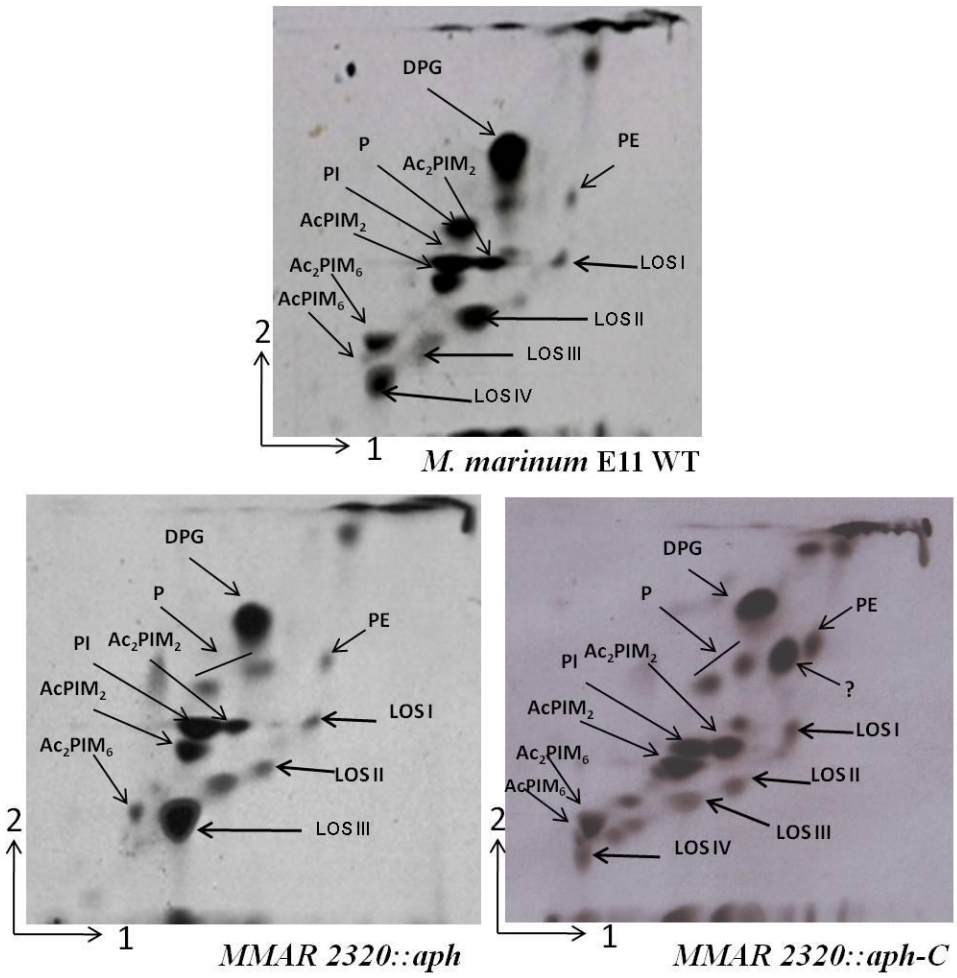


Figure 4.2. Autoradiograph of a 2D-TLC showing labelled polar lipids from *M. marinum* E11 (wild type), *MMAR2320::aph*, *MMAR2320::aph*-C grown in Middlebrook 7H10 broth. Direction 1- chloroform: methanol: water, 60:40:6 (v/v/v), direction 2- chloroform: acetic acid: methanol: water, 40:25:3:6 (v/v/v/v). *MMAR2320* shows accumulation LOS-III. $AcPIM_2$ and Ac_2PIM_2, mono and di-acyl phosphatidyl-inositol dimannosides; $AcPIM_6$ and Ac_2PIM_6, mono and di-acyl phosphatidyl-inositol hexamannosides; LOS I-IV, lipoologosaccharides; PI, phosphatidylinositol; DPG, diphosphatidylglycerol; PE. Phosphatidylethanolamine; P, unknown phospholipids.

Biochemical analysis of the accumulating LOS biosynthesis intermediates was performed to ascertain the sugar compositions and to relate it to the associated gene function. This was ascertained by determining the chemical nature of the accumulating LOS-III from the mutant strain *MMAR2320::aph*. The LOS-III was purified using a combination of column chromatography and preparative TLC, and per-*O*-methylated LOS was analysed by MALDI-MS and ES. A prominent signal was obtained at *m/z* 1915.9 [M + Na] + (Figure 4.3), for the LOS species isolated from *MMAR2320::aph* which corresponds to the mass of the tetraglucose core, methylated rhamnose, xylose with 2 caryophyllose residues.

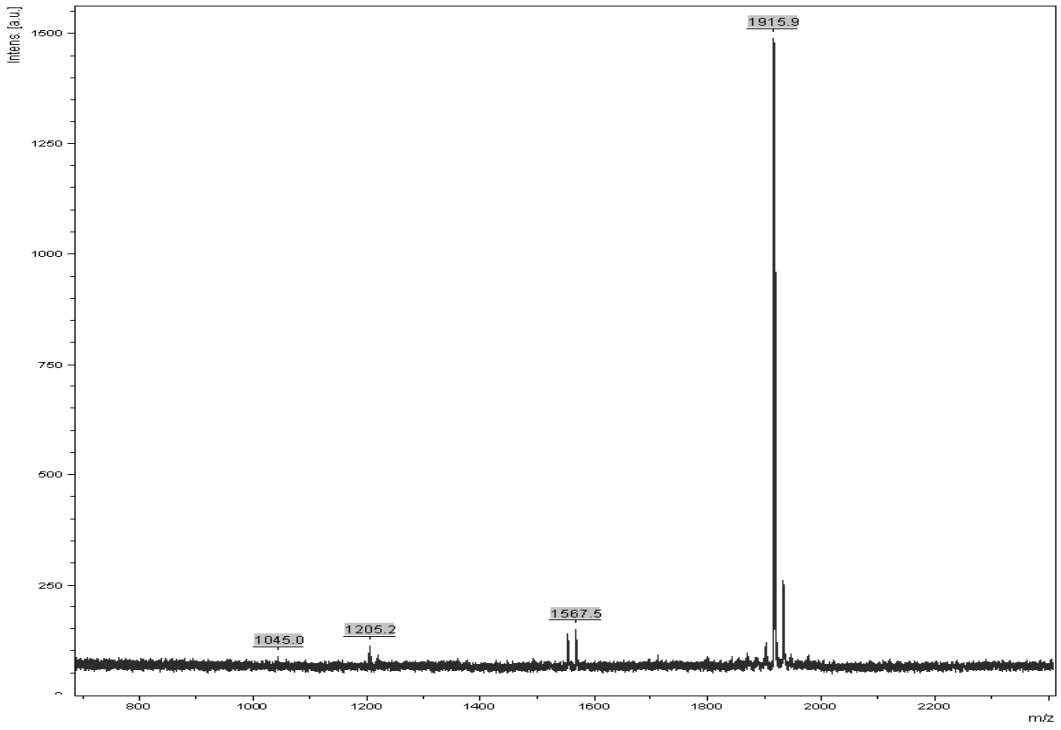


Figure 4.3. Mass spectrometric analysis of per-O-methylated LOS-III isolated from *MMAR 2320::aph.* The accumulating LOS species afforded a signal at *m/z* 1915.9 (M+Na), which corresponds to the mass of the tetraglucose core, methylated rhamnose, xylose with 2 caryophyllose residue; LOS-III.

These data indicates that mutant strain with a disruption in *MMAR2320* (WecE) is unable to synthesise LOS-IV, which has an additional N-acyl 4,6 dideoxygalactose added to the LOS-III structure. In other words, the addition of the N-acyl 4, 6 dideoxygalactose residues to the **D**-Xyl*p*-glycan-caryophyllose core did not take place in the Δ*MMAR2320* mutant. *MMAR2320* is a sugar transaminase and is believed to play a major role in the synthesis of the N-acyl 4,6 dideoxy galactose residue. Other glycosyltransferases maybe involved in the later stages of transferring a lipid-bound N-acyl sugar to the LOS-III moiety on the extracytoplasmic side.

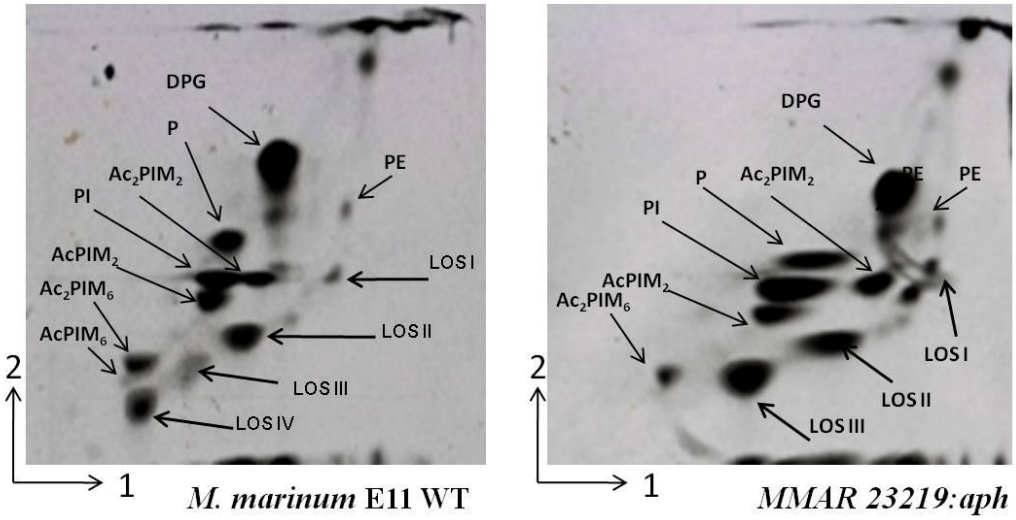


Figure 4.4 Autoradiograph of a 2D-TLC showing labelled polar lipids from *M. marinum* E11 (wild type) and *MMAR2319::aph* grown in Middlebrook 7H10 broth. Direction 1- chloroform: methanol: water, 60:40:6 (v/v/v), direction 2- chloroform: acetic acid: methanol: water, 40:25:3:6 (v/v/v/v). *MMAR2319* shows over production of LOS-II and LOS-III.

Another mutant strain *MMAR2319::aph* had a disruption in the gene downstream of the *wecE* gene, *MMAR2319*, which encodes a hypothetical transmembrane protein. Similar to the *MMAR2320::aph* strain, 2D-TLC analysis showed the strain to be deficient in LOS-IV biosynthesis. However, in contrast to

the *MMAR2320::aph* strain, it was found to accumulate two LOS species that migrated to a position similar to both LOS-II and LOS-III (Figure 4.4).

4.3.2.2 Mutant strains defective in production of LOS-III and LOS-IV

Other mutants in the LOS region also showed various defects in LOS biosynthesis. Mutant strain *MMAR2327::aph*, which was disrupted in *MMAR2327*, a gene encoding a multiple transmembrane protein, accumulated large amounts of a LOS species with TLC migration patterns similar to LOS-II and the strain was devoid of LOS-III and LOS-IV (Figure 4.5).

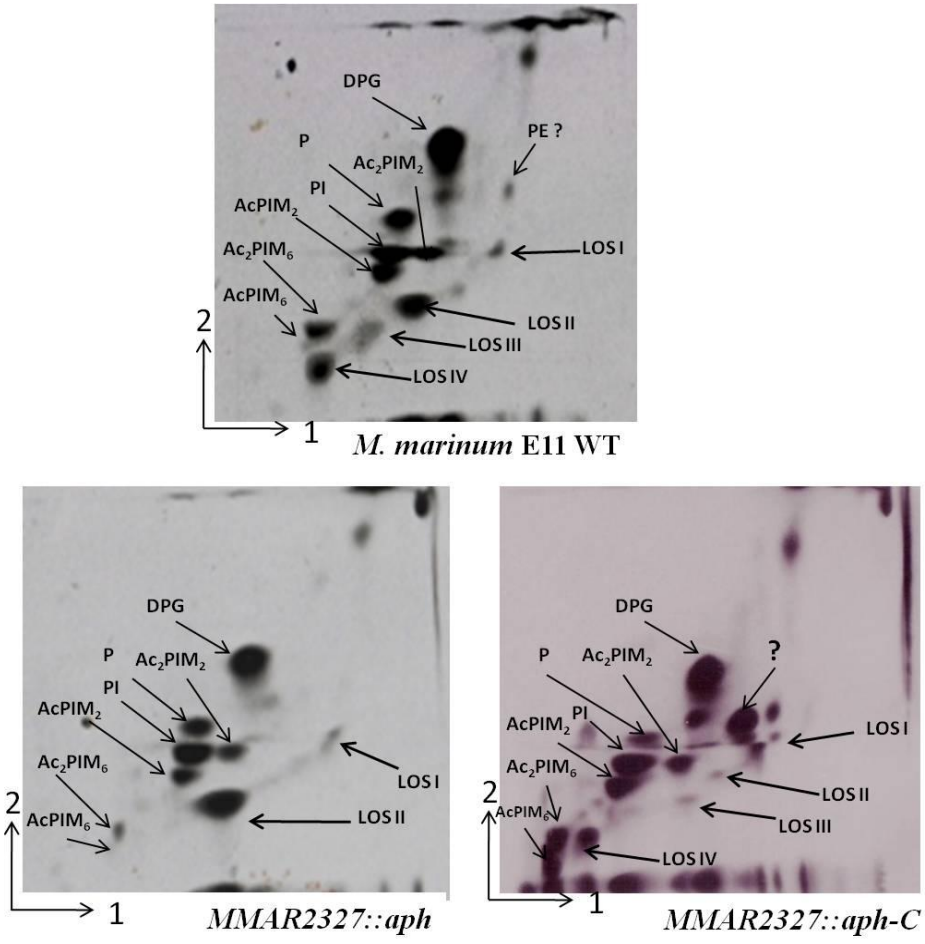


Figure 4.5 Autoradiograph of a 2D-TLC showing labelled polar lipids from *M. marinum* E11 (wild type), *MMAR2327::aph*, *MMAR2327::aph-C* grown in Middlebrook 7H10 broth. Direction 1- chloroform: methanol: water, 60:40:6 (v/v/v), direction 2- chloroform: acetic acid: methanol: water, 40:25:3:6 (v/v/v/v). *MMAR2327* shows over production of LOS-II.

Introduction of a plasmid borne copy of the wild type gene into this strain restored the LOS profile to that of the wild type strain (Figure 4.5). MS - analysis of the accumulating LOS species in the mutant strain Δ*MMAR2327* revealed a mass corresponding to LOS-II, previously reported from the wild type species (Burguière et al., 2005). A signal was obtained at *m/z* 1567.9 (M+Na) which corresponds to the mass of the tetraglucose core, methylated rhamnose, xylose and one caryophyllose residue (Figure 4.6). The protein MMAR2327 is conserved across mycobacterial species and has a high identity to a transmembrane protein in "*M. canettii*" and *M. tuberculosis* gene *Rv1508*. Rv1508 is a highly conserved protein with similarities to glycosyltransferases from various mycobacteria and has 42% identity in a 105 amino acid overlap (http://tuberculist.epfl.ch). The mutant strain with a disruption in the gene *MMAR2327* was not able to produce LOS-III, or add a caryophyllose residue onto the LOS-II* moiety (described in Chapter 2).

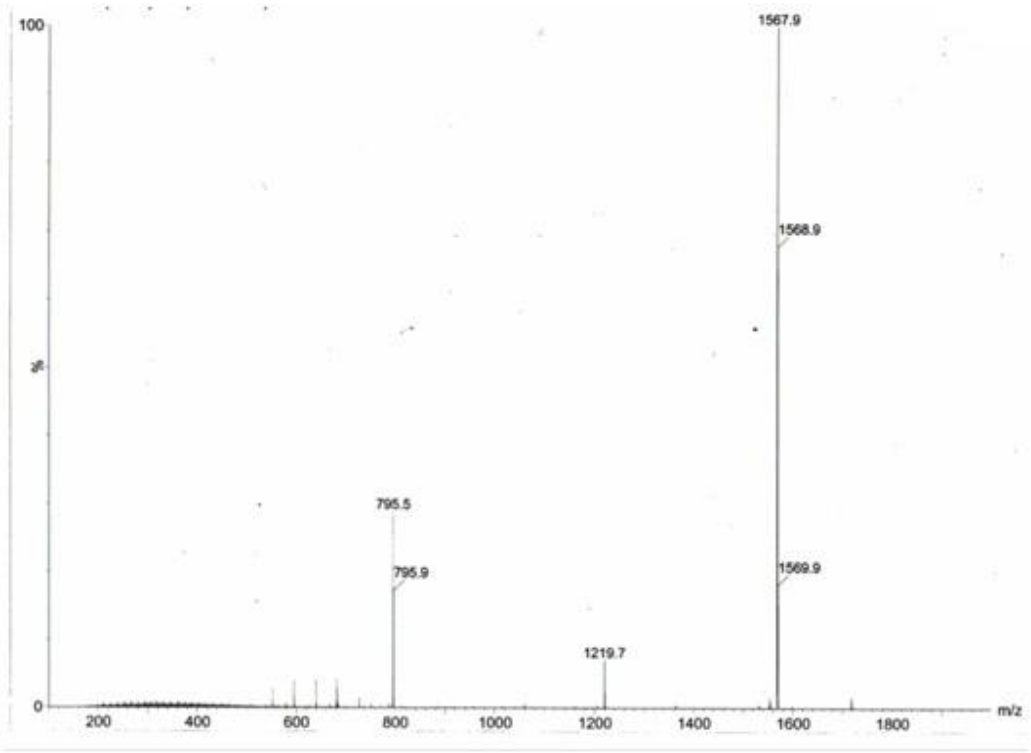


Figure 4.6. Mass spectrometric analysis of per-O-methylated LOS-II isolated from *MMAR 2327::aph.* The accumulating LOS species afforded a signal at *m/z* 1567.9 (M+Na), which corresponds to the mass of the tetraglucose core, methylated rhamnose , xylose with one caryophyllose residue ; LOS-II.

4.3.2.3 Mutant strains defective in production of LOS-II, LOS-III and LOS-IV

The mutant strain *MMAR2336::aph* was disrupted in *MMAR2336* a gene encoding a putative UDP-glucose 4-epimerase. An intermediate between LOS-I and LOS-II, named LOS-II* was observed in two other mutant strains Δ*MMAR2333* (Chapter 2) and Δ*MMAR2332* . The LOS intermediate detected in this strain had the same migrating pattern as the LOS-II* from above two mutant strains. Complementation of this mutant strain with a copy of the gene *MMAR2336* on the replicative plasmid pVV16 restored LOS biosynthesis patterns similar to the wild type strain (Figure 4.7).

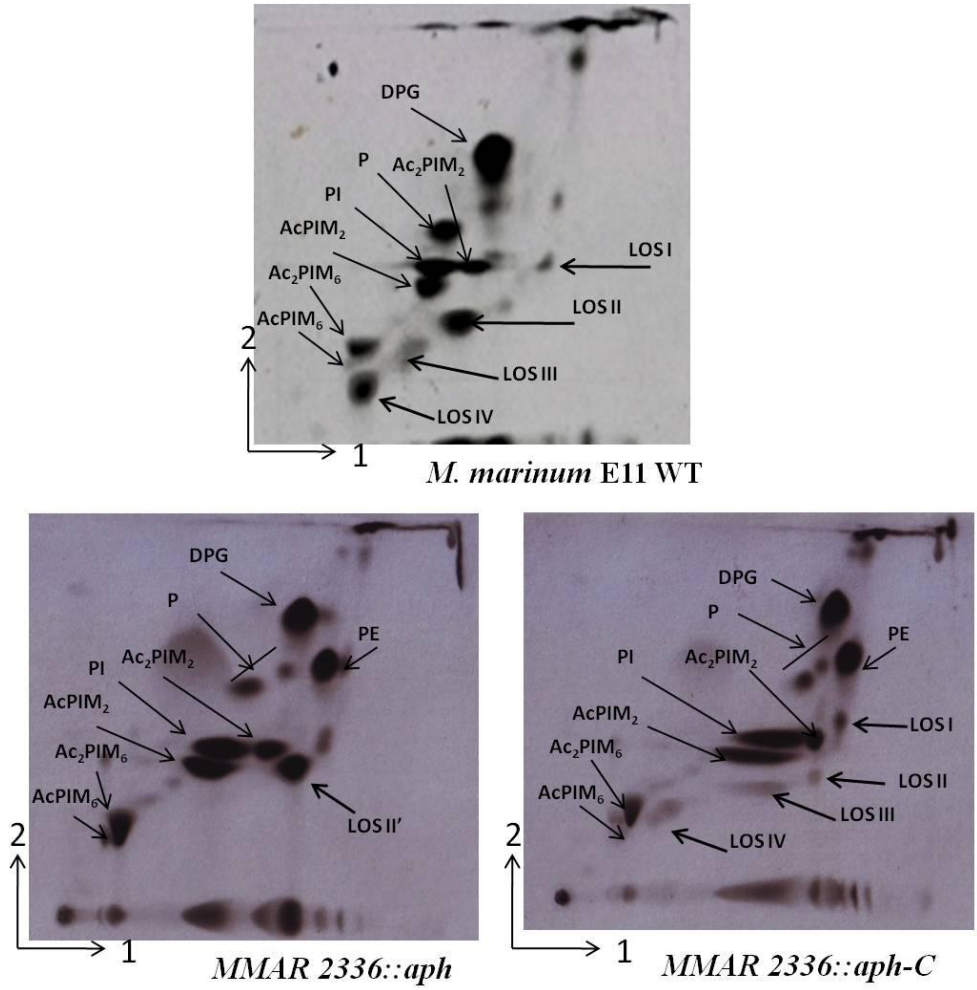


Figure 4.7. Autoradiograph of a 2D-TLC showing labelled polar lipids from *M. marinum* E11 (wild type), *MMAR2336::aph*, *MMAR2336::aph-C* grown in Middlebrook 7H10 broth. Direction 1- chloroform: methanol: water, 60:40:6 (v/v/v), direction 2- chloroform: acetic acid: methanol: water, 40:25:3:6 (v/v/v/v).

MMAR2336 encodes a UDP-glucose 4 epimerase, which is involved in catalysing the conversion of a UDP-glucose to UDP-galactose. These groups of enzymes have a structurally conserved Rossmann fold, an NADP (H) binding region and a diverse C-terminal region. 2D-TLC analysis of polar lipids extracted from this mutant strain revealed a LOS species, LOS-II' which migrated to a position similar to that of an intermediate between LOS-I and LOS-II,. This sugar lacked the unique caryophyllose residue that is characteristic of LOS-II and LOS-

III. Complementation of the mutant strain with a plasmid borne copy of the wild-type gene completely restored the phenotype to wild type. Mass spectrometric analysis afforded a signal at *m/z* 1175.7 (M+Na), which corresponds to 43 units less than the mass of the tetraglucose core, methylated rhamnose and xylose (Figure 4.8). These enzymes are known to catalyse the conversion of a nucleotide bound sugar to a nucleotide nucleotide bound keto-deoxy sugar. The inability of the *MMAR2336* disrupted strain, to produce LOS-II indicates a possible role in production of the caryophyllose sugar essential for producing LOS-II.

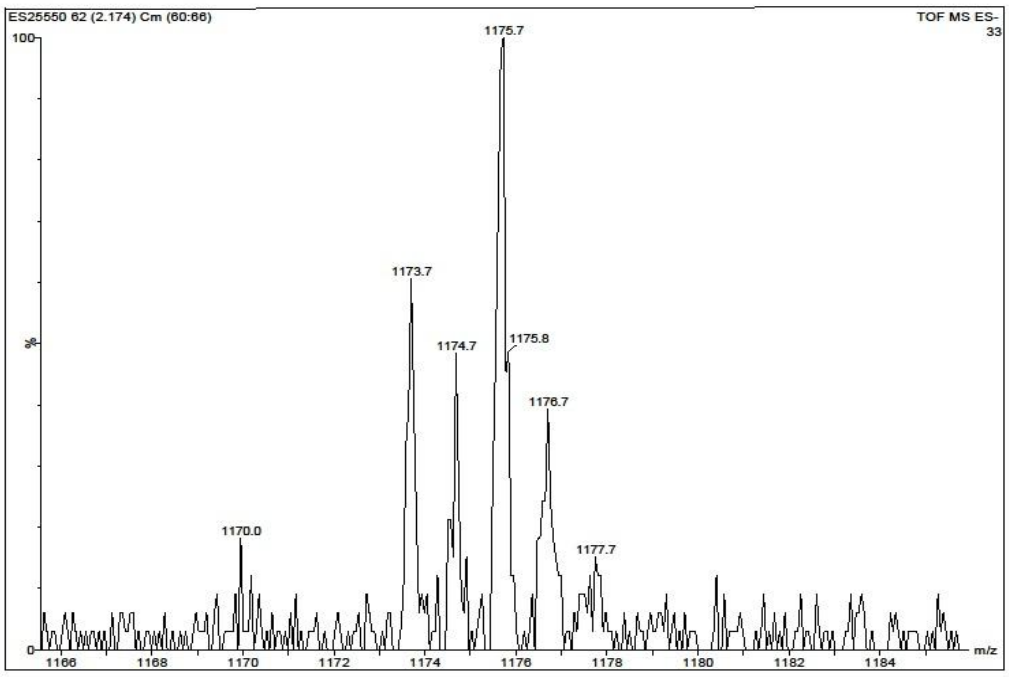


Figure 4.8. Mass spectrometric analysis of per-*O*-methylated LOS isolated from *MMAR 2336::aph.* The accumulating LOS species afforded a signal at *m/z* 1175.7 (M+Na).

The M^{USA} mutant strain was disrupted in the gene *MMAR2307*. *MMAR2307* encodes a hypothetical transmembrane protein and *in silico* analysis reveals no conserved domains. The closest identity is with a hypothetical

transmembrane domain of *M. kansasii MkanA1_010100001085c* which lies in the LOS biosynthesis cluster of *M. kansasii*. *MMAR2307* shows a LOS profile similar to the earlier reported mutant strain with a disruption in MMAR2309 (Ren et al., 2007). The mutant strain accumulated a species migrating to the position of LOS-I (Figure 4.9).

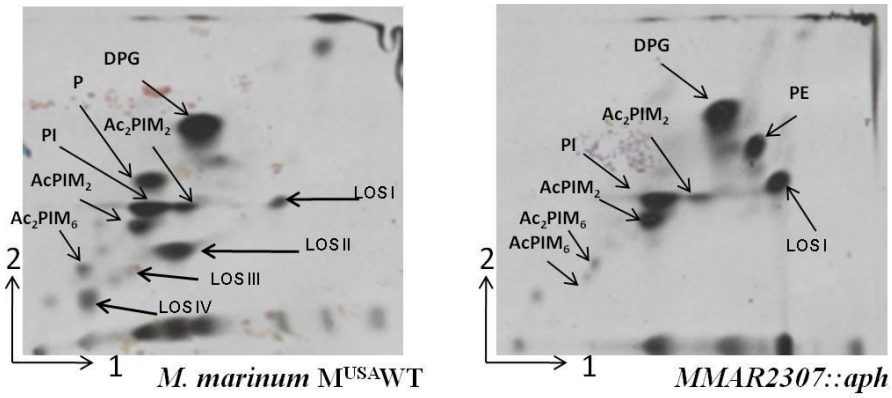


Figure 4.9. Autoradiograph of a 2D-TLC showing labelled polar lipids from *M. marinum* M^{USA} (wild type) and *MMAR2307::aph* grown in Middlebrook 7H10 broth. Direction 1- chloroform: methanol: water, 60:40:6 (v/v/v), direction 2- chloroform: acetic acid: methanol: water, 40:25:3:6 (v/v/v/v). *MMAR2307::aph* shows accumulation of LOS-I.

A mutant strain was generated in the *M. marinum* strain M^{USA}. This mutant had a disruption in the gene *MMAR2307*. This gene encodes a transmembrane protein and no conserved domains were detected. 2D–TLC analysis revealed that the mutant strain accumulated a polar lipid that migrated to the position of LOS-I. MALDI-MS analysis of the accumulating LOS species afforded a signal at 1059.3 *m/z* which corresponds to the tetraglucose core present in *M. marinum* LOSs (Figure 4.10)

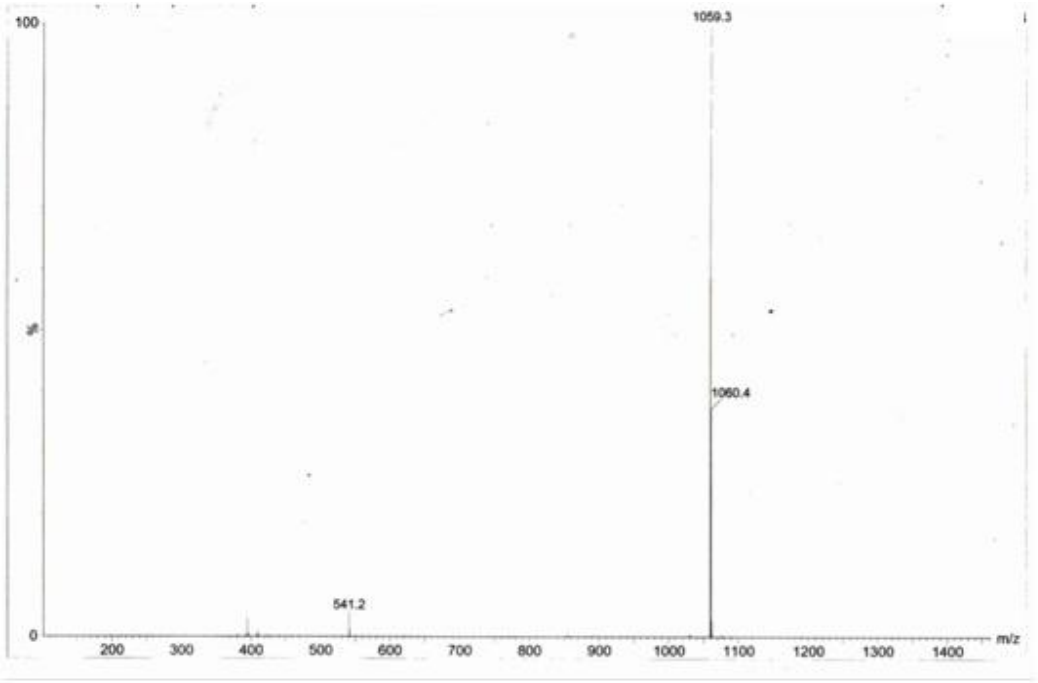


Figure 4.10 Mass spectrometric analysis of per-*O*-methylated LOS-I isolated from *MMAR 2307::aph.* The accumulating LOS species afforded a signal at *m/z* 1059.3 (M+Na) which corresponds to the tetraglucose core.

4.3.2.4 Mutant strains defective in production of LOS-I to LOS-IV.

The gene *MMAR2340* encodes a polyketide synthase, *pks5*. MMAR2340 shares 76% identities with MKAN1200 (*M. kansasii* Pks5), 74% identity with "*M. canettii*" Pks5, *M. tuberculosis* Rv1527 and *M. bovis.* MMAR2340 also shares 63% identity with MSMEG4727/Pks5, a MAS-like pks responsible for synthesis of methyl branched fatty acyl chains in LOSs from *M. smegmatis* (Etienne et al., 2009). The 2090 amino acid protein comprises of a ketoacyl synthase domain with catalytic sites at N-terminal and C-terminal, acyltransferase domain, Gro-ES like alcohol dehydrogenase domain, Zinc-binding dehydrogenase domains, ketoreductase and phosphopantetheine binding.

The 2D-TLC patterns for polar lipids of the mutant strains disrupted in the polyketide synthase 5 genes, involved in the biosynthesis of the fatty acyl chain *MMAR2340*::*aph* revealed that the strain is completely defective in LOS production, as none of the four *M. marinum* LOS structures were visible (Figure 4.11). A null mutant of *MMAR2340,* generated by specialised transduction in the *M. marinum* 1218R strain, described in Chapter 3 also showed the similar phenotype. Repeated attempts at generating a complementation construct for this mutant strain was unsuccessful. This suggests that the gene plays a role in the biosynthesis of the core acylated trehalose structure of LOS.

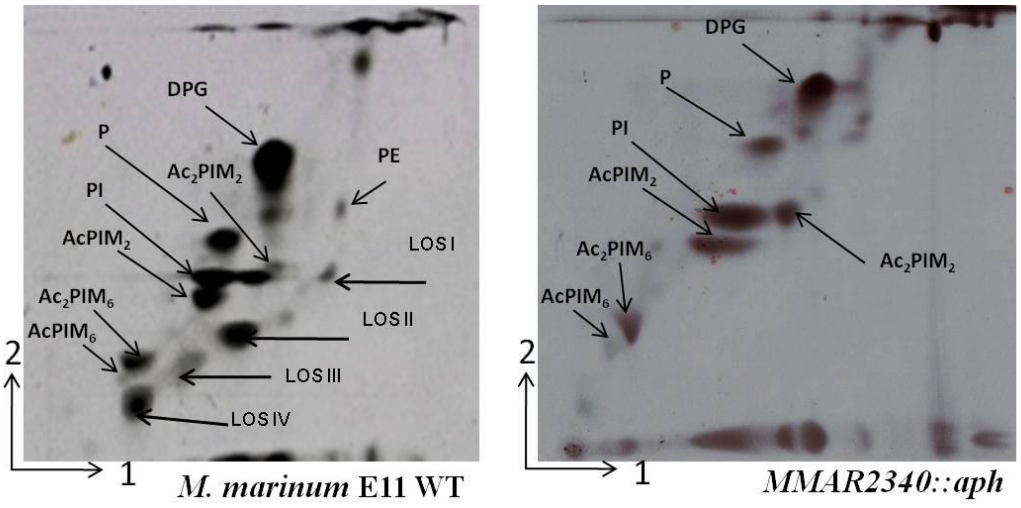


Figure 4.11. Autoradiograph of a 2D-TLC showing labelled polar lipids from *M. marinum* E11 (wild type) and *MMAR2340::aph* grown in Middlebrook 7H10 broth. Direction 1- chloroform: methanol: water, 60:40:6 (v/v/v), direction 2- chloroform: acetic acid: methanol: water, 40:25:3:6 (v/v/v/v).

The gene downstream of the *pks5* gene is *MMAR2341* is annotated as a fatty acyl AMP ligase, *fadD25*. *MMAR2341* has a proposed role in activation of fatty acyl-AMP intermediates and loading the adenylated metabolite onto the Pks5

multienzyme for extension. Disruption of this gene also resulted in a similar phenotype to the *pks5* mutant (Figure 4.12).

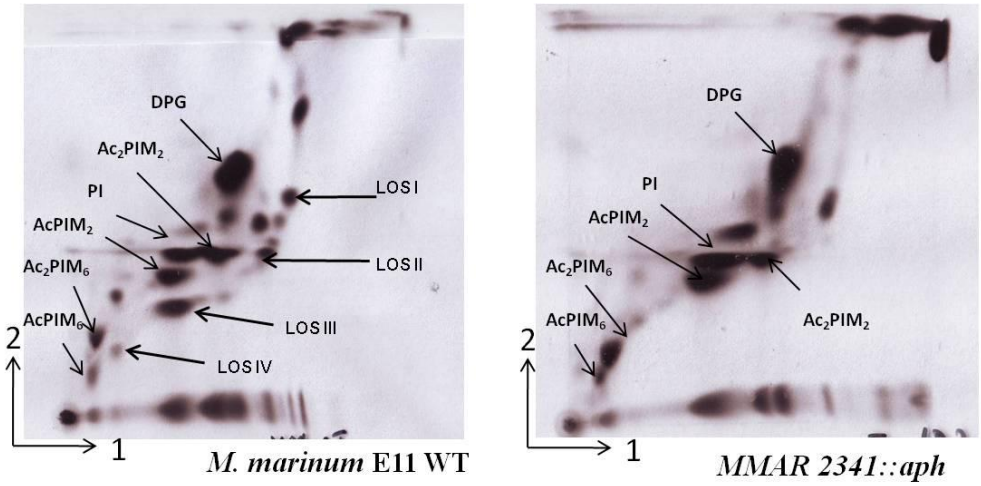


Figure 4.12. Autoradiograph of a 2D-TLC showing labelled polar lipids from *M. marinum* E11 (wild type) and *MMAR2341::aph* grown in Middlebrook 7H10 broth. Direction 1- chloroform: methanol: water, 60:40:6 (v/v/v), direction 2- chloroform: acetic acid: methanol: water, 40:25:3:6 (v/v/v/v). *MMAR2341::aph* is devoid of all the 4 LOS species.

2D-TLC analysis of mutant *MMAR2355::aph* revealed that this mutant strain was not able to produce LOS's (Figure 4.13). This gene encodes the conserved polyketide synthase-associated protein PapA3. PapA3 might function as an acyltransferase associated with Pks5, explaining its role in the biosynthesis of the LOS core structure of acylated trehalose.

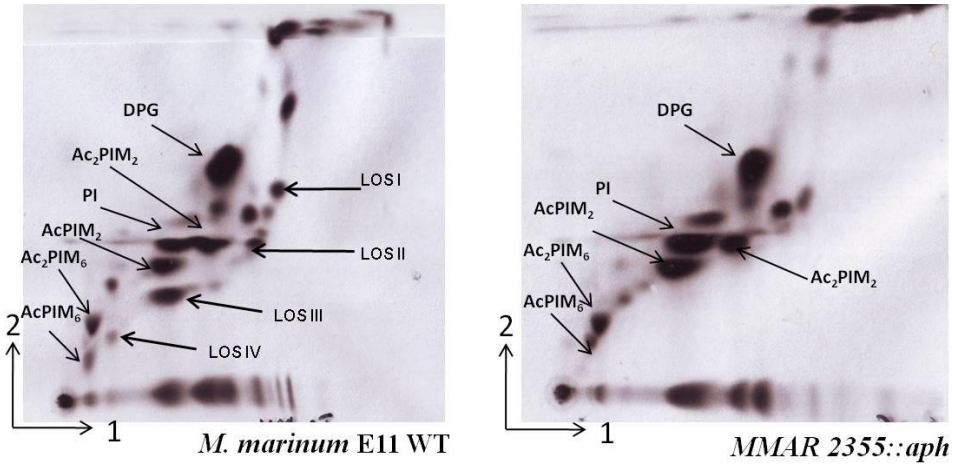


Figure 4.13. Autoradiograph of a 2D-TLC showing labelled polar lipids from *M. marinum* E11 (wild type) and *MMAR2355::aph* grown in Middlebrook 7H10 broth. Direction 1- chloroform: methanol: water, 60:40:6 (v/v/v), direction 2- chloroform: acetic acid: methanol: water, 40:25:3:6 (v/v/v/v). *MMAR2355::aph* is devoid of all LOSs.

MMAR2356 encodes an isoleucyl-tRNA-synthetase. It has a conserved ATP-binding domain. Disruption in this gene resulted in a strain which was devoid of any of LOSs.

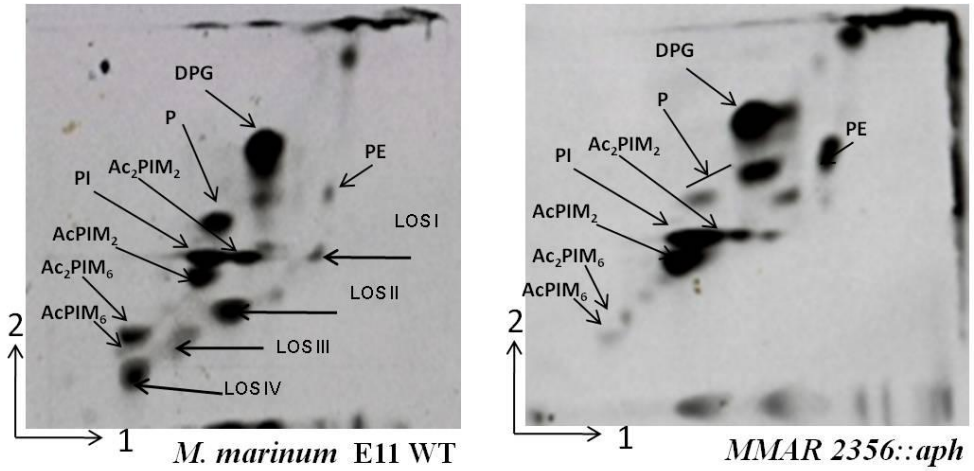


Figure 4.14. Autoradiograph of a 2D-TLC showing labelled polar lipids from *M. marinum* E11 (wild type) and *MMAR2356::aph* grown in Middlebrook 7H10 broth. Direction 1- chloroform: methanol: water, 60:40:6 (v/v/v), direction 2- chloroform: acetic acid: methanol: water, 40:25:3:6 (v/v/v/v). *MMAR2356::aph* is deficient in production of all LOSs.

4.3.2.5 Mutant strains with no difference in LOS production profile

From the transposon library generated in *M. marinum 1218R* strain in-house, a mutant strain with altered colony morphology was selected and genetic analysis revealed a disruption in the gene *MMAR2351*. *MMAR2351* encodes a type II glycosyltransferase and shares 83% amino acid identity with a glycosyltransferase in *M. kansasii* LOS biosynthesis cluster. These groups of enzymes catalyze the transfer of sugar moieties to form glycosidic bonds. 2D-TLC analysis of polar lipids extracted from this strain revealed that a disruption in this gene did not affect the LOS biosynthesis in *M. marinum*.

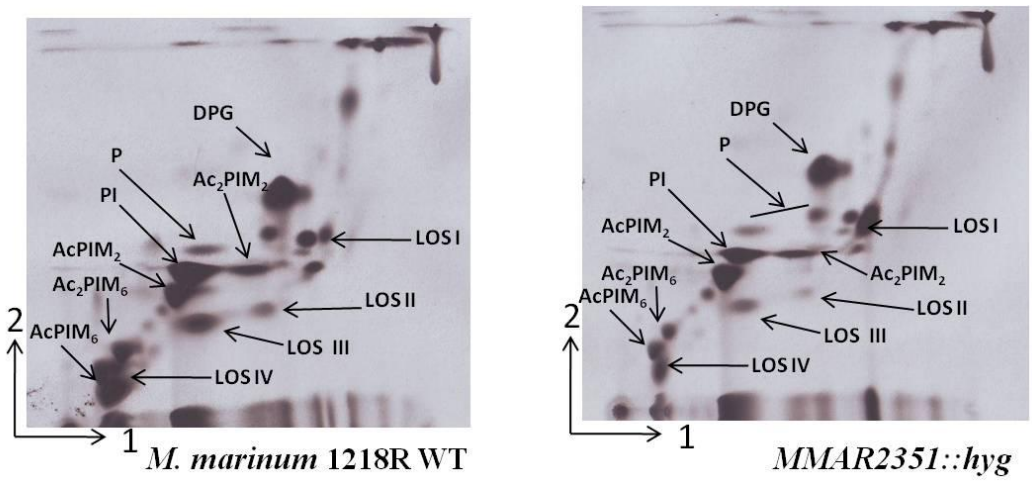


Figure 4.15. Autoradiograph of a 2D-TLC showing labelled polar lipids from *M. marinum* 1218R (wild type) and *MMAR2351::hyg* grown in Middlebrook 7H10 broth. Direction 1- chloroform: methanol: water, 60:40:6 (v/v/v), direction 2- chloroform: acetic acid: methanol: water, 40:25:3:6 (v/v/v/v). *MMAR2351::hyg* does not differ in LOS production as compared to the wildtype.

Rough colony morphology is a characteristic phenotype of strains lacking the presence of LOSs. Rough-dry phenotype observed for some mutants located downstream of the proposed LOS biosynthesis region, *i.e.* in genes *MMAR2355*, and for one mutant strain located far from the LOS biosynthesis region, in gene *MMAR5170*. To examine whether these mutants strains have a role in LOS production, their polar lipid contents were analysed.

The mutant strain *MMAR2353::aph* was disrupted in the gene *MMAR2353*, located downstream of the glycosyltransferase *MMAR2351* in the same operon. *MMAR2353* encodes a UDP-glycosyltransferase. Glycosyltransferases play an important role in synthesis of the LOSs. Loss of *MMAR2315 (losA)* revealed that the strain was defective in production of LOS-IV (Burguière et al., 2005) and loss of another glycosyltransferase *MMAR2333* was responsible for a strain in deficient in LOS-II to LOS-IV (Sarkar et al., 2011). 2D-TLC analysis of the mutant strain *MMAR2353::aph* showed no effect on LOS production as shown in Figure 4.16.

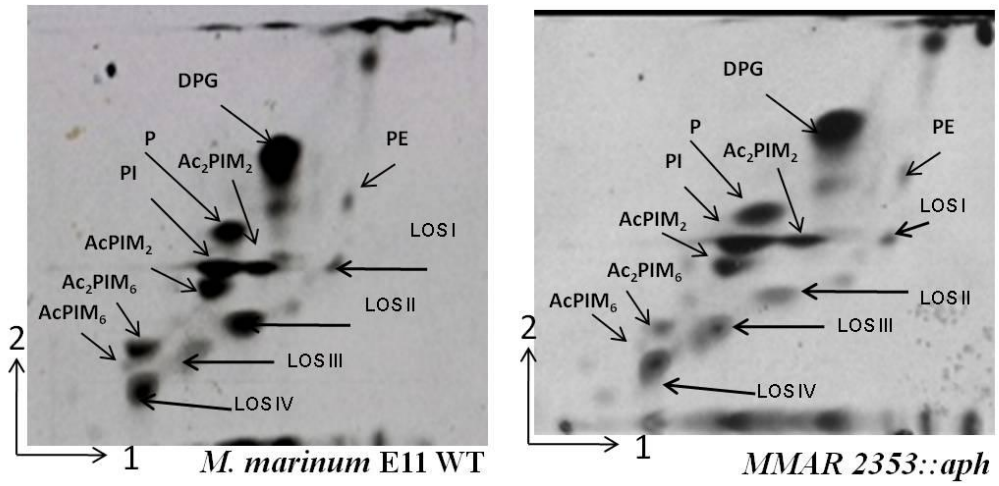


Figure 4.16 Autoradiograph of a 2D-TLC showing labelled polar lipids from *M. marinum* E11 (wild type) and *MMAR2353::aph* grown in Middlebrook 7H10 broth. Direction 1- chloroform: methanol: water, 60:40:6 (v/v/v), direction 2- chloroform: acetic acid: methanol: water, 40:25:3:6 (v/v/v/v). *MMAR2353::aph* does not differ in LOS production as compared to the wildtype.

4.3.2.6 Mutant strains with diminished LOS production

The mutant strain Δ*MMAR5170::aph* was disrupted in the gene *MMAR5170* which encodes for the transcriptional regulatory protein WhiB4. 2D-TLC analysis of polar lipids of this mutant showed that LOS production was highly diminished, although some traces of LOS, especially LOS-III, seem to be present (Fig 4.17). Wildtype phenotypes were restored upon complementation with vector pUCintCat containing the *whiB4* gene.

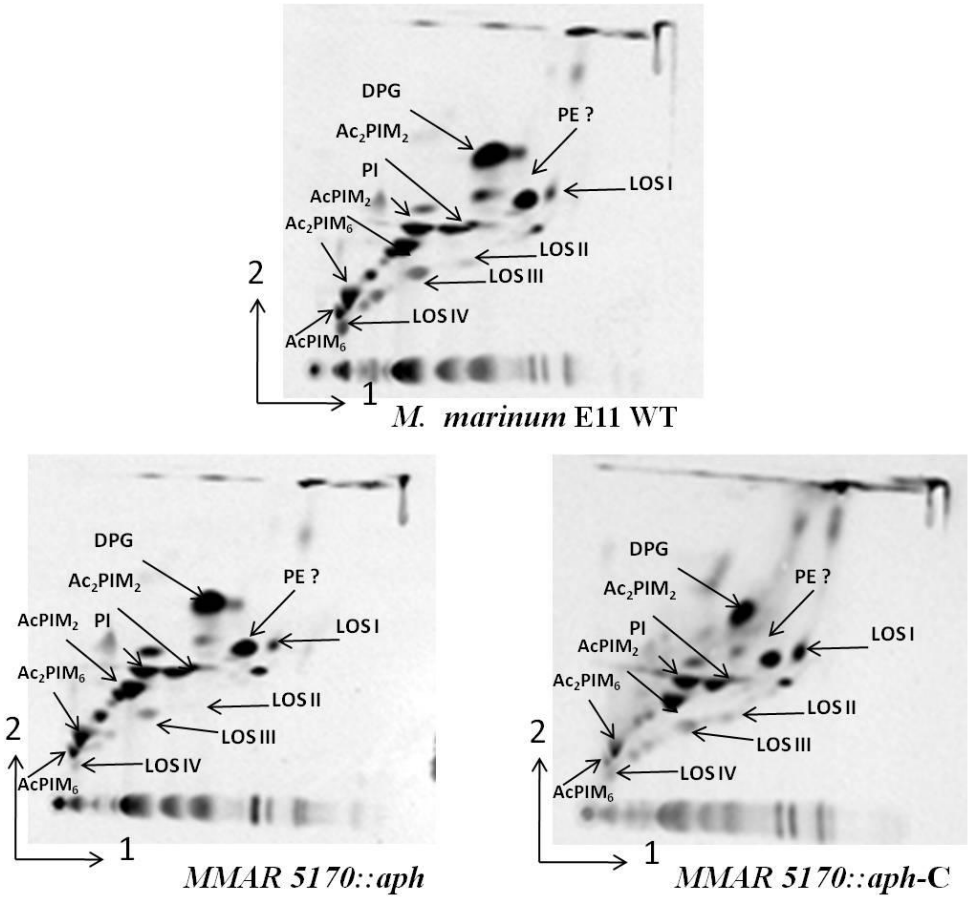


Figure 4.17. Autoradiograph of a 2D-TLC showing labelled polar lipids from *M. marinum* E11 (wild type) and *MMAR5170::aph* grown in Middlebrook 7H10 broth. Direction 1- chloroform: methanol: water, 60:40:6 (v/v/v), direction 2- chloroform: acetic acid: methanol: water, 40:25:3:6 (v/v/v/v).

This result together with the characteristic rough colony morphology suggests that WhiB4 has a role in LOS biosynthesis, which could be regulation of the LOS gene cluster. Together, these results show that the LOS biosynthesis region is even more extended than proposed by Ren et al. (2007) (Figure 4.24).

4.3.3 Intracellular survival in bone marrow derived macrophages

M. marinum mutant strains defective in LOS-IV production were previously reported to have an inability in entering entering bone marrow derived murine macrophages (Burguière et al., 2005). We selected three mutant strains characterised in this study for infecting murine macrophages - *MMAR2327::aph* which accumulated LOS-II, *MMAR2336::aph* which accumulated an intermediate LOS-II* and the mutant strain *MMAR2340::aph* which was deficient in production of all LOSs. These strains would allow us to assess the effects of :

1) Complete loss of all LOSs.

2) Loss of LOS-IV

3) Loss of LOS-III and LOS–IV.

4) Loss of LOS-II and the effect of accumulation of the intermediate LOS- II* and LOS-II'.

In order to assess the roles of *MMAR2327, MMAR2336* and *MMAR2340* disrupted strains in virulence, an intracellular survival assay was performed with bone marrow derived murine macrophages. Activated macrophages were infected with the mutant strains using a multiplicity of infection of 10 to determine the ability to enter and survive within the macrophages. The survival of intracellular bacteria was followed over a period of 1, 3 and 5 days. The number of intracellular bacteria (colony forming units) was enumerated by lysing the macrophages and plating on 7H10 agar supplemented with 10% OADC.

In a previous study it was reported that transposon-mediated disruption of *MMAR2332*, which led to the accumulation of LOSII* was not altered in the ability to survive inside cultured murine J774 macrophages. We wanted to check the ability of the transposon mutant strains generated in this study to enter and survive in murine bone marrow derived macrophages. Also, as reported in Chapter 2 of this thesis, a knockout strain of *MMAR2333* was not affected in the ability to enter and survive inside the bone marrow derived macrophages.

In this study we observed that the mutant strains *MMAR2327::aph* and *MMAR2336::aph* were not altered in their abilities in entering macrophages as observed from the colony forming units/ml counts obtained after lysing the infected BMDMs (Figures 4.18 and 4.19). The strain *MMAR2327::aph* produced only LOS-I and LOS-II and was deficient in producing LOS-III and LOS-IV, while the strain *MMAR2336::aph* accumulated an intermediate, LOS-II' that migrated to positions between LOS-I and LOS-II on a 2D-TLC. The intermediate species had the same migration patterns as LOS-II* on 2D-TLC but differed in mass.

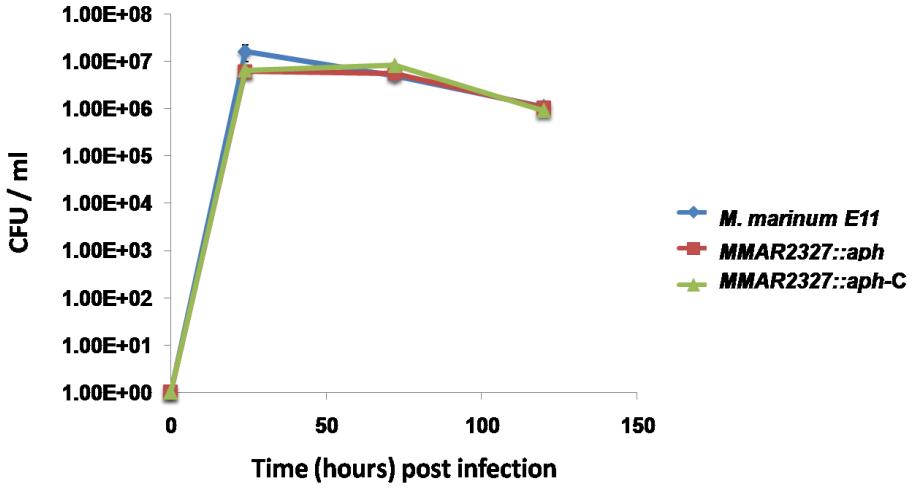


Figure 4.18. Survival of *M. marinum* strains in infected murine (Balb/c) bone marrow derived macrophages. BMDM cells were lysed and plated on 7H10 agar plates and the colony forming units /ml were enumerated.

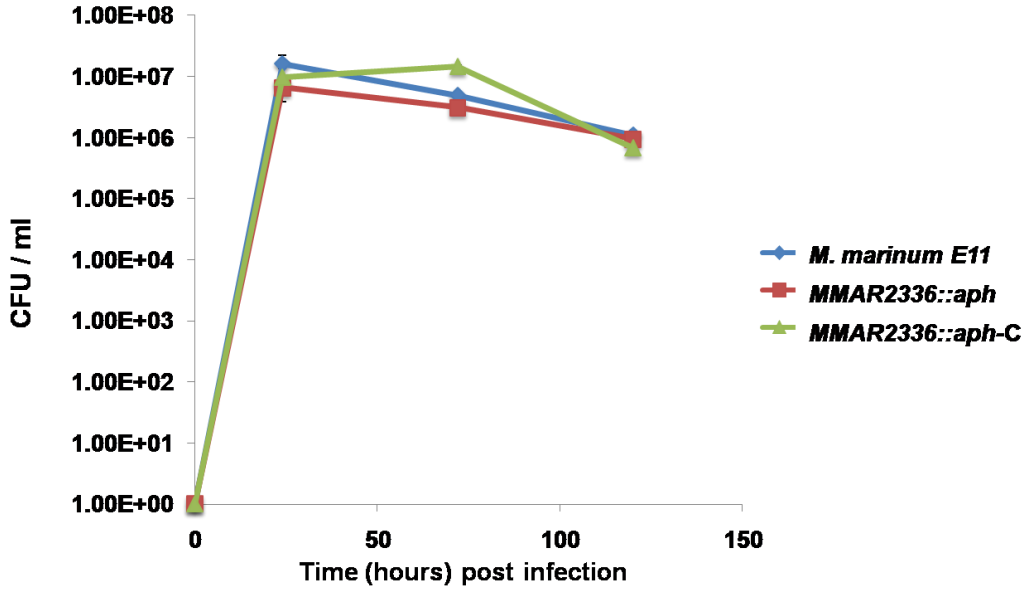


Figure 4.19. Survival of *M. marinum* strains in infected murine (Balb/c) bone marrow derived macrophages. BMDM cells were lysed and plated on 7H10 agar plates and the colony forming units /ml was enumerated.

MMAR2340 encodes a polyketide synthase Pks5 and a disruption in this gene resulted in a strain lacking all the four classes of LOSs. In case of infection

with the mutant strain *MMAR2340::aph*, no change was detected in the ability of the mutant strain in entering the macrophages as shown in Figure 4.20.

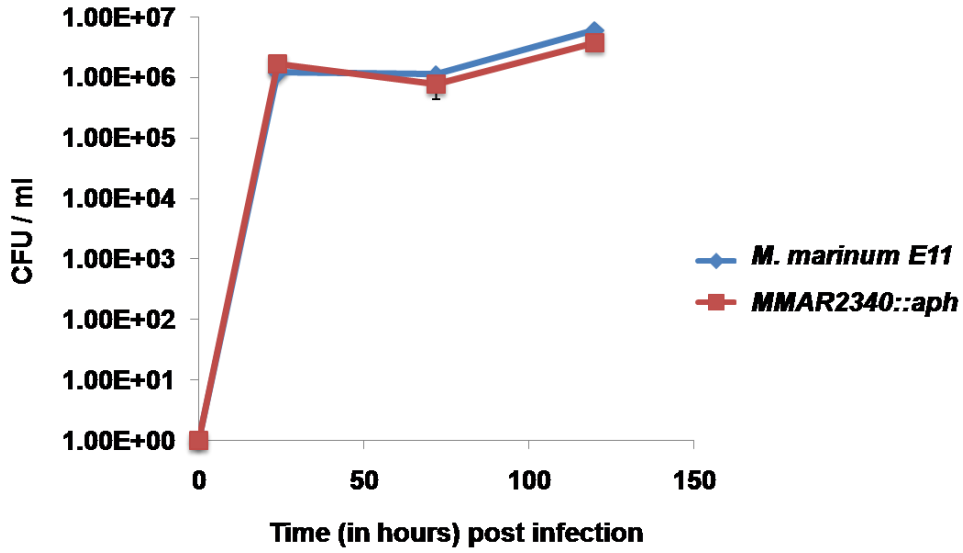


Figure 4.20. Survival of *M. marinum* strains in infected murine (Balb/c) bone marrow derived macrophages. BMDM cells were lysed and plated on 7H10 agar plates and the colony forming units /ml were enumerated.

Cell counts obtained from macrophages infected by different mutant strains did not show any significant difference in terms of entry and survival of the bacterium. In a previous study (Rao et al., 2005; Glickman et al., 2000) it was shown that a *pcaA* mutant of *M. tuberculosis* did not show any defects in entry and persistence in cultured macrophages, but was deficient in granuloma formation and also induced lower levels of the proinflammatory cytokine TNF-α as compared to the wild type strain. Thus, mutant strains that do not show any differences in cell counts may still affect the pro-inflammatory response.

Tumour necrosis factor–alpha (TNF-α) belongs to a superfamily of proinflammatory cytokines that play an important role in regulating inflammation,

host defense, adaptive immunity, apoptosis, autoimmunity and organ development. Macrophages are the primary source of TNF-α production but TNF-α is also produced by many different cell types; T and B cells, osteoblasts, smooth muscle cells etc. (Levine et al., 1995). TNF –α production can be stimulated by a variety of substances for e.g., bacterial lipopolysaccharide, or cytokines like interferon-gamma (IFN-γ). Studies have reported that *M. tuberculosis* infection induces production of the proinflammatory cytokine TNF-α in macrophages and dendritic cells (Orme, 2004). Based on these, TNF- α levels released by infected bone marrow deived macrophages were measured.

Studies using *M. kansasii* to infect murine models have reported that strains, which had a glossy colony morphology, were readily cleared from the mice models while the rough appearing strains were able to survive and produce a systemic infection in mice (Collins and Cunningham, 1981). The glossy and rough colony morphologies were later attributed to production of LOSs. LOS producing strains had smooth / glossy colonies while LOS negative strains had rough colonies (Belisle and Brennan, 1989). Previous reports of studies on *M. marinum* mutant strains devoid of LOS-III and LOS-IV showed that the strains were impaired in entering macrophages (Ren et al., 2007). In contrast to these, it has been shown in another study that purified LOS-IV was responsible for inhibition of TNF-α secretion in activated macrophages in a dose dependant fashion (Rombouts et al., 2009). Also, it was reported that loss of TNF signalling resulted in accelerated bacterial growth and granuloma formation (Clay et al., 2008).

TNF is an important regulator of immune responses and crucial to host defense mechanisms to *M. tuberculosis* infection (Flynn et al., 1995; Zganiacz et al., 2004). On the contrary it has also been shown that TNF can facilitate early growth of *M. tuberculosis* in macrophages (Byrd, 1997;Engele et al., 2002). In this study we observed that the *MMAR2340::aph* strain was efficient in entering and surviving within bone marrow macrophages but the TNF-α levels detected in the infected cell supernatants were much higher than the levels obtained using the wild type strains (Figure 4.21) indicating a pro-inflammatory response accompanied by cytokine release and establishment of infection.

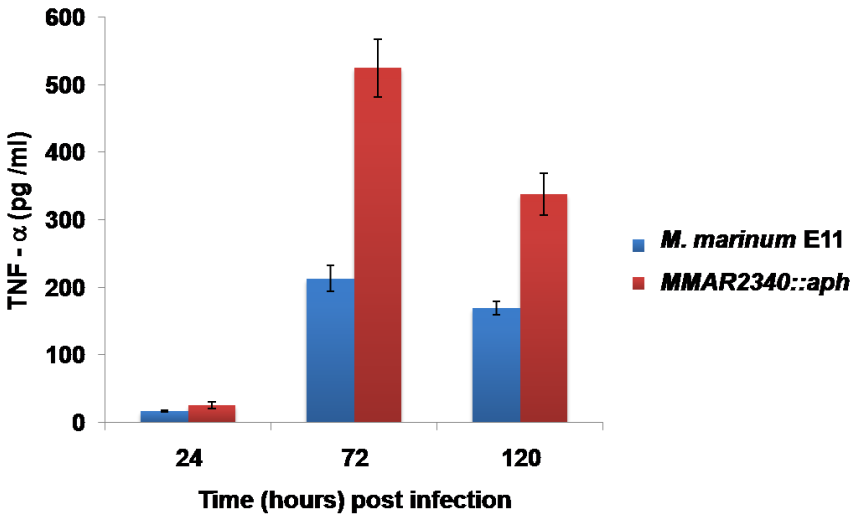


Figure 4.21 TNF- α production by murine (Balb/c) bone marrow derived macrophages infected with different *M. marinum* E11 strains.

Absence of all the four classes of LOSs from this strain might be responsible in interfering with the host immune pro-inflammatory responses which is crucial to the events involved in granuloma formation. A similar cytokine profile was observed in an infection model using a knockout strain of Δ*MMAR2340* in *M. marinum* 1218R, detailed in Chapter 3. Unfortunately due to

the unavailability of a complemented strain and the BMDM being infected with whole cells, further insights into the role of Pks5 in virulence could not be obtained.

It has been proposed that LOSs mask the virulent activities of other cell wall glycolipids like, LAM and PGL (Belisle and Brennan, 1989). The elevated levels of TNF-α due to the loss of all the four classes of LOSs agree with the above hypotheses. The TNF-α levels detected in this experiment were obtained from activated macrophages infected with the mutant bacterium, hence in this case, macrophage cell surface antigen presentation needs to be taken in account. Further research is required to study the exact mechanism of the observed virulence in the absence of LOSs.

Cell supernatant analysis for TNF-α release in infection experiment with the strain *MMAR2327::aph* revealed a slight lower levels in the mutant strain as compared to the wild type and complemented strains. Post infection at 72 hours the wildtype strain infected BMDM TNF-α levels were recorded at 366.5 pg/ml while it was 299 pg /ml in case of the mutant strain. Post infection at 120 hours the values recorded was 221 pg /ml incase of the wildtype and 91.5 pg /ml for the strain *MMAR2327::aph* (Fig 4.22).

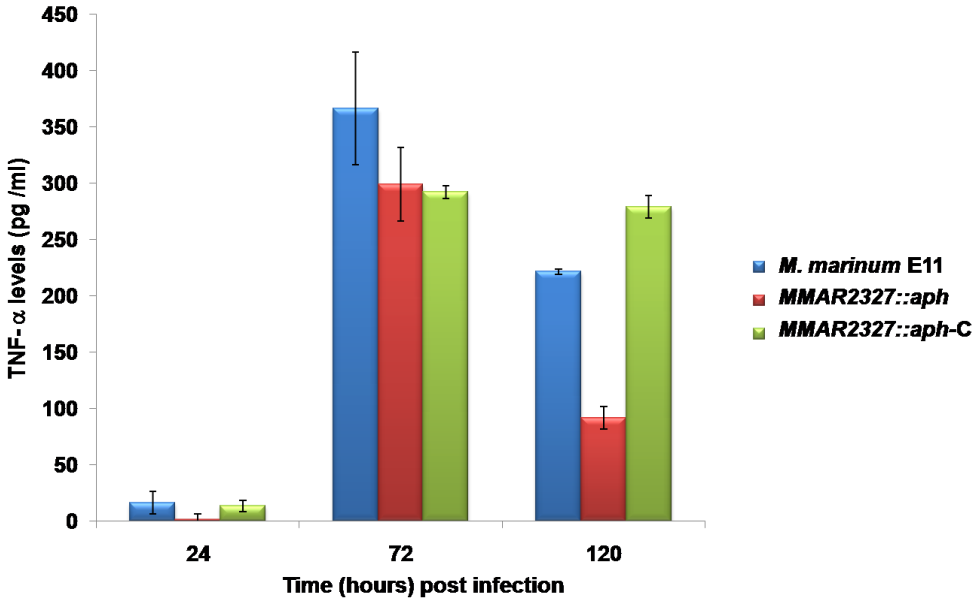


Figure 4.22. TNF- α production by murine (Balb/c) bone marrow derived macrophages infected with different *M. marinum* E11 strains.

TNF-α released by infected BMDM supernatants in case of infection using the strain *MMAR2336::aph* were 251.5 pg /ml as compared to 366.5 pg /ml 72 hours post infection. The levels of TNF-α released decreased to 54 pg /ml 120 hours post infection as compared to 221 pg /ml in the wild type infection set (Figure 4.23).

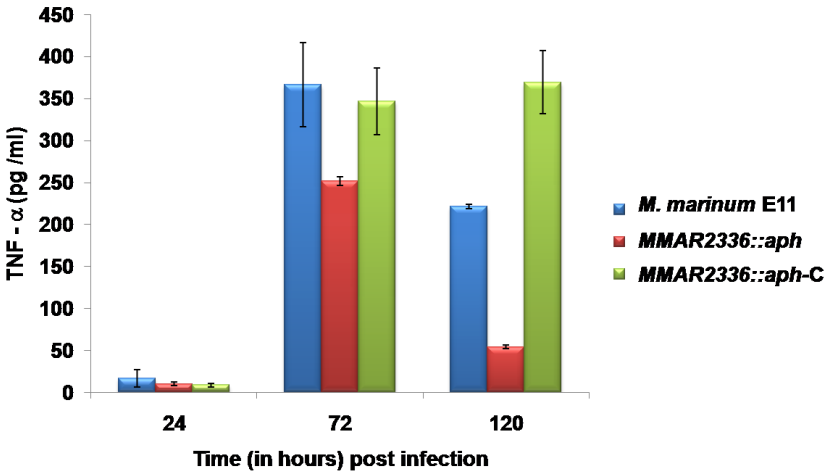


Figure 4.23 TNF-α production by murine (Balb/c) bone marrow derived macrophages infected with different *M. marinum* strains.

Table 4.5 Effects on TNF-α levels in the presence of the different LOS classes.

Type of LOSs	**Effect on TNF-α levels**
No LOS	increased levels of TNF-α
LOS-I , LOS-II'	decreased TNF levels-α
LOS-I , LOS-II *	increased levels of TNF-α
LOS-I, LOS-II	Slight decrease levels of TNF-α
LOS-I, LOS-II and LOS-III	-

Table 4.5 lists the effects on the levels of TNF-α release in the presence of the different LOS classes. TNF-α is a proinflammatory cytokine and has been shown to e released by macrophages upom infection with *M. tuberculosis* (Orme, 2004). It has been proposed that LOSs act as a mask for the cryptic virulent glycolipids like the LM and TDM in the mycobacterial cell wall (Belisle and Brennan, 1989). A study using purified LOS-IV seconds these hypotheses. It was shown that purified LOS-IV was able to inhibit release of TNF-α (Rombouts et al., 2009). Also in out study we show that a strain deficient in production of all the LOSs *MMAR2340::aph* is able to enter and persist in activated BMDM and shows increased production of TNF-α from macrophages indicating infective stage.

Infection with strains of *M. marinum* producing only LOS-I and LOS-II, *MMAR2327::aph* did not affect the TNF-α production levels. While a strain producing LOS-I and LOS-II* (Δ*MMAR2333*, Chapter 2) showed an increase in TNF-α levels. These findings hints at a possibility that the higher LOSs, LOS-III and LOS-IV probably supress inflammatory responses during mycobacterial infection. Surprisingly it was noticed that infection with a mutant strain *MMAR2336::aph* that produces LOS-II' and an intermediate species

between LOS-I and LOS-II was able to reduce TNF-α levels as compared to infection with wild type *M. marinum.*

4.4 Discussion

Presence and absence of LOSs have always been linked to rough-dry /smooth colony morphology in LOS producing mycobacteria. Based on the colony morphology, the *Tn*-mutants were selected for analysis and the gene disruptions in the mutant strains were identified. Polar lipid analysis of these strains led to the identification of ten genes in the LOS biosynthesis pathway, which greatly extends our knowledge of LOS biosynthesis in *M. marinum*.

The LOS biosynthesis gene cluster was identified to extend from *MMAR2302* to *MMAR2340* in a previous study by Ren et al., (2007). In this study mutant strains with disruptions in genes on either side of the above cluster were isolated and characterised. The LOS mutants detected in this screen show a specific deficit in higher order LOS production and a concomitant accumulation of the lower order LOS structures. The genes affected in these mutants also show a specific spatial genomic clustering according to their role in LOS biosynthesis. LOS-I accumulation, indicating a deficiency to synthesize, attach or transport the xylose unit to the LOS structure, is found at the beginning of the LOS biosynthesis cluster in genes *MMAR2307* and *MMAR2309.* The region responsible for the synthesis and attachment of caryophyllose to produce LOS-II and LOS-III seems to be localised roughly between genes *MMAR2327* and *MMAR2336.* Also a rather large part of the genome cluster, genes *MMAR2313* to *MMAR2320,* seems to be reserved for the synthesis and transfer of the terminal 4.6 dideoxy N acyl

galactopyranose to produce LOS-IV. Together, these results greatly contribute to understanding the genes involved in LOS biosynthesis.

The region downstream of *losA (MMAR2313), MMAR2314-2317*, have been described earlier to have a role in the biosynthesis of LOS-IV, hence it is predicted that the gene cluster extending from *MMAR2313 (LosA) to MMAR2320 (WecE)* is possibly responsible for LOS-IV production. An accumulation of LOS-I and loss of LOS-II to LOS-IV was attributed to the loss/disruption of the genes *MMAR2307* and *MMAR2309*. Loss of *MMAR2327* resulted in accumulation of LOS-II and absence of LOS-III and LOS-IV, while the ORF *MMAR2332* to *MMAR2336* was shown to be responsible for loss of LOS-II to LOS-IV and accumulation of an intermediate LOS-II*. The gene cluster *MMAR2340* to *MMAR2406* was shown to be involved in pivotal steps in LOS biosynthesis. As this cluster have genes necessary for the synthesis of the core LOS structure. *MMAR2340* and *MMAR2341* encode Pks5 and FadD25 which are involved in the synthesis and activation of the acyl chains linked to the trehalose core. The gene disruption of *MMAR2353* encoding PapA3 also resulted in a LOS deficient phenotype. Although this gene is located downstream of the putative LOS region, the role of an acyltransferase in the synthesis of the LOS core structure is not surprising. In *M. smegmatis*, the putative acyltransferase *MSMEG4728* was also postulated to have a role in LOS biosynthesis.

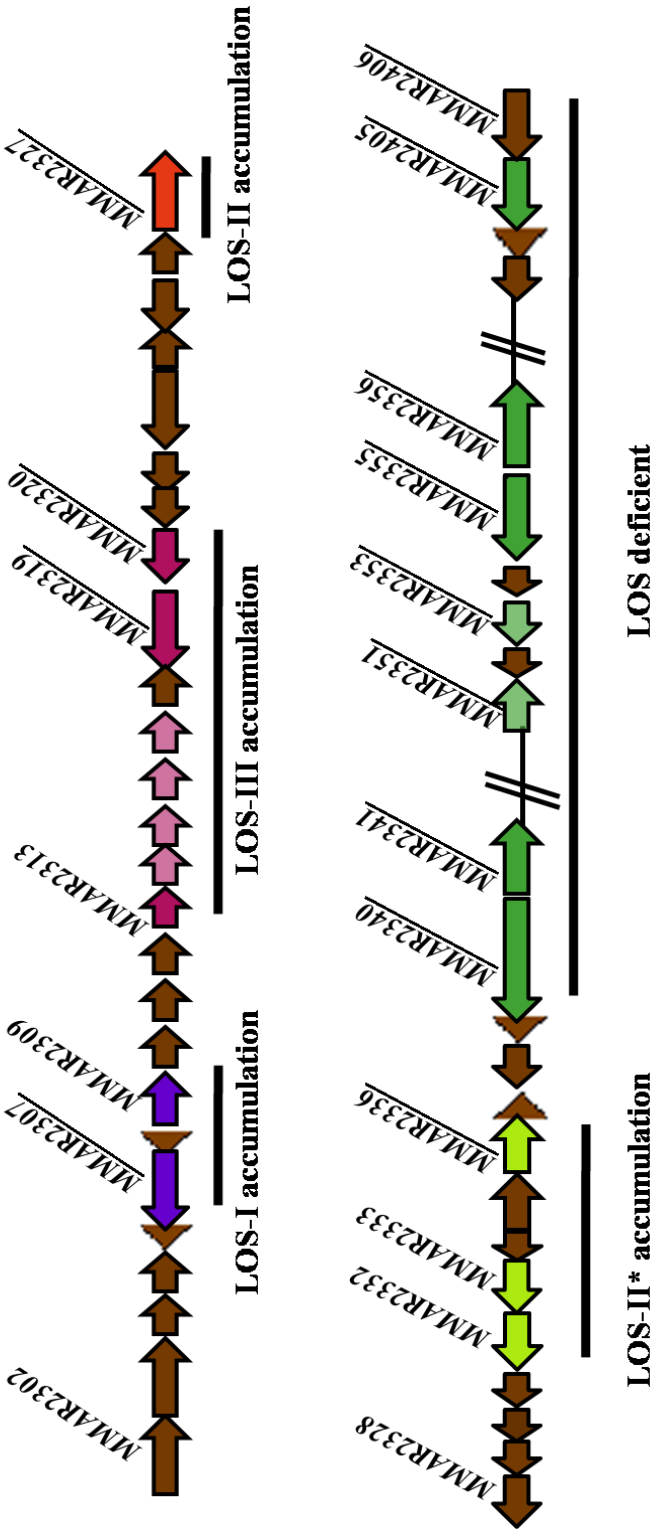


Figure 4.24. Genetic locus involved in LOS biosynthesis. Defects in LOS biosynthesis are indicated under arrows. Genes involved in LOS biosynthesis obtained in this screen and characterised are underlined. Genes with similar LOS profiles are shown in colours, whilst the other genes in the same cluster with no changes in LOS biosynthesis are depicted in lighter colours.

The decrease in LOS production of the *whiB4* mutant suggests that this regulatory protein also has a role in LOS biosynthesis regulation. The paralogue WhiB3 was shown to regulate lipid biosynthesis by regulation of *pks2* and *pks3* expression (Singh et al., 2009). Therefore, it seems likely that WhiB4 regulates LOS biosynthesis, perhaps by regulation of *pks5* expression. The distinctive phenotype of this mutant on filter assay suggests that WhiB4 could have additional functions.

A disruption in the *MMAR2320* gene of *M. marinum,* resulting in LOS-IV deficiency and concomitant LOS-III accumulation, leads to significantly increased early granuloma formation in zebrafish embryos (A.van der woude, communicated). A disruption in the *Pks5* gene lead a strain which was deficient in production of all the four LOSs and higher levels of the proinflammatory cytokine TNF-α was detected in murine bone marrow derived macrophages infected with whole cell of the *M. marinum* mutant strain. Also in our study we show that a strain deficient in production of all the LOSs, *MMAR2340::aph* is able to survive in activated BMDM and shows increased production of TNF-α from macrophages indicating infective stage while infection with *MMAR2327::aph* (producing only LOS-I and LOS-II) did not affect the TNF-α production levels. These findings hints at a possibility that the higher LOSs, LOS-III and LOS-IV probably supress inflammatory responses during mycobacterial infection. The only deviation was observed with *MMAR2336::aph, a* strain that produces LOS-II' and an intermediate species between LOS-I and LOS-II. Suppression was observed in TNF-α levels as compared to infection with wild type *M. marinum.*

It has been proposed that the presence of LOS might act as a mask for other surface-associated factors, such as LAM and PGL (Belisle and Brennan, 1989). LOSs may express key effector molecules capable of interfering with the host immune response which is key to the pathophysiological events that culminate into granuloma formation The fact that LOS production is absent in most species of the *M. tuberculosis* complex, except for *M. canettii*, also fits within this hypothesis. Further research is required to study the exact mechanism of the observed virulence in infection caused by the absence of LOS-IV, and deficiency of LOSs, which would help understand more about the interaction of mycobacteria with its host.

5

Lipooligosaccharide biosynthesis in *Mycobacterium kansasii*

5.1 Introduction

Mycobacterium kansasii was first described in by (Buhler and Pollak, 1953). It is slow growing, photochromogenic and has a characteristic yellow colour and was hence termed 'yellow bacillus'. The bacterium was the first to be shown to cause nontuberculous mycobacterial infections in humans and is the second-most common opportunistic non-tuberculous mycobacteria (Choudhri et al., 1995; Bittner et al., 1996). Five subtypes of *M. kansasii* have been identified based on the genetic analyses of the *hsp65* gene, intergenic region between the 16S and 23S rRNA gene and the 16S rRNA gene of *M. kansasii* (Alcaide et al., 1997; Picardeau et al., 1997; Ross et al., 1992; Richter et al., 1999, Rogall et al., 1990). Type I is the most common subtype associated with human infections (Taillard et al., 2003). *M. kansasii* infections have been reported throughout the world and immunocompromised individuals are at a higher risk of infection. The resemblance of *M. kansasii* infections to tuberculosis and high incidence of occurrence in immunocompromised individuals revealed the importance of nontuberculous mycobacteria as important human pathogens (Wolinsky, 1979).

The most common site of infection is the lungs and symptoms are clinically indistinguishable from pulmonary tuberculosis as they are accompanied by similar histopathological observations such as tubercles and caseation. The symptoms of pulmonary *M. kansasii* infections include cough, sputum production, chest pain, breathlessness, weight loss, fever and sweats. *M. kansasii* also causes skin infections and disseminated disease. Cutaneous infections include formation of nodules, pustules, erythematous plaques, abscesses and ulcers.

Patients with AIDS/ immunocompromised individuals usually present a disseminated infection (Sherer et al., 1986; Selik et al., 1987). Patients with HIV infection, who develop *M. kansasii* infection, can develop *M. kansasii* meningitis similar to *M. tuberculosis* meningitis, bacteraemia, oral ulcers, chronic sinusitis, scalp abscess and disseminated infection. Cutaneous *M. kansasii* in immunocompromised hosts does not show granuloma formation and is sometimes the cause for delayed diagnosis.

M. kansasii has a cell envelope lipid profile similar to that of *Mycobacterium tuberculosis* and *Mycobacterium gastri* (Ortalo-Magne et al., 1996). These bacteria have a unique characteristic of expressing variations in colony morphology ranging from rough forms to smooth/ glossy forms. These variations in physical appearances are attributed to the presence of polar antigenic glycolipids on the cell wall of the bacterium. Presence of immunogenic glycolipids is central to the cell wall of mycobacteria. Most of these are common to the mycobacterial species while some are species specific. Two distinct classes of these glycolipid surface antigens have been identified in *M. kansasii* – phenolic glycolipids and the trehalose containing lipooligosaccharides (Hunter et al., 1983; Hunter et al., 1985; Hunter et al., 1984; Belisle and Brennan, 1989).

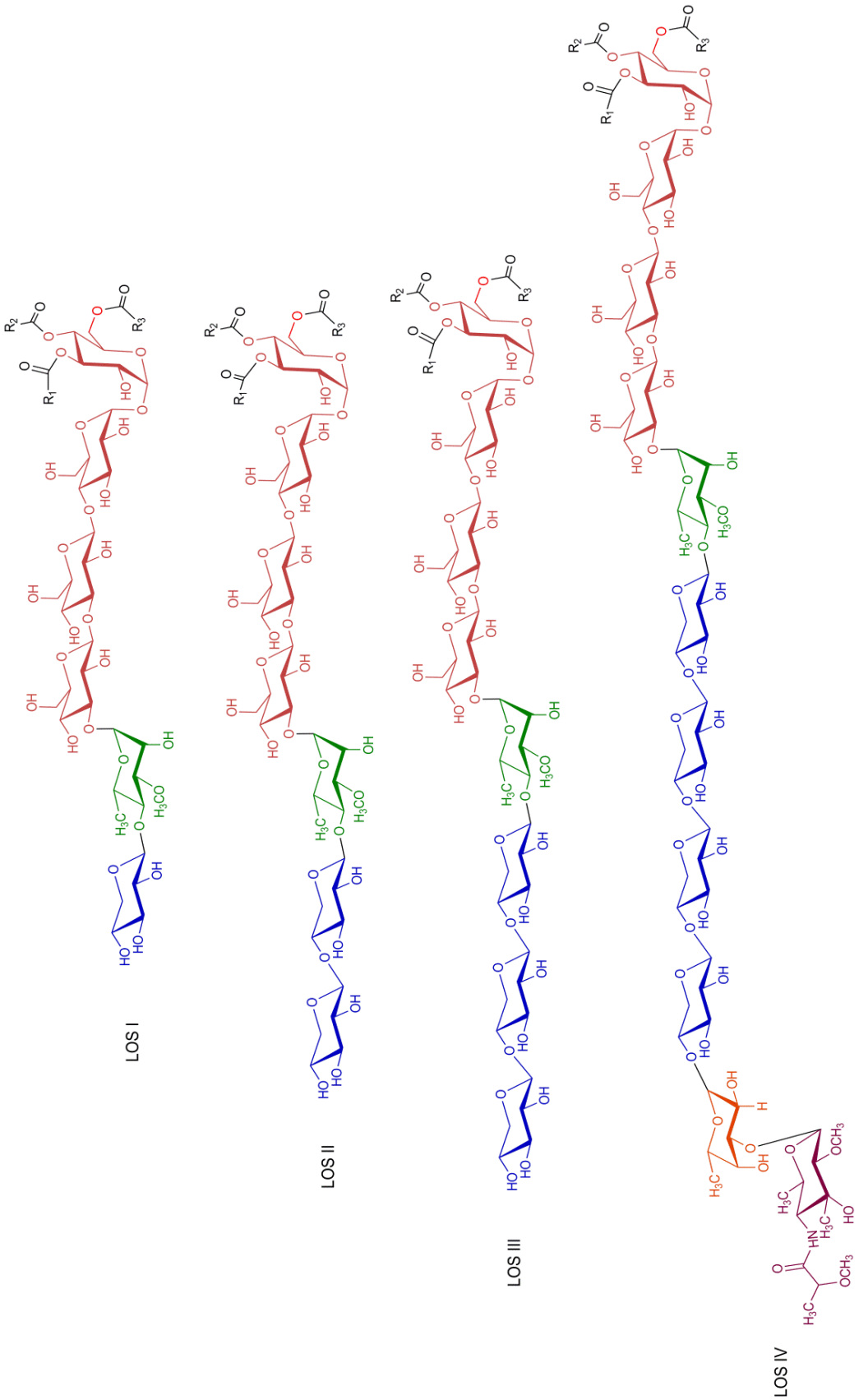
Phenolic glycolipids have been reported and extensively characterised in slow growing pathogenic mycobacteria such as *Mycobacterium leprae, M. tuberculosis, M. kansasii, M. tuberculosis* strain *Canettii, Mycobacterium bovis* , *Mycobacterium marinum, M. gastri,* and *Mycobacterium ulcerans* (Daffé et al., 1987; Puzo, 1990). PGLs consist of a conserved lipid core with varying carbohydrate residues (Minnikin et al., 1982; Chatterjee et al., 1988). The lipid

core is made up of long chain β-diols (C_{33}-C_{41}) known as phenolpthiocerols. The β-diols are further esterified to poly-methyl branched fatty acids. The sugar moiety of phenolic glycolipid consists of one to four sugar residues, most of which are O-methylated deoxysugars. These vary between species. The structure of the *M. kansasii* major phenolic glycolipid differs considerably from the above and was reported to be 2,6-dideoxy-4-*O*-methyl-α-**D**-arabino-hexopyranose (Gilleron et al., 1990), although similarities have been established with glycolipid antigens from *M. gastri*. Phenolic glycolipids have been reported to have immunomodulatory properties (Prasad et al., 1987), for example inhibition of inflammatory cytokine release (Reed et al., 2004). It has also been reported that a loss of phenolic glycolipids resulted in attenuation of *M. bovis* in a Guinea pig infection model (Collins et al., 2005).

The trehalose containing lipooligosaccharides (LOSs) have been reported to play a role in virulence of *M. kansasii*. *M. kansasii* produces seven classes of LOSs, LOS-I to LOS-VII. Chemically these antigenic molecules contain a tetraglucose core, glycosidically linked to a triacyl trehalose unit. The core is common to all the seven classes and is **D**-Glc*p* (β1→ 3) D-Glc*p* (β1→ 4) D-Glc*p*(α1→ 1)**D**-Glc*p*. In addition to this, the LOSs have a 3-O-methylrhamnose residue and varying amounts of xylose. The higher (IV-VII) and more polar LOSs are characterized by the presence of fucose and a novel species-specific N-acylamino sugar {4, 6-dideoxy-2-*O*-methyl-3*C*-methyl-4-(2'-methoxypropionamido) hexopyranose}, now known as N-Acylkanosamine (Hunter et al., 1984). The LOSs from *M. kansasii* are acylated at the 3,4 and 6 position of the terminal glucose by 2,4-dimethyl tetradecanoic methyl esters. In

contrast *M. marinum* has four classes of LOSs - LOS-I, LOS-II, LOS-III and LOS-IV, each containing a common glycan core consisting of four glucose residues and one methylated rhamnose (Burguière et al., 2005).They are acylated by two different classes of polymethylbranched fatty acids – 2,4 dimethyl hexadecanoate and 2,4 dimethyl 2-pentadecenoate.

Analysis of the rough and smooth strains of *M. kansasii* showed that the phenolic glycolipid profile did not differ between the two but LOS production was only observed in the smooth strains of *M. kansasii* but not in the rough strains (Belisle and Brennan, 1989). This indicated that the variations in colony morphology were caused by presence of LOSs in the cell envelope. This variation also translated into differences in virulence observed in the rough strains. It has been reported that the rough strains were able to develop a systemic infection within the infected host while the smooth strains were cleared away (Collins and Cunningham, 1981).


LOS I
LOS II
LOS III
LOS IV

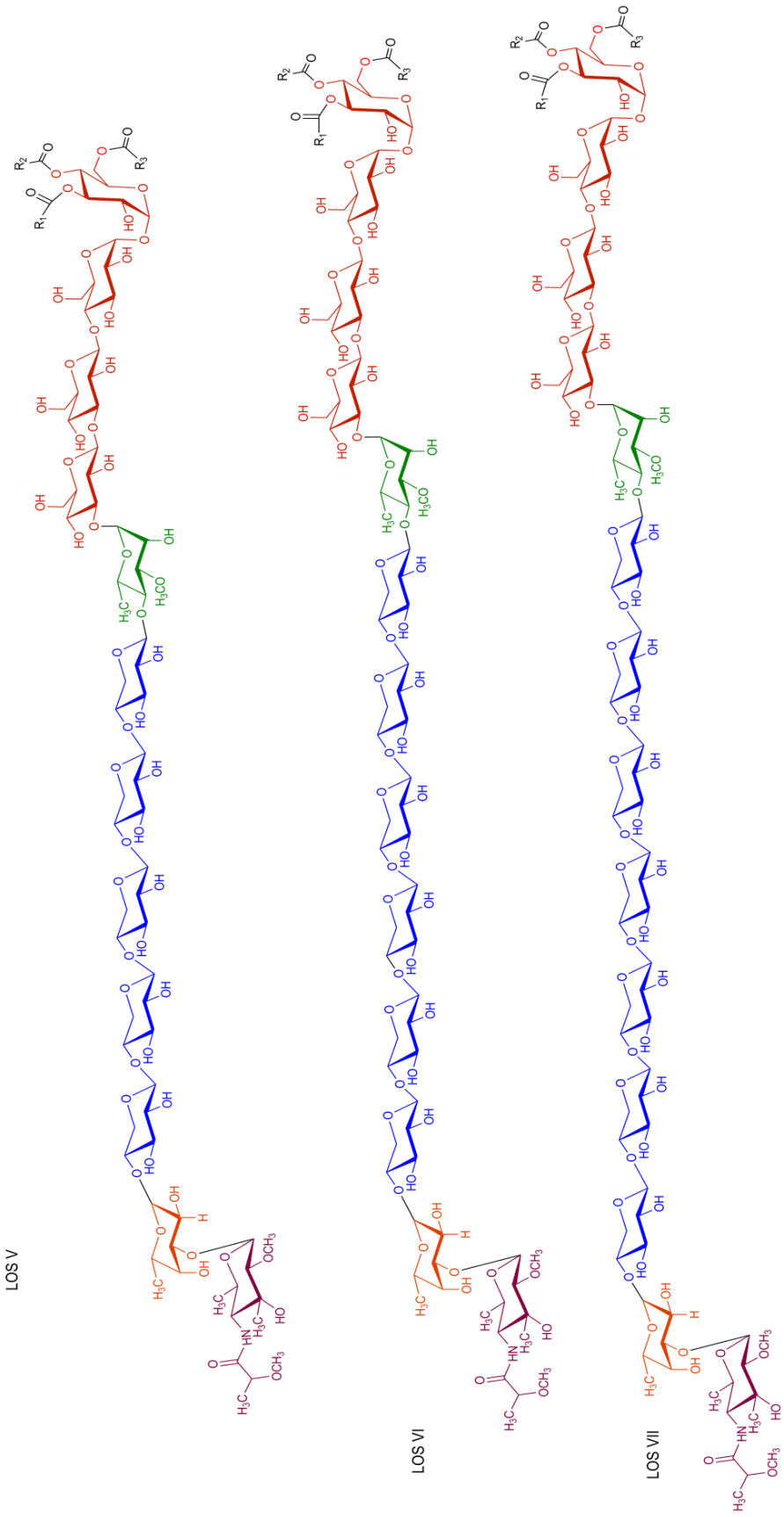


Figure 5.1. Structure of LOS –I to LOS-VII from *M. kansasii*. The rhamnose is shown in green, xylose residues in blue, fucose in orange and the N-acylkanosamine in purple. R_1, R_2, R_3 denote 2, 4, dimethyl tetradecanoic acid.

The LOS biosynthesis gene cluster of *M. marinum* has been identified in a study by Ren et.al. (2007). The aim of this project was to identify the LOS gene cluster in *M. kansasii*. The first step involved analyses of the homologous regions between *M. marinum* and *M. kansasii* and annotation of the LOS cluster from *M. kansasii* based on protein identities. The next step was to generate strains defective in these to further study their effects on LOS biosynthesis. Transposon mutagenesis was used for generation of mutant strains in *M. kansasii*.

5.2 Materials and methods

5.2.1 Bacterial strains, phages and growth conditions

All *M. kansasii* strains were grown in 7H9 broth supplemented with 10% OADC (oleic acid/albumin/dextrose/catalase, BD) and 0.05% Tween 80 at 37°C, (5% CO_2). Hygromycin B was added at a final concentration of 50μg/ml for selection of *M. kansasii* strains where required. *Escherichia coli* cc118λpir strains were used for rescue of the transposon and was grown in LB broth or on LB agar at 37 °C, using 150 μg/ml hygromycin B where required.

Mycobacterium smegmatis strain mc^2155 was used for generation and propagation of mycobacteriophages and was routinely grown at 37 °C either in Middlebrook 7H9 or Tryptic Soy Broth (TSB) / agar.

Table 5.1 Bacterial strains, plasmids and phages used in this study

Strains and phages	Description	Reference
Bacteria		
E. coli cc118λpir		
M. smegmatis mc^2155	Wild type strain, *Ept* mutant of *M.smegmatis* strain mc^26	(Snapper et al., 1990)
M. kansasii	Wild type strain	
Phages		
pHAE181	Conditionally replicating phage TM4 derivative carrying *Tn-5371*	(Kriakov et al., 2003)

5.2.2 Transposon mutagenesis

M. kansasii cultures were grown in 50ml of 7H9+OADC with 0.05% Tween 80 to an optical density (OD_{600nm}) of 0.8. The cells were harvested by centrifugation at 4500g for 10 minutes and washed twice with 50 ml MP buffer. The cell pellet was then gently resuspended in 2 ml MP buffer and mixed with 1 ml of high titre phage lysate (10^{-8} to 10^{-10} pfu/ml). Separately, 500 µl cells were mixed with 500 µl of MP buffer to serve as control. The cell – phage mix was incubated at 37°C overnight (static), the cells were then harvested by centrifugation and resuspended in 10 ml 7H9 broth with OADC and 0.05% Tween 80. The transduced *M. kansasii* cells were recovered by overnight incubation at 37°C. Cells were centrifuged and resuspended in 1 ml fresh media and plated on 7H10+OADC agar plates (100 µl per plate) with 50µg/ml hygromycin B. Plates were incubated at 37°C (5% CO_2) for 2 to 3 weeks.

5.2.3 Isolation and sequencing of *Tn* insertion sites

Transductants obtained were selected on the basis of altered colony morphology. Selected mutants were grown in 10ml of 7H9+OADC + 0.05% Tween 80 +50μg/ml hygromycin B, and genomic DNA was extracted. The genomic DNA was then digested with *Bss*HII (NEB) and ligated with T4 ligase (NEB). The ligation mix was used to transform *E. coli* cc118λpir competent cells. To select self-ligated genomic DNA fragments containing the transposon, the transformation mix was plated on LB-plates with 150μg/ml hygromycin B and incubated overnight at 37°C. Colonies obtained were inoculated in LB-broth with hygromycin to obtain plasmid DNA. Plasmid DNA isolated from the hygromycin resistant transformants was analysed by *Bss*HII digestion and sequenced using the primers HOPS1 (5' GCTTACAATTTAGGTGGCACT 3') and KMN1 (5' AGTGCCACCTAAATTGTAAGC 3') to get the sequence flanking the left and right of the transposon insertion sites.

5.3 Results

5.3.1 Identification of the LOS gene cluster in *M. kansasii* by alignment with *M. marinum* LOS genes

M. kansasii produces seven different types of LOSs with unique sugar moieties. Hence it was presumed that the *M. kansasii* LOS cluster would encompass a large region in the genome as compared to *M. marinum* which synthesises only four LOSs. As some of the genes would be common between *M. kansasii* and *M. marinum*, I used the gene sequences from the *M. marinum* LOS cluster to probe the *M. kansasii* genome in an attempt to identify the *M. kansasii* LOS cluster. An

alignment was produced using the region between *MMAR2302* to *MMAR2367*. The genetic region was compared using the gene information and the BLAST search tool provided by the following website - http://www.ncbi.nlm.nih.gov/sites/entrez?db=genome&cmd=Retrieve&dopt=Overview&list_uids=6107 and also comparing the translated protein products of the genes. The website address has now moved and the information is available on http://www.xbase.ac.uk/genome/mycobacterium-kansasii-atcc-12478 (whole genome shotgun sequence NZ_ACBV01000002, 200.4KB, 166 CDS). The comparison search is presented Table 5.2

Table 5.2. List of genes of *M. kansasii* with a homologue in *M. marinum* LOS cluster.

***M. kansasii* gene ID**	**Function**	***M. marinum* homologue**	**Function**
MKanA1_010100001025	cobalmin biosynthesis	no homology	
MKanA1_010100001030	Permease	no homology	
MKanA1_010100001035c	hypothetical protein	no homology	
MKanA1_010100001040	hypothetical protein	no homology	
MKanA1_010100001045c	PE_PGRS	no homology	
MKanA1_010100001050c	PE_PGRS	no homology	
MKanA1_010100001055c	hypothetical protein	*MMAR2301*	conserved membrane protein of unknown function
MKanA1_010100001060	methylmalonyl-CoA mutase small subunit, MutA	*MMAR2302*	methylmalonyl-CoA mutase small subunit, MutA
MKanA1_010100001065	methylmalonyl-CoA mutase	*MMAR2303*	methylmalonyl-CoA mutase large subunit, MutB
MKanA1_010100001070	ATPase	*MMAR2304*	LAO/AO transport system kinase
MKanA1_010100001075	Esterase LipL	*MMAR2305*	conserved hypothetical esterase LipL
MKanA1_010100001080c	hypothetical protein	*MMAR2306*	conserved hypothetical protein

MKanA1_010100001085c	hypothetical protein	*MMAR2307*	hypothetical trans-membrane protein
MKanA1_010100001090c	hypothetical protein (GtrA domain)	*MMAR2308*	hypothetical membrane protein
MKanA1_010100001095	dehydrogenase	*MMAR2309*	UDP- glucose/GDP-mannose dehydrogenase
MKanA1_010100001100	UDP glucose epimerase	*MMAR2310*	UDP-glucose 4-epimerase
MKanA1_010100001105	Glycosyltransferase	*MMAR2311*	Glycosyltransferase
MKanA1_010100001110	hypothetical protein	*MMAR2802*	conserved membrane protein
MKanA1_010100001115	hypothetical protein	*MMAR2307*	hypothetical trans-membrane protein
MKanA1_010100001125c	hypothetical protein (Gtr domain)	*MMAR2337*	conserved hypothetical membrane protein
MKanA1_010100001130c	hypothetical protein	*MMAR2924*	Methyltransferase
MKanA1_010100001135	hypothetical protein	No homology	
MKanA1_010100001140	Glycosyltransferase ,GT-A type (DPM-DPG)	No homology	
MKanA1_010100001145c	NAD-dependent epimerase/dehydrat ase family protein	No homology	
MKanA1_010100001150	Glycosyltransferase (GTA)	No homology	
MKanA1_010100001155	glucose-1-phosphate cytidylyltransferase	No homology	
MKanA1_010100001160	NAD-dependent epimerase/dehydrat ase family protein	No homology	
MKanA1_010100001165	Rhamnose epimerase	No homology	
MKanA1_010100001170	methyltransferase	No homology	
MKanA1_010100001175	perosamine synthetase	No homology	
MKanA1_010100001180	carbamoyl phosphate synthase	No homology	
MKanA1_010100001185		No homology	
MKanA1_010100001190	methyltransferase	No homology	
MKanA1_010100001195	SAM methyltransferase	*MMAR2339*	SAM methyltransferase

MKanA1_010100001200	Pks5	*MMAR2340*	Pks5
MKanA1_010100001205	acyl CoA synthetase	*MMAR2341*	FadD25
MKanA1_010100001210c	hypothetical conserved protein	No homology	Oxidoreductase
MKanA1_010100001215	hypothetical conserved protein	*MMAR4618*	hypothetical conserved protein
MKanA1_010100001230	WhiB4		
MKanA1_010100001240	hypothetical conserved protein		
MKanA1_010100001250c		*MMAR2343*	hypothetical conserved protein
MKanA1_010100001255c	Pks5_1	*MMAR2344*	Pks5_1
MKanA1_010100001260c		No homology	
MKanA1_010100001265		*MMAR2345*	hypothetical conserved protein
MKanA1_010100001270c	hypothetical conserved protein	No homology	
MKanA1_010100001275	Dioxygenase	*MMAR2346*	hypothetical conserved protein
MKanA1_010100001280	transcription regulator		
MKanA1_010100001285			
MKanA1_010100001290	rhamnosyl transferase	*MMAR2349*	Wbbl2
MKanA1_010100001295	Glycosyltransferase	*MMAR2351*	Glycosyltransferase
MKanA1_010100001300c	Methylase	*MMAR2350*	methylase /SAM-dependent methyltransferase
MKanA1_010100001305c	hypothetical conserved protein	*MMAR2352*	hypothetical conserved protein
MKanA1_010100001310c	UDP glycosyltransferase	*MMAR2353*	hypothetical conserved protein
MKanA1_010100001315c	hypothetical conserved protein	*MMAR2354*	hypothetical conserved protein
MKanA1_010100001320c	PapA3	*MMAR2355*	Polyketide associated protein PapA3
MKanA1_010100001325c	hypothetical conserved protein,		
MKanA1_010100001330	isoleucyl t-RNA synthase	*MMAR2356*	hypothetical conserved protein

MKanA1_010100001335	Arylsulfatase	*MMAR1375*	hypothetical conserved protein
MKanA1_010100001340c	hypothetical conserved protein, vls	*MMAR2358*	hypothetical conserved protein
MKanA1_010100001345	DNA polymerase IV	*MMAR2359*	DNA polymerase IV, DinP_1
MKanA1_010100001350	L-Asparginase	*MMAR2360*	L-Asparginase
MKanA1_010100001355	lipoprotein signal peptidase	*MMAR2361*	lipoprotein signal peptidase LspA
MKanA1_010100001360	Rlu family pseudouridine synthase	No homology	
MKanA1_010100001370	RNA polymerase ECF-subfamily sigma factor	No homology	
MKanA1_010100001375c	putative haemoglobin	No homology	
MKanA1_010100001380	fatty acyl-CoA reductase	*MMAR2366*	Fatty-acyl-CoA reductase
MKanA1_010100001385	ketoacyl reductase	*MMAR2367*	ketoacyl reductase

The expanse of the LOS gene cluster in *M. kansasii* is much larger in comparison to *M. marinum*, accounting for the more complex and higher LOSs in *M. kansasii*. The LOS cluster in *M. marinum* has five glycosyltransferase genes – *MMAR2311*, *MMAR2313*, *MMAR2333*, *MMAR2349* and *MMAR2351*. Earlier studies have shown that *MMAR2313* annotated as *LosA* is essential for biosynthesis of the tetraglucose core as the *M. marinum* strain defective in *MMAR2313* was unable to produce LOS-IV. Deletion of *MMAR2333* results in a strain producing a LOS intermediate LOS-II* and is deficient in production of LOS-II to LOS-IV. LOS-II* is essentially the LOS-I molecule with an additional xylose residue but lacking the unique caryophyllose sugar present in higher LOSs (Chapter 2). A disruption in *MMAR2351* did not alter LOS biosynthesis and the strain was efficient in producing all the four LOSs, as detailed in Chapter 4. The LOS gene cluster in *M. kansasii* extends from *MKanA1_010100001060* to

MKanA1_010100001330 based on the homology with *M. marinum* genes as shown in Table 5.2. Various genes encoding glycosyltransferases were identified in the *M. kansasii* gene locus homologous to *M. marinum*. Some of these genes were unique to *M. kansasii* and did not posess any conserved domain. The identities of these genes with other *M. marinum* genes were ≤65%.

5.3.1.1 Glycosyltransferases (GTFs) –

The LOS gene locus of *M. kansasii* contains many more GTFs compared to *M. marinum*, which also explains the different sugars in *M. kansasii* LOSs. The genes *MKanA1_010100001085c* and *MKanA1_010100001090c* share ≥81% identity to the *M. marinum* genes *MMAR2307* and *MMAR2308.* The gene *MKanA1_010100001085c* encodes a hypothetical protein and no conserved domains were detected. Loss of *MMAR2307* generated a strain with an inability in the production of the LOSs II to IV in *M. marinum.* The gene downstream of this, *MKanA1_010100001090c* possesses a GtrA domain and is unique to *M. kansasii*. These enzymes are thought to be involved in the translocation of an undecaprenyl phosphate linked sugar across the cytoplasmic membrane. The next GTF identified is MKanA1_010100001105 (86% identities with *MMAR2311*, type 2 GTF). It posesss a DPM-DPG synthase domain and a S-adenosyl methionine dependant transferase domain. The gene MKanA1_010100001125c has a glycosyltransferase GtrA type domain and shares 83% identitity to MMAR2337 (hypothetical protein with no conserved domains). The role of MMAR2337 is not yet known in LOS biosynthesis, but strains of *M. marinum* with loss of *MMAR2333* or a disruption in *MMAR2336* resulted in an inability to synthesise LOS-II to LOS-IV (Chapter 2 & 4).

The region between *MKanA1_010100001140* and *MKanA1_010100001190* show no matches with the *M.marinum* genome. Analysis of the protein sequences reveal presence of unique *M. kansasii* specific GTFs and methyltransfearses. The closest matches on a BLAST search with *MKanA1_010100001140* are the plant pathogens *Clavibacter michiganensis sp. michiganensis* and *sp. sependonicus*. A characteristic GT-2 structural fold is detected with bacterial dolichol phosphate mannose (DPM)-like domains. Eukaryotic DPM synthases are members of the GTF-2 super family that catalyse the transfer of nucleotide sugars to dolichol phosphate. In bacteria, homologues of DPM synthases use polyprenol phosphate, rather than dolichol phosphate. *MKanA1_010100001150c* and *MKanA1_010100001155* encode a type-2 GTF with a bacterial DPM1-DPG synthase domain and a glucose-1-phosphate cytidyltransferase domain, respectively. The bacterial dolichol-phosphate glucosyltransferase (DPG) is involved in the transfer of the glucose from a UDP-glucose to a polyprenol carrier. The nucleotide transferase enzymes transfer nucleotides onto phosphosugars. In *M. marinum* a caryophyllose sugar is present in LOS-II to LOS-IV and a type-2 GTF with a bacterial DPM1-DPG synthase domain MMAR2333 is likely involved in the transfer of the caryophyllose to a polyprenol carrier and by actions of other GTFs the sugar is added to the growing LOS chain (Sarkar et al., 2011). The protein product of *MKanA1_010100001290* is a rhamnosyltransferase which shares 89% identity to a type-2 GTF in *M. marinum* MMAR2349 *(Wbbl2). MKanA1_010100001295* encodes a GTA type glycosyltransferase and the amino acid sequence has a significant match to a GTF in *M. marinum,* MMAR2351. Disruption of *MMAR2351* did not affect LOS

biosynthesis in *M. marinum* (Chapter 4). *MKanA1_010100001310c* encodes a UDP-glycosyltransferase and the protein shares 83% identity to MMAR2353. A disruption in *MMAR2353* did not seem to affect LOS biosynthesis as shown in Chapter 4.

5.3.1.2 Other classes of enzymes

MKanA1_010100001160 encodes a NAD-dependant epimerase/dehydratase. It shares 44% amino acid identity with the *M. marinum* MMAR2336 which encodes a UDP-glucose 4-epimerase and both the enzymes possess conserved NAD binding motifs. This family of proteins use NAD as a cofactor and catalyse various reactions using a nucleotide sugar substrate. Disruption in *MMAR2336* resulted in a mutant strain which failed to produce LOS-II to LOS-IV and accumulated an intermediate similar to LOS-II* (Chapter 4), which lacked the nucleotide sugar caryophyllose. *MKanA1_010100001170* encodes a methyltransferase and the protein posess methyltransferase motifs at both N and C terminus. *MKanA1_010100001175* encodes a perosamine which belongs to the WecE superfamily (3-amino, 5-hydroxy benzoic acid synthase / aspartate amino transferase family). These enzymes are pyridoxal phosphate dependant and are predicted to be involved in cell wall biosynthesis. A sugar transaminase present in the LOS cluster in *M. marinum, MMAR2320 (WecE)* was shown to be involved in the partial biosynthesis of the N-acylated dideoxy galactose found in LOS-IV of *M. marinum* (Rombouts et al., 2010). The higher LOSs in *M. kansasii* has a unique N-acyl-kanosamine (N-acyl propionamido hexose sugar) and the gene product MKanA1_010100001175 maybe unique to *M. kansasii* and involved in biosynthesis of the N-acyl kanosamine. *MKanA1_010100001195* encodes a S-

adenosyl methionine dependant methyltransferase and shares 83% amino acid sequence identity with the *M. marinum* LOS cluster methyltransferase *MMAR2339.* The genes between *MKanA1_010100001205* and *MKanA1_010100001250* are annotated as conserved hypothetical proteins and maybe unique to *M. kansasii*. Amongst this region, *MKanA1_010100001245c* encodes a transport family protein mmpl (*m*ycobacterium *m*embrane *p*rotein, *l*arge*)* which is identical to MMAR2342 (MmpL). These are large proteins involved in transport of various classes of molecules and further characterization is required to decipher if the protein is possibly involved in transport of the LOSs across the cell membrane.

5.3.1.2.1 Polyketide synthases (Pks) and Pks associated genes

MKanA1_010100001200 encodes polyketide synthase 5 and has considerable identity to the Pks5 from *M. marinum MMAR2340.* Loss of *MMAR2340* resulted in a strain deficient in production of all the LOSs in *M. marinum.* The terminal glucose in the LOS is acylated. It is predicted that the Pks5 genes are involved in acylating the glucose core in LOSs. Two copies of Pks5 are present in both *M. marinum* and *M. kansasii*. *MKanA1_010100001255c* encodes the other Pks5 gene in *M. kansasii*, known as Pks5_1. *MKanA1_010100001205* encodes an acyl-CoA synthetase which is similar to *MMAR2341 (FadD25).* The protein product of *MKanA1_010100001320c* is a polyketide associated protein PapA3. *M. kansasii* PapA3 shares 90% identitiy to PapA3 from *M. marinum* and posesses a phosphopantetheine binding motif and is predicted to be involved in catalysing condensation reactions. Disruption in *PapA3 (MMAR2355)* resulted in a strain

lacking all the four LOSs in *M. marinum.* The conserved domains in this protein and phenotype predict that PapA3 is involved in condensing the fatty acyl chains generated by the Pks5.

5.3.2 Generation of *Tn*-mutants in *M. kansasii* using phAE181

A mariner-based transposon *Tn5371* was used to generate a transposon library of *M. kansasii* using the recombinant phage phAE181 (Kriakov et al., 2003) and the data included in this chapter describe the isolation and identification of *M. kansasii Tn*-mutants with altered colony morphology. phAE181 has not been reported to be used earlier with *M. kansasii*, hence prior to transduction it was tested for ability to form plaques on a *M. kansasii* lawn (Figure 5.2)

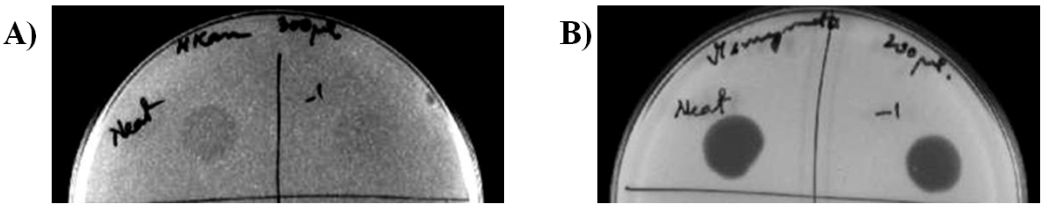


Figure 5.2. Plaque formation on a lawn of *M. kansasii* and *M. smegmatis.* A) 10µl spot of phAE181 on overaly of *M. kansasii* on 7H10 + OADC soft agar, incubated at 30°C for 5 days. B) 10µl spot of phAE181 on overaly of *M. smegmatis* on overlay of 7H10 + OADC soft agar, incubated at 30°C for 5 days.

5.3.3 Isolation of *M. kansasii Tn*-mutants with altered colony morphology

Variations in colony morphology of mycobacteria have been reported from very early literature (Steenken, 1950). Variations reported were usually smooth and rough colony morphology and have been associated with immunospecificity (Petroff and Steenken, 1930). Changes in LOS patterns in *M. marinum* often led to altered colony morphology (Chapter 2). *M. kansasii* strains showed distinct

variations in colony morphology (Fregnan and Smith, 1962) and the variations were attributed to the presence and absence of LOSs (Belisle and Brennan, 1989). This basis of variation was used to distinguish between *hyg*-resistant *M. kansasii* transductants obtained from the transposon mutagenesis. From the library of ~700 Tn-mutants, 196 colonies were selected by visual determination of rough /smooth phenotype. These were inoculated into 96-well culture plates in 7H9 complete media with Hygromycin B and grown till mid-log phase. In order to view single colony morphology, the cultures of the bacterial strains were then used to generate serial dilutions of upto10^{-8}. The neat culture and the dilutions were spotted 7H10 agar and 7H10-T (0.05% Tween 80) agar plates. The plates were incubated for 2 weeks at 37°C (5% $CO_{2)}$ and isolated single colonies were studied under a binocular microscope. The same was done for the wild type strain. Some mutants showed altered colony morphology as compared to the wild type strain. Also under the same growth conditions and equal volume of inoculum, some had slower growth and smaller colonies whilst few had larger colonies as compared to the wild type strain.

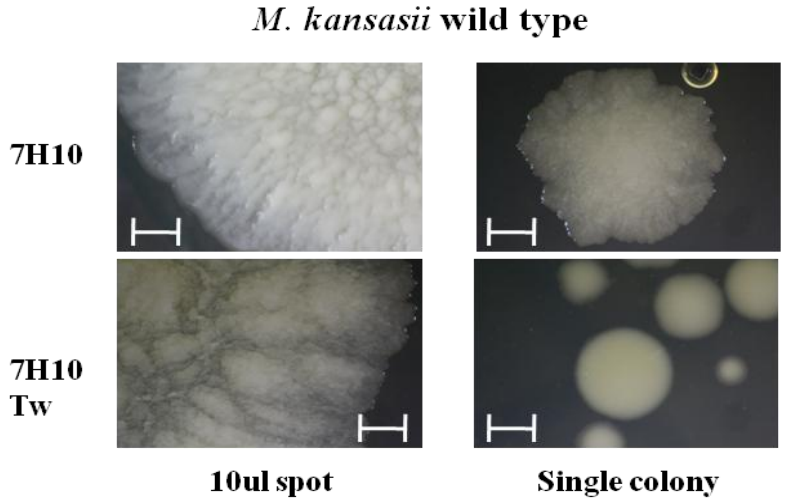
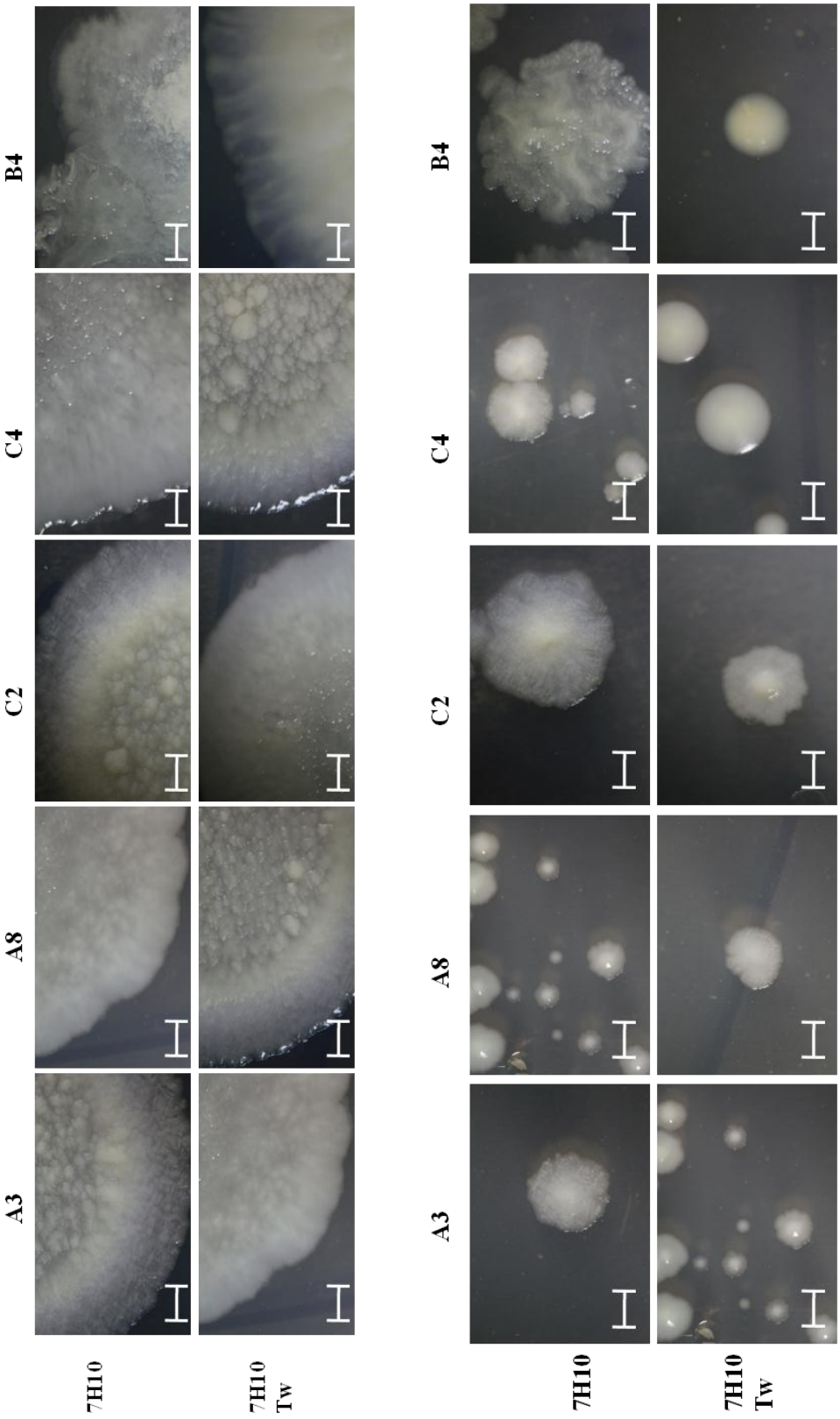


Figure 5.3. Colony morphology of *M. kansasii* wild type. 10µl spot of a mid-log phase culture and single colony. 7H10Tw; 7H10 agar containing 0.05% Tween 80.


A3
A8
C2
C4
B4
7H10
7H10 Tw
A3
A8
C2
C4
B4
7H10
7H10 Tw

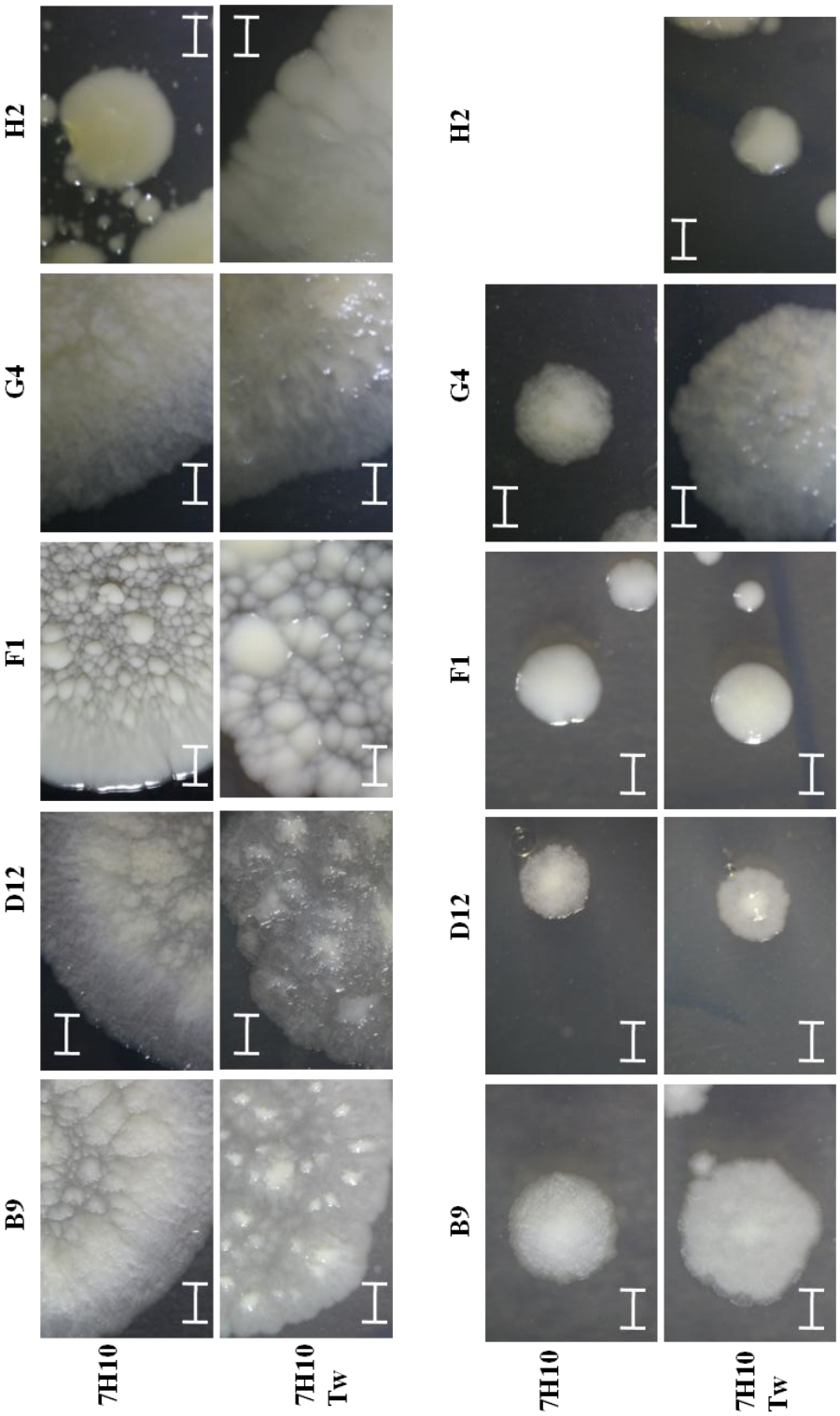


Figure 5.4. Colony morphology of *M. kansasii Tn*-mutant strains. A) and C) 10µl spot of a mid-log phase culture; B) and D) Single colony of each *Tn*-mutant. 7H10Tw - 7H10 agar containing 0.05% Tween 80. Scale bar represents 1mm. Single colony image of mutant strain *H2* was not available.

5.3.4 Isolation of transposon insertion sites and identification of disrupted genes in *M. kansasii* mutants with altered colony morphology.

The restriction enzyme *Bss*HII has no recognition sites in the transposon *Tn-5371*. This enzyme is hence used for digesting genomic DNA obtained from the *Tn*-mutant. When a fragment spanning the *Tn*-disrupted DNA circularizes by ligation, it can replicate as a plasmid as *Tn-5371* has a R6K *ori*. The transposon insertion sites were rescued in *E. coli* cc118λ pir in this way. The isolated plasmids were then sequenced using primers KMN1 5' AGTGCCACCTAAATTGTAAGC 3' and HOPS1 5' GCTTACAATTTAGGTGGCACT 3'. The sequence obtained was used as query for a BLAST (Altschul et al., 1990) search on the Xbase genome database http://www.xbase.ac.uk/mycodb/blast.

The Table 5.3 shows the list of genes disrupted in the *Tn*-mutants isolated from the colony morphology screen. The aim of this research project was to generate mutants in the LOS biosynthesis pathway. Surprisingly, only one of the mutants isolated in this screen has a gene disruption in an ORF in the putative LOS cluster. The mutant strain F1 showed a disruption in the *M. kansasii* gene *MKanA1_010100001215* which falls in the gene cluster homologous to the *M. marinum* LOS cluster. This mutant was further analysed to detect changes in LOS profiles.

Table 5.3 Genes disrupted by Transposon insertion in *M. kansasii* mutants

Mutant no	Gene no	Gene name	Function	Homologue in *M. marinum*
A3	*MKanA1_010100012848*	hypothetical protein	4-hydroxybenzoate 3-monoxygenase	*MMAR4271*
A8	*MKanA1_010100011352*	Acyl-CoA synthetase	involved in fatty acid biosynthesis	*MMAR4476 (pks16)*
C2	*MKanA1_010100001510*	putative transmembrane protein	unknown function	*MMAR2388*
C4	*MKanA1_010100024653*	hypothetical protein	translation incomplete on carboxyl end	*MMAR0482*
B4	*MKanA1_010100025520*	hypothetical protein	zinc peptidase like superfamily	*MMAR3123*
B9	*MKanA1_010100011087*	hypothetical protein	Acyl-ACP thioesterase , involved in lipid metabolism	*MMAR0791*
D12	*MKanA1_010100011032*	putative peptidase	amino acid transport and metabolism	*MMAR0779 (peptidase)*
F1	*MKanA1_010100001215*	hypothetical protein	unknown function	*MMAR4618*
G4	*MKanA1_010100003997*	integral membrane acyltransferase	cellular metabolism	*MMAR0477*
H2	*MKanA1_010100019271*	PPE family protein	unknown function	*MMAR1849*

5.3.5 LOS profile analysis of selected *M. kansasii* F1-mutant

M. kansasii F1::hyg had a transposon insertion in the gene *MKanA1_010100001215*, which encodes a protein with no conserved domains. Bioinformatics analysis shows that the protein product shares 55% identity with *M. marinum* protein MMAR4618. *MKanA1_010100001215* lies downstream to the Pks5 ORF and also falls in the gene cluster homologous to the *M. marinum* LOS gene cluster as shown in Table 5.2. Since the *M. kansasii* LOS gene cluster involves more genes as compared to *M. marinum* and presence of mor LOS types in this strain, the mutant strain *M. kansasii F1::hyg* was selected for further biochemical analysis of the cell wall polar lipids to study the effect of deletion of *MKanA1_010100001215* .

Cultures of the wild type and mutant strains were grown upto an O.D. of 0.6 and pulsed with [^{14}C]-acetate to label lipids. Polar and apolar lipids were extracted and analysed by 2D-TLC as described by Dobson et al. (1985). This study was primarily focussed on detecting any alteration in the LOS profiles, hence the polar lipids were analysed using solvent system E- chloroform / methanol / water : 60/30/6 (v/v) in direction 1 and chloroform / acetic acid/ methanol / water : 40/25/3/6 (v/v) in direction 2.

There were no detectable changes in the LOS profile between the wild type and mutant strain. The following figure shows the LOS pattern in the wild type strain and the mutant F1 (Figure 5.5).

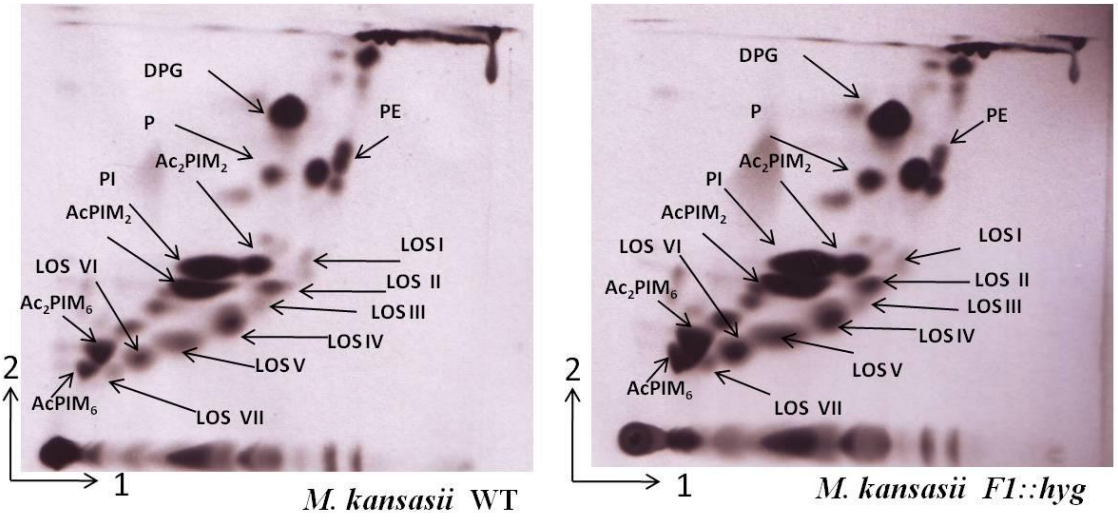


Figure 5.5. 2D-TLC autoradiograph showing labelled polar lipids from *M. kansasii* wild type and *M. kansasii F1::hyg* grown in Middlebrook 7H9 broth. Direction 1- chloroform: methanol: water, 60:40:6 (v/v/v), direction 2- chloroform: acetic acid: methanol: water, 40:25:3:6 (v/v/v/v). *M. kansasii F1::hyg* does not differ in LOS production as compared to the wildtype.

5.4 Discussion

Mycobacteria are characterised by the presence of species or type specific glycolipid antigens. *M. kansasii* is an opportunistic pathogen causing tuberculosis - like disease in humans. The cell wall of *M. kansasii* has been reported to be varied in terms of smooth and rough types. The smooth and rough morphologies have been reported to govern infection establishment in murine models (Collins and Cunningham, 1981). Later, it was shown that the smooth and rough forms are due to the presence or absence of the highly polar immunogenic LOS (Belisle and Brennan, 1989).

Based on the amount of sugar residues in the LOSs of *M. kansasii*, the involvement of many glycosyltransferases is predicted in their biosynthesis. *M. marinum* LOS biosynthesis

cluster has five glycosyltransferases (GTFs). In comparison, the LOS cluster (from *in-silico* analysis of the genome based on amino acid identity search) reveals the presence of nine GTFs in *M. kansasii*. Out of these five GTFs are unique to *M. kansasii* and the four others share high identity with GTFs in the *M. marinum* LOSs gene cluster. This relates to the diversity of the glucose core in *M. kansasii* LOSs.

The aim to this project was to identify the LOS gene cluster by bioinformatics analysis and alignment of *M. kansasii* genes with *M. marinum* genes. Due to unavailability of complete sequence information, generation of targeted knockouts in this species was a hindrance. We used mycobacteriophage mediated transposon mutagenesis for generation of mutants in *M. kansasii*. The transposon mutants were further screened based on their colony morphology and were sequenced. Unfortunately, this study failed to detect any mutant defective in the LOS biosynthesis cluster. A large library of mutants was generated but due to time constraints in this thesis project, the work could not be carried any further. Future plans would involve screening of the remaining transposon mutants and also generation of targeted knockout strains (using specialised transduction) of the GTFs in *M. kansasii* and studying the effects of loss of these genes on the LOS profiles. Availability of the complete genome information of *M. kansasii* will accelerate this process.

6

Mycolic acid processing and transport in *Mycobacterium*

6.1 Introduction

Mycolic acids constitute a major group of cell wall lipids and are one of the defining features of *M. tuberculosis.* They are a vital component of the cell wall and essential to the survival of the organism. They also play a critical role in virulence (Glickman et al., 2000; Dubnau et al., 2000). These long chain α-alkyl-β-hydroxy fatty acids are comprised of 60 – 90 carbons and consist of a meromycolic acid chain (upto C_{56}; which varies between types of mycolates and the species they originate from), and a saturated α- branch consisting of 24 – 26 carbons (Brennan and Nikaido, 1995). Mycolic acids are found in various families of the suborder *Corynebacterineae*, chiefly in *Mycobacteriaceae*, *Corynebacteriaceae*, *Nocardiaceae* and *Gordoniaceae* families which are closely related. The mycolic acids of the pathogenic mycobacteria differ from those of other related genera such as *Corynebacterium*, and *Nocardia* in that they are the longest (C_{70} to C_{90}) (Watanabe et al., 2001; Watanabe et al., 2002). Mycolic acids confer unique properties to mycobacteria, such as unusually low permeability and consequent resistance to common antibiotics (Glickman et al., 2001; Bhatt et al., 2007b; Daffé and Draper, 1998). As most genes involved in the mycolic acid biosynthesis are essential, pathways involved in mycolate biosynthesis can be exploited as potential drug targets. Existing front-line anti-tubercular drugs, such as INH and ethionamide, owe their therapeutic properties to the inhibition of enzymatic components of the mycolic acid biosynthesis pathway. Research in the past two decades has successfully deciphered the biosynthetic pathway for mycolic acids. However, the processing and transport of mycolic acids are not yet clearly understood. A review by Takayama et al. (2005) gave an insight into the possible pathways in which the mycolic acid processing and transport may take place.

These hypothesized pathways were based on findings from a mix of bioinformatics analysis and experimental data.

Mycobacteria contain two fatty acid synthases (FAS) systems - FAS-I and FAS-II. FAS-I is a multidomain peptide found both in eukaryotes and prokaryotes; mycobacterial FAS-I works in a bimodal fashion, catalysing the formation of 'housekeeping' fatty acids (C_{18}) as well as the α-branch of mycolic acids (Bloch and Vance, 1977) thus contributing to (Zimhony et al., 2004). FAS-II on the other hand is a multienzyme complex similar to fatty acid synthases from other prokaryotes and plants, and consists of four discrete enzymatic components (Figure 6.1).

6.1.1 Role of FAS-I and FAS-II in mycolic acid biosynthesis

FAS-I catalyses *de novo* fatty acid biosynthesis using acetyl-CoA and malonyl-CoA primers. After transacylation of these substrates to the enzyme, elongation takes place by condensation of the two starter units to generate a β-ketobutryl-S-Enzyme (covalently linked to the FAS-I enzymes via a thioester linkage). This intermediate product undergoes β-ketoacyl reduction, dehydration and enoyl-reduction to give rise to a butryl-S-enzyme product. Continuous elongation of the C_4-S-enzyme product fatty acids produces long-chain fatty acids. The C_{16} and C_{18} fatty acids are used to the synthesis of membrane phospholipids and the C_{20} and C_{26}-CoA products are fed to the FAS-II cycle (Takayama et al., 2005).

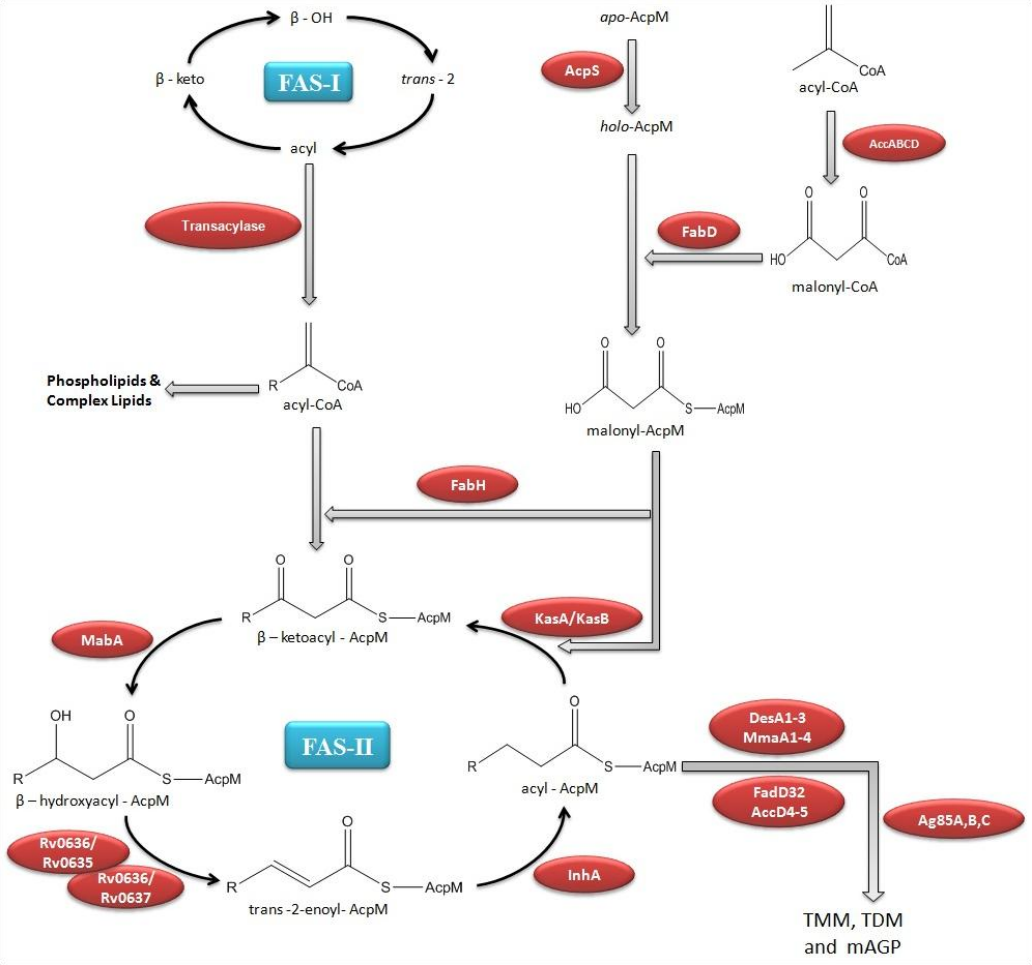


Figure 6.1. Mycolic acid biosynthesis in *M.tuberculosis* adapted from Bhowruth et al. (2008). Malonyl-AcpM is generated from malonyl-CoA by mtFabD, which is then ligated to FAS-I synthesized C_{14}-CoA by mtFabH. The product of this is a C_{16} acyl-AcpM which is further processed by the FASII to generate meromycolates (C_{56}). These meromycolates are ligated to the C_{26} fatty acids synthesized by the FAS-I to form the α-branch of mycolic acids. The final step is catalyzed by Pks13 which involves condensation of the meromycolates to the the α-branch to produce mycolic acids.

FAS-II is only able to elongate CoA derivatives of long chain fatty acids generated by the FAS-I cycle and employs an acyl carrier protein (ACP, termed AcpM in *M. tuberculosis*) to shuttle the substrates between each enzyme component (Kremer *et al*. 2001). The initial

elongation reaction requires the substrate malonyl-AcpM. This is generated by the transacylation reaction of holo-ACP with malonyl-CoA and is catalysed by the enzyme *mt*FabD (malonyl-CoA: ACP transacylase) (Kremer et al., 2001). The acyl-CoA primer from FAS-I is then condensed with malonyl-AcpM by the enzyme *mt*FabH (β-ketoacyl ACP synthase) and is channelled into the FAS-II system. Within the FAS-II cycle, the acyl-AcpM undergoes keto-reduction by MabA (FabG1) (Marrakchi et al., 2002), dehydration by a β-hydroxyacyl-AcpM dehydratase and enoyl-reduction by an enoyl-AcpM-reductase, InhA (Quemard et al., 1995; Bhatt et al., 2007b). The product, a fatty acyl-AcpM with two more carbons added, is repeatedly fed back into the cycle to elongate the chain upto 56 carbons. During this chain elongation process, *mt*FabH activity is replaced by the β-ketoacyl AcpM synthases KasA and KasB which catalyses the subsequent acyl extension steps (Bhatt et al., 2007a;Bhatt et al., 2005; Slayden and Barry, 2002; Schaeffer et al., 2001). *Mt*FabH is the initiator of mycolic acid elongation and structural studies reveal that it possesses the catalytic triad (Cys^{112}, His^{224} and Asn^{274}) which is conserved amongst β-ketoacyl ACP synthase III. Recombinant *mt*FabH also showed sective preference for acyl-CoA as a substrate than acyl-ACP primers (Choi et al., 2000).

The penultimate step in the synthesis of mycolic acids is the Claisen-type condensation reaction catalysed by Pks13 (*Rv3800*), a polyketide synthase which links the α-branch from FAS-I ($C_{20\ -26}$ acyl- CoA) with the meromycolate chain (C_{56} acyl-AcpM) from FAS-II to generate a α-alkyl, β-keto acid, reduction of which yields an oxo-mycolic acid product (Portevin et al., 2004), which is subsequently reduced by a reductase, Rv2509 (Lea-Smith et al., 2007; Bhatt et al., 2008) to yield a mature mycolic acid.

Pks13 is an essential gene in mycobacteria and is very attractive as a drug target. A *C. glutamicum* pks-*13* deletion mutant strain was unable to synthesize corynomycolics acids and produced fatty acid precursors (Gande et al., 2004). The meromycolyl-S-AcpM derived from the the FAS-II is converted to meromycolyl-AMP by a specific fatty acid AMP-ligase FadD32 (Trivedi et al., 2004). The C_{26}-S-CoA generated by FAS-I is carboxylated by the actions of acyl-CoA carboxylases, AccD4 and AccD5 to produce 2-carboxyl- C_{26}-S-CoA. These two products are substrates for the condensation reaction catalysed by pks13 to yield the β-keto ester. This is then converted to a mature mycolate by the action of a reductase, proposed to be *Rv2509*.The majority of the mycolates are then esterified to arabinogalactan of the PG-AG layer, and some end up in the cell wall as trehalose monomycolate (TMM), trehalose 6.6'-dimycolate (TDM) and glucose monomycolate (GMM) (Minnikin et al., 2002). These mycolate derivates are thought to intercalate with the mycolates attached to the PG-AG layer.

6.1.2 Processing and transport of mycolic acids

Mycolic acid biosynthesis is well established but pathways undelying the processing of newly synthesized mycolic acids remain unknown. The mycolyltransferases activity of the Ag85 complex (Belisle et al., 1997; Puech et al., 2002) and role of Myc-PL as a carrier bound intracellular intermediate (Besra et al., 1994) remain the only known components of these pathways. Based on experimental and *in silico* analysis, Takayama et al. (2005), proposed a series of reactions that could occur after the newly synthesised mycolic acid is released from Pks13.These reactions are hypothesised to occur partly inside the cell and are completed outside the cell (Figure 6.2). The enzymes proposed to be involved in this pathway are

mycolyltransferase-I and mycolyltransferase-II. Takayama *et al.*, (2005) reported the presence of 2 classes of mycolyltransferases in mycobacteria – a cytoplasmic mycolyltransferase I and membrane associated mycolyltransferase II.

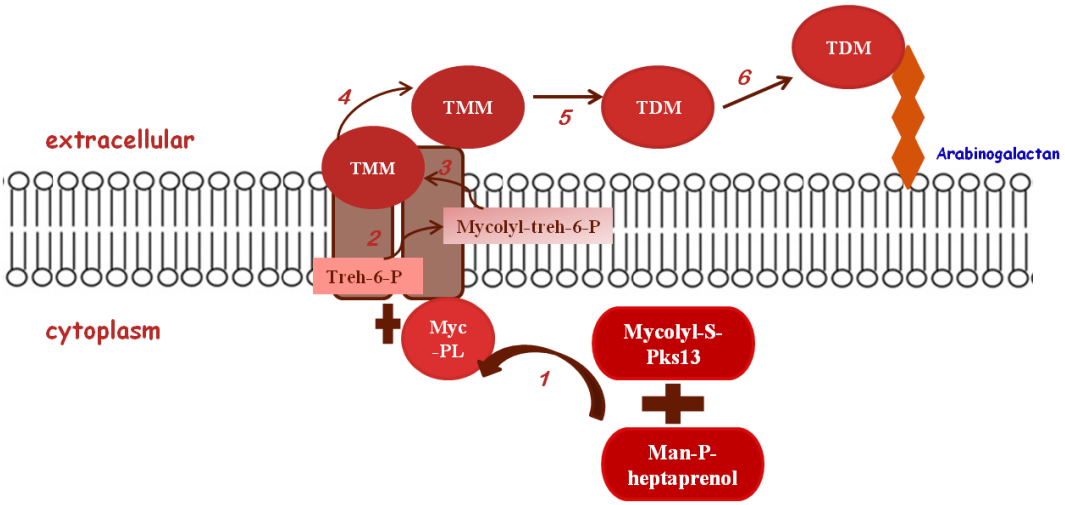


Figure 6.2. Schematic representation of processing of newly synthesized mycolic acids in *M. tuberculosis* (Takayama et al., 2005). Enzymes involved: 1 - mycolyltransferase I, 2- mycolyltransferase II, 3 - TMM-P-phosphatase, 4 - TMM transporter, 5 and 6 - FbpA, FbpB and FbpC.

Mycolic acids from mycolyl-S-Pks13 are transferred to **D**-mannopyranosyl-1-phosphoheptaprenol (MPH) by a cytoplasmic mycolyltransferase-I, to produce MycPL. MycPL then migrates to the inner surface of the cell membrane is proposed to dock next to an ABC transporter. Inside the ABC transporter the mycolyl group is transferred to trehalose 6-phosphate by mycolyltransferase-II which results in a TMM phosphate. The phosphate group is removed by trehalose 6-phosphate phosphatase and TMM is transported outside the cell by the ABC transporter (Takayama et al., 2005). After transport, TMM is then used as a substarte for mycolate transfer to the AG and to transferred to the arabinogalactan and to form TDMs by

the antigen-85 complex to produce arabinogalactan-mycolate and trehalose dimycolate, respectively (Takayama et al., 2005; Sathyamoorthy and Takayama, 1987).

The final stages in the formation of the mycobacterial cell wall involve transport and attachment of the mycolic acids to the peptidoglycan-arabinogalactan and formation of the TDM. Trehalose is a disaccharide formed by a α,α-1,1-glucosidic linkage of two α-glucose units. The fibronectin binding proteins of the antigen-85 complex, Ag85A, Ag85B and Ag85C (known as FbpA, FbpB and FbpC respectively) are thought to be involved in this last step of cell wall assembly (Kremer et al., 2002). These are major secretory proteins of *M. tuberculosis* and posess high affinity towards fibronectin, hence abbreviated fibronectin binding protein (Fbp) - FbpA (Rv3804c), FbpB (Rv1886c) and FbpC (Rv0129c). These proteins have a signal peptide at the N-terminus and a carboxylesterase at the C -terminus and have been shown to possess mycolyltransferases activities *in vitro.* They catalyze the transfer of mycolyl residues from α,α'- trehalose monomycolate to another resulting in α α' trehalose dimycolate (Belisle et al., 1997).

It is quite likely that the mycolyltransferases might have an activity similar to the esterase activity of Ag85 on TMM. Based on the presence of the catalytic triad of serine, histidine and glutamic acid, esterase D domain, protein sequence identities to Ag85 and homology across many mycobacterial genomes, the involvement of *Rv3802c* (mycolyltransferase-I) and *Rv1288, Rv0519c, Rv0774c* (mycolyltransferases-II) as potential candidates for mycolyltransferases in mycolic acid processing was predicted (Takayama et al., 2005). *Rv3802c* is located in the gene cluster responsible for mycolic acid biosynthesis, upstream of *fadD32* (*Rv3801*) and *pks13* (*Rv3800*), encodes a phospholipase/thioesterase

(Parker et al., 2009) and is an essential protein (Sassetti and Rubin, 2003; Meniche et al., 2009) . However, these studies did not show the *in vivo* role of *Rv3802c* in Mycobacterium. This study focussed on the potential role of *Rv1288* and *Rv0519c* as the mycolyltransferases-II gene and investigate their roles and essentiality in survival of the bacterium.

Rv1288 encodes a 456 amino acid protein and shares ~26% sequence homology and ~41% identity at amino acid levels with the FbpA of the Ag85 complex. The protein is characterized by the presence of 3 LysM (lysine domains) motifs at the N-terminus. LysM domains were identified in cell wall degrading enzymes and also have been shown to mediate peptidoglycan binding (Birkeland, 1994). The C-terminal amino acids match to an esterase D family protein. This family of proteins includes Ag85 mycolyltransferases from *C. glutamicum*. Further the active site residues forming the catalytic triad –serine, histidine and glutamic acid are conserved in this protein.

Rv0519c encodes a conserved membrane protein and shares ~29% sequence homology and ~48% amino acid identity to *Rv1288*. It encodes a 300 amino acid membrane protein and also possesses the active site catalytic triad of serine, histidine and glutamic acid. Both *Rv1288* and *Rv0519c* have homologous genes in *Mycobacterium bovis*, *Mycobacterium smegmatis*, *Mycobacterium marinum*, *Mycobacterium leprae* and *Mycobacterium avium*.

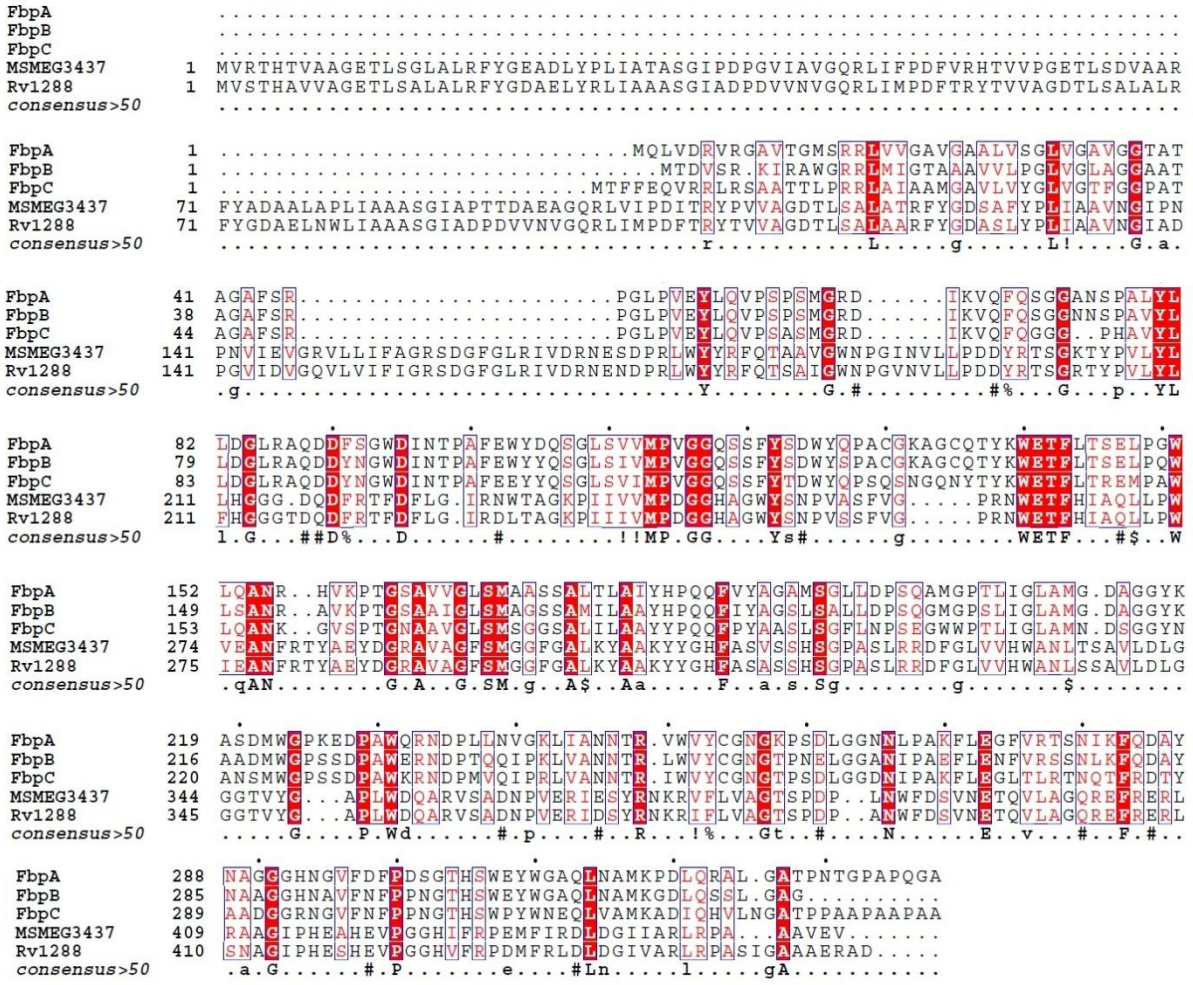


Figure 6.3. Multiple sequence alignment of the *M. tuberculosis* Fbp antigens with Rv1288 and its orthologue in *M. smegmatis* MSMEG3437. Numbers indicate the amino acid co-ordinates of MSMEG3437. Dots indicate gaps. Red boxes with white letters indicate identical amino acid sequences at the aligned position for all three proteins. White boxes indicate similar or identical residues for two of the three proteins at the aligned position; the bold sequence letters in these boxes indicate identical or similar residues at the aligned position.

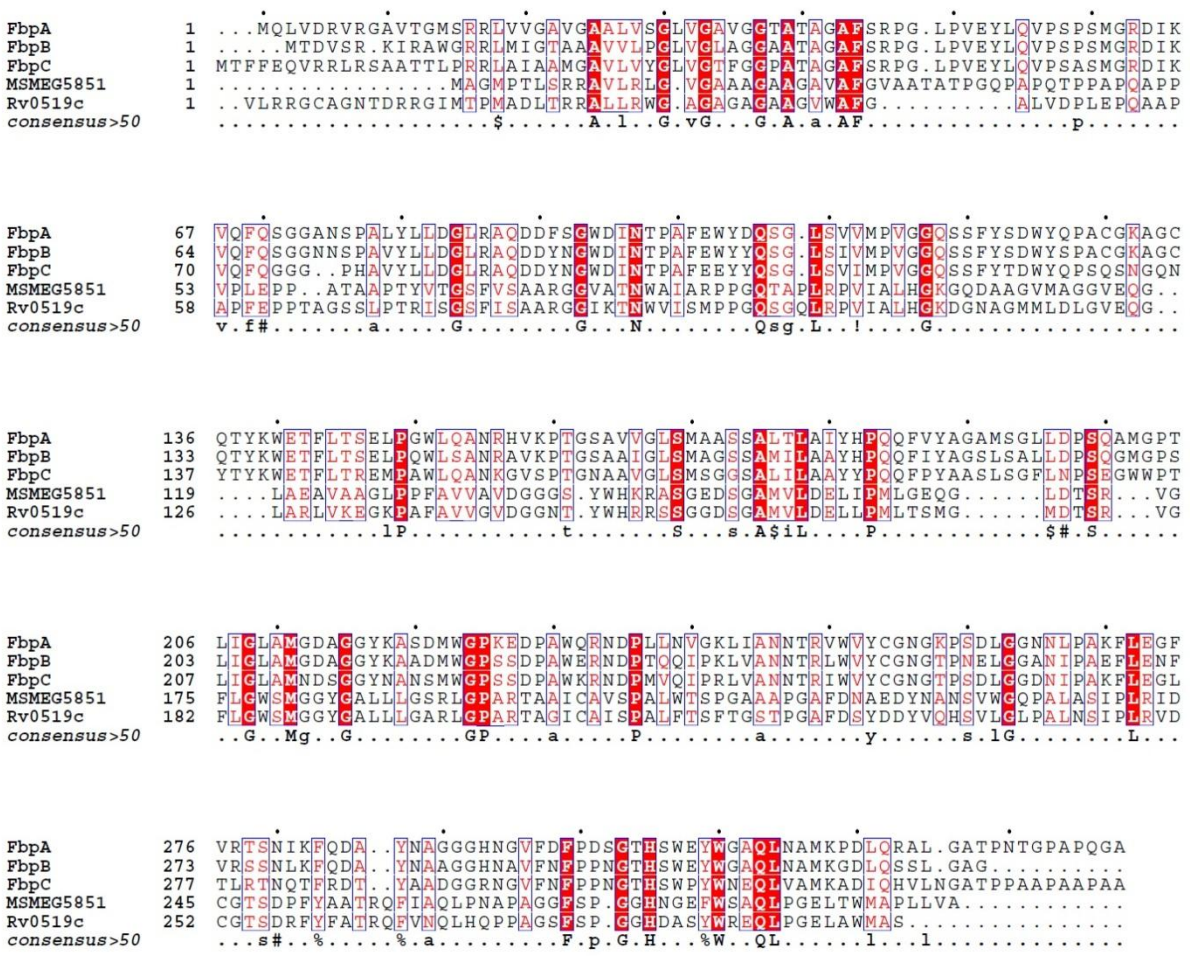


Figure 6.4. Multiple sequence alignment of the *M. tuberculosis* Fbp antigens with Rv0519c and its orthologue in *M. smegmatis* MSMEG5851. Numbers indicate the amino acid co-ordinates of MSMEG5851. Dots indicate gaps. Red boxes with white letters indicate identical amino acid sequences at the aligned position for all three proteins. White boxes indicate similar or identical residues for two of the three proteins at the aligned position; the bold sequence letters in these boxes indicate identical or similar residues at the aligned position.

Transport of mycolic acids and attachment to the cell wall remains unclear to date. Takayama et al.(2005), proposed a hypothetical pathway involving the transfer of a mature mycolate to an isoprenoid carrier to form Myc-PL and consequent transfer to a trehalose to generate trehalose monomycolate. There are two different hypotheses post this event – the

TMM is transported outside the cell by an unknown mechanism followed by which it is used as a substrate by the mycolyltransferases enzymes of the Ag85 complex (Belisle et al., 1997; Puech et al., 2002) to transfer the mycolate residue to another TMM to form TDM or to the arabino-galactan to form the mAG-complex. The other theory suggests intracellular synthesis of TMM and flipping of Myc-PL to the outside for use as a substrate for extracellular TDM generation.

The genome of *M. tuberculosis* has thirteen genes encoding a family of RND proteins (resistance, nodulation and cell division) proteins, (Cole et al., 1998; Domenech et al., 2005). These are a family of multidrug resistance pumps and mediate transport of various drugs, dyes and fatty acids across the cytoplasmic membrane and have been reportedly present in all kingdoms of life. However none of the thirteen MmpLs *M. tuberculosis*, MmpL1 – MmpL12, unlike their functions in other organisms have been reported in drug transport and resistance. Instead in mycobacteria, these proteins are predicted to have a role in transport of complex cell wall lipids and are known as MmpL (*M*ycobacterial *m*embrane *p*roteins *L*arge). MmpLs are large proteins and are proposed to serve as a scaffold for localized synthesis of cell wall lipids, pairing with an ABC transporter (Sonden et al., 2005) or an MmpS protein (Deshayes et al., 2010). MmpL8 has been shown to be involved in the synthesis of sulfolipid-I by transporting an intermediate of the molecule to extracytoplasmic enzymes (Converse et al., 2003; Domenech et al., 2004). Thus, if mycolates were transported as sugar-bound substrates the involvement of an MmpL protein in the translocation of a bound mycolate molecule outside the cell is possible. The mycolate-moiety transported outside maybe then used as a substrate for mycolylation of the cell wall.

Many of the MmpL genes occur in the same gene cluster involved in biosynthesis of the polyketide synthase genes, cell wall associated glycolipids like lipoologosaccharides, sulfolipids, glycopeptidolipids and complex lipids like phthiocerol dimycocerosate which indicate their involvement in the transport of complex lipids in *M. tuberculosis* (Domenech et al., 2004; Domenech et al., 2005; Cole et al., 1998; Converse et al., 2003; Sonden et al., 2005; Rombouts et al., 2011; Cox et al., 1999). Insertion mutations in MmpL7, involved in the final stages of biosynthesis of pthiocerol dimycolates (Jain and Cox, 2005), revealed the strains had impaired growth patterns and lethality. While a *M. tuberculosis* strains with mutation in MmpL8, which transports a sulfolipid precursor, and a MmpL11, reported to be involved in the transport of an unknown substrate was able to establish infection but interestingly was attenuated in virulence in 'time to death' studies in murine models (Domenech et al., 2005).

The genes MmpL3 and MmpL11 are closely related and occur in the gene cluster involved in mycolic acid transport. Further there is a synteny between the two genes across all mycobacterial species (Figure 6.5). Mycolic acids are essential for the survival of mycobacteria (Bhatt et al., 2005; Portevin et al., 2004; Vilcheze et al., 2000). The synteny between *mmpL3* and *mmpL11* is conserved across all mycobacterial genomes. *M. leprae*, possess a minimal genome but makes mycolic acids. Hence it is an useful model to find out genes involved in mycolic acid biosynthesis. The synteny between the *mmpL3* and *mmpL11* is also also observed in the genome of *M. leprae* which has homologues of five *M. tuberculosis* MmpL genes which include MmpL 3 and MmpL11. Thus it was likely that these two genes are involved in mycolic acid transport in *M. tuberculosis*. MmpL3 has been shown to be essential while MmpL11 has been shown to be involved in virulence. Due to MmpL 3 being

essential and time constraints in generation of a conditional mutant, this study proceeded with characterisation of MmpL11.

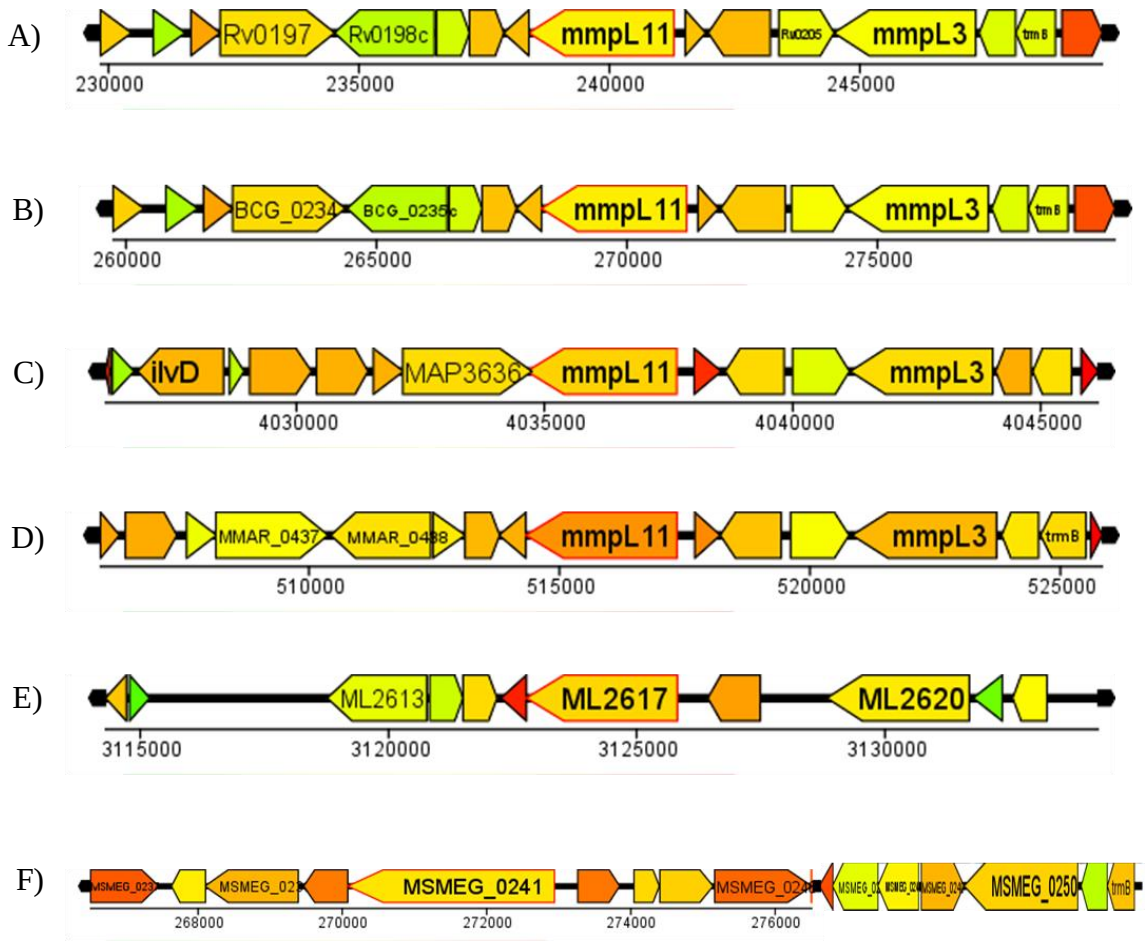


Figure 6.5 Genomic region comparison around MmpL11 and MmpL3 in A) *M. tuberculosis* H37Rv, B) *M. bovis*, C) *M. avium*, D) *M. marinum*, E) *M. leprae* and F) *M. smegmatis* , showing synteny between the two genes across all mycobacterial species.

The primary aim of this project was to understand mycolic acid processing and transport pathways in mycobacteria. Based on the report by Takayama et al. (2005), mycolyltransferase *Rv1288* and *Rv0519c* were selected as candidates to study their possible role in mycolic acid

processing. In this study we investigated the roles of the *Mycobacterium smegmatis* genes *MSMEG3437* and *MSMEG5851*, homologues of Rv1288 and *Rv0519c*. The advantages of using *M.smegmatis* as a surrogate system for *M. tuberculosis* research are its non pathogenic nature and faster growth rates. In order to study the role of mycolyltransferases, I planned to first attempt the generation of knockout strains of *MSMEG3437* and *MSMEG5851*. If either/both were found to be essential, CESTET (Conditional expression-specialised transduction essentiality testing) would be used to generate conditional mutant strains.

Additionally, this study probed the role of *MSMEG0241*, the *M. smegmatis* homologue of *mmpL11*, which shares 72% sequence identity with the *M. tuberculosis* gene.

6.2 Materials and Methods

6.2.1 In silico analysis of M. smegmatis MmpL11 (*MSMEG0241*)

The *MSMEG0241* (*Ms-MmpL11*) gene sequence was obtained from the Comprehensive Microbial Resource website (http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi). CLUSTALW and ESPript were used to produce multiple sequence alignments of MmpL11 from different mycobacterial species. The Expasy server (http://expasy.ch/) and the Centre for Biological Services server (http://www.cbs.dtu.dk) were used to predict the transmembrane domains.

6.2.2 Plasmids, strains and DNA manipulation

Plasmids, bacterial strains and phages used in this study are listed in Table 6.1. *Escherichia coli* strains were routinely cultured in Luria-Bertani (LB) broth at 37ºC. *M. smegmatis* strains

were grown at 37ºC in tryptic soy broth (TSB) or 7H9 broth or on TSB agar or Middlebrook 7H10 medium, supplemented with 0.02% Tween 80. Hygromycin B (Roche) and Kanamycin sulfate (Sigma) were used at concentrations of 100μg/ml and 25μg/ml respectively for *M. smegmatis* and Hygromycin B (Roche) at 150μg/ml for *E.coli*. For experiments involving the usage of phages, Tween 80 was not used in media. Mycobacteriophages were routinely propagated on Middlebrook's 7H9 with 0.2% glycerol. Phage high titres were generated using protocols described by Larsen et al. (2007).

Table 6.1 Bacterial strains, plasmids and phages used in this study

Plasmids, phages and strains	Description	Reference
Plasmids		
p0004s	Cosmid containing the Hygr-SacB cassette	Larsen et. al., 2007
pΔMSMEG3437	Derivative of p0004S obtained by cloning the right and left PCR flanks of *MSMEG3437*	This work
pΔMSMEG5851	Derivative of p0004S obtained by cloning the right and left PCR flanks of *MSMEG5851*	This work
pΔMSMEG0241	Derivative of p0004S obtained by cloning the right and left PCR flanks of *MSMEG0241*	This work
pMV261	Kan^R , *E. coli*-mycobacterial shuttle vector (ColE1 *oriM* P*hsp60*)	Stover et al., 1991
Phages		
phAE159	Conditionally replicating shuttle phasmid derived from lytic mycobacteriophage TM4	Larsen et al., 2007
phAEΔ*MSMEG3437*	Derivative of phAE159 obtained by cloning *pΔMSMEG3437*into its PacI site	This work
phAEΔ*MSMEG5851*	Derivative of phAE159 obtained by cloning *pΔMSMEG5851*into its PacI site	This work

phAEΔ*MSMEG0241*	Derivative of phAE159 obtained by cloning *pΔMSMEG0241*into its PacI site	This work
Bacterial strains		
E.coli TOP 10	F– *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen
HB101	*E. coli* K-12 *F__*(*gpt-proA*)62 leuB1 glnV44 ara-14 galK2 lacY1 hsdS20 rpsL20 xyl-5 mtl-1 recA13	Stratagene
M. smegmatis mc^2155	Wild type strain, E*pt* mutant of *M.smegmatis* strain mc^26	Snapper *et al.,* 1990
Δ*MSMEG3437*	*M. smegmatis* disrupted in gene *MSMEG3437*	This work
Δ*MSMEG5851*	*M. smegmatis* disrupted in gene *MSMEG5851*	This work
Δ*MSMEG0241*	*M. smegmatis* disrupted in gene *MSMEG0241*	This work
Δ*MSMEG0241*-C	Δ*MSMEG0241, c*omplemented with wild type copy of the gene *MSMEG0241*	This work
Δ*MSMEG0241*-RvC	Δ*MSMEG0241, c*omplemented with wild type copy of the gene *Rv0202c*	This work

6.2.3 Generation of recombinant knockout phages

Allelic exchange plasmids *pΔMSMEG0241, pΔMSMEG3437, pΔMSMEG5851* were generated using PCR amplified upstream and downstream flanks of *MSMEG0241*, *MSMEG3437* and *MSMEG5851* cloned on either sides of a hygromycin resistance cassette in

the vector p0004S. Left and right flank sequences of were PCR amplified using primers listed in Table 6.2.

Table 6.2 Primers used for generation of knockout constructs in *M. smegmatis*

Primer	Sequence 5' -3'	Region amplified	Size
MSMEG0241_LL	TTTTTTTTCCATAAATTGGTGGTTGGTCTGCGGGTTCTC	left side flank sequence of *MSMEG0241*	970 bp
MSMEG0241_LR	TTTTTTTTCCATTTCTTGGCTTCTGGCGCAGCTGTTTGG		
MSMEG0241_RL	TTTTTTTTCCATAGATTGGTTCTCATACGGCAGCTCTGG	right side flank sequence of *MSMEG0241*	925 bp
MSMEG0241_RR	TTTTTTTTCCATCTTTTGGGTTCGGTCAGTCACGAGTAG		
MSMEG3437_LL	TTTTTTTTCAGAAACTGACTAGGCCCAGACACAAC	left side flank sequence of *MSMEG3437*	830 bp
MSMEG3437_LR	TTTTTTTTCAGTTCCTGCACGAAGTCGGGAAAGATG		
MSMEG3437_RL	TTTTTTTTCAGAGACTGCGAGAGCTACCGGAACAAG	right side flank sequence of *MSMEG3437*	998 bp
MSMEG3437_RR	TTTTTTTTCAGCTTCTGTGGCGCACACCGATTAC		
MSMEG5851_LL	TTTTTTTTCCATAAATTGGCTCGTCCACCAGTGATG	left side flank sequence of *MSMEG5851*	855 bp
MSMEG5851_LR	TTTTTTTTCCATTTCTTGGAACGAACCCGTCACGTAGG		
MSMEG5851_RL	TTTTTTTTCCATAGATTGGCACCAGCGATCCCTTCTAC	right side flank sequence of M*SMEG5851*	864 bp
MSMEG5851_RR	TTTTTTTTCCATCTTTTGGTCCCGAGGTCTCACTGTTC		

The PCR products were gel purified (QIAGEN gel purification kit) and digested with *Van*91I (left and right flanks of *MSMEG0241* and *MSMEG3437*) and *AlwN*I (*MSMEG5851*). The digested fragments were ligated with P0004S-*Van*91I digested fragments using T4 DNA ligase. The ligation mix was then transformed into chemically competent *E. coli* Top10 cells and transformants were selected on LB agar with Hygromycin at 37°C. Plasmids obtained by miniprep (Qiagen miniprep kit) were digested with *Van*91I and *Pac*I and sequenced to confirm presence of the left and right flanks. The PacI digested knockout plasmids *pΔMSMEG0241, pΔMSMEG3437* and *pΔMSMEG5851* was then ligated with PacI digested phAE159 DNA. The ligation mix was then packaged in the temperature sensitive mycobacteriophage phAE159 λ phage heads and transduced into *E.coli* HB101 and selected on LB agar with Hygromycin at 37°C.

6.2.4 Generation of *M. smegmatis* deletion mutants.

The cosmids recovered from *E. coli* HB101 were confirmed by digestion with PacI digestion. The positive cosmids were transformed by electroporation into *M. smegmatis* at 1800V and recovered at 30°C for ~4 hours in TSB. The recovered cells were then harvested and resuspended in 200µl of MP buffer. This was mixed with 200µl of freshly growing *M. smegmatis* and 5ml molten soft agar (50°C) and poured on 7H9 basal agar plates and incubated at 30°C for 2 -3 days and allowed to form plaques. The plates were soaked in minimum amount of MP buffer for 5-6 hours and the solution containing phages was filtered and stored at 4°C. This generated recombinant phages - phΔ*MSMEG0241,* phΔ*MSMEG3437* and phΔ*MSMEG*5851 designed to replace the genes *MSMEG0241, MSMEG3437* and *MSMEG5851* respectively with *hyg*.

M. smegmatis cultures were grown in 50ml of TSB (0.05% Tween 80) to an OD_{600} of about 0.8 and harvested by centrifugation at 4500xg for 10 minutes. The cell pellet was washed twice with 50ml of MP buffer. Finally the pellet was resuspended in 2ml of MP buffer and 2ml of high titre (10^{-10} pfu /ml) phage lysate was mixed with the cells. A control was set up where 0.5 ml of resuspended cell was mixed with 0.5ml of MP buffer. The mix was incubated overnight at 37°C followed by harvesting and recovery with 10ml TSB with Tween-80 for 12 -24 hours at 37°C. This was plated onto TSB agar plates with hygromycin B and plates were incubated at 37°C for 1 – 2 weeks. Hygromycin resistant colonies obtained after transduction of *M. smegmatis* mc^2155 (wild type strain) were inoculated in 10ml TSB-Tween 80 with hygromycin B for genomic DNA extraction and further characterization. Once confirmed by Southern blot, one strain of each Δ*MSMEG0241,* Δ*MSMEG3437* and Δ*MSMEG5851* were picked for further biochemical analysis.

6.2.5 Southern blot to confirm knockouts

Restriction enzymes were selected based on the sequence of the knockout plasmids. For Δ*MSMEG0241* and Δ*MSMEG5851* the restriction enzymes used was *Kpn*I, and Δ*MSMEG3437* genomic DNA was digested with *Nco*I. The wildtype genomic DNA was also digested with respective enzymes listed above. Following digestion, the genomic DNA fragments were separated by gel electrophoresis.In the mutant strains the gene is replaced by a *hyg-SacB* gene, thus after digestion the pattern of fragment produced will differ from the wildtype in either size or number of fragments. PCR products of the left and right flanks of the respective genes were used as probes.

The procedure was performed as described in DIG High Prime DNA Labelling and Detection Starter Kit II (cat no – 11585614910, Roche). This kit uses digoxigenin, a steroid to label DNA probes by random priming. The hybridized probes are then immunodetected by anti-digoxigenin-AP (Fab fragments), which are visualised by chemiluminescence.

6.2.6 Complementation of the Δ*MSMEG0241* mutant.

Complemented strains were constructed by cloning a copy of *MSMEG0241* in the mycobacterial replicative plasmid pMV261 (kanamycin resistant) and introduced in the mutant strain by electroporation. The *M. tuberculosis* MmpL11 gene *Rv0202c* was also cloned in pMV261 and introduced into the Δ*MSMEG0241* strain to generate an Rv–complemented strain.

Table 6.3 Primers used for generation of complemented strains

Name	Sequence (5' → 3')	Product	Size
MSM0241C_ F	GATCGATCGAATTCCATGATGCGCTTGAGCAGCACT*	MSMEG0241 gene sequence	2865 bp
MSM0241C_ R	GATCGATCAAGCTTTCATTCGCCTCCTCCAGCATT#		
Rv0202C_ F	GATCGATCGAATTCCATGATGCGCTTGAGCCGCAA*	Rv0202c (Mmpl11) gene sequence	2901 bp
Rv0202C_ R	GATCGATCAAGCTTTCACCTCGCCTCCAACA#		

*Underlined sequence shows *Eco*RI restriction site, # Underlined sequence shows *Hind*III restriction site

6.2.7 Growth curve

Growth of the wild type mc^2155, mutant *MSMEG0241* and *MSMEG0241-C* complemented strain was monitored at O.D. 600nm over a period of 0 to 48 hours at intervals of 6, 12, 24, 36

and 48 hours. All the strains were grown in Tryptic soy broth with 0.05% Tween 80 and appropriate antibiotics at 37° C. The starting O.D. for all the cultures was 0.2.

6.2.8 Colony morphology and sliding motility

Mid-log cultures of the bacterial strains were streaked on TSB agar plates with and without tween. The plates were incubated for 2-3 days at 37°C and isolated single colonies were studied under a microscope. The sliding motility assay was adapted from Mathew et al (2006). Middle-brook 7H9 was supplemented with 60ml /l glycerol and 10% OADC was solidified using 0.3% high grade agarose (Bioline). To check the effect of detergent on biofilm formation, 0.05% Tween 80 was added to a set of plates, and another set of plates were made with no glycerol.

5µl culture ($O.D_{600}$ = 0.5) was spotted at the centre of the plates and allowed to dry for 1hour. Biofilm formation was evaluated after incubation at 37º C for 3-5 days in a humidified incubator.

6.2.9 Analysis of cell envelope lipids

M. smegmatis cultures were grown to an OD_{600} nm of 0.4 in Tryptic soy broth and 7H9 medium in the presence and absence of 0.05% Tween 80 at 37°C with appropriate antibiotics where required in a shaking incubator, following which 1 mCi/ml [1,2-^{14}C]acetate (57 mCi/mmol, GE Healthcare, Amersham Bioscience) was added to the culture and the incubation was continued for another for 12 hours. The labelled bacterial cells were harvested, washed, and dried. The dried cells were initially resuspended in 2 ml of petroleum ether (60–

80°C), and mixed for 30 minutes. The upper layer was collected in a fresh tube and the remaining cell suspension re-extracted with 2ml of petroleum ether (60–80°C). The upper layers were pooled and dried. This petroleum ether extract contains surface exposed, non-covalently bound lipids. Apolar and polar lipids from the remaining cell pellet were extracted as described in Chapter 8.

The apolar lipid extract (50,000 cpm) was applied to the corners of 6.666 cm plates of silica gel 60 F254 (Merck 5554) TLC plates. The plates were then developed in solvent system C, using direction 1, chloroform/methanol /water (100:14:0.8, v/v/v) and direction 2, toluene/acetone (80:20, v/v) to separate ^{14}C-labelled lipids (free fatty acids and mycolic acids). Lipids were visualized by autoradiography by overnight exposure of Kodak XOmat AR film to the TLC plates to reveal [^{14}C]labelled lipids and compared to known standards.

To study the effect of loss of genes *MSMEG0241, MSMEG3437* and *MSMEG5851* on the wall bound mycolates and fatty acids, delipidated cells were subjected to alkaline hydrolysis using 5% aqueous tetrabutylammonium hydroxide (TBAH) at 100°C overnight, followed by the addition of 4 ml of CH_2Cl_2, 500 µl of CH_3I, 2 ml of water, followed by mixing for 30 min. The upper aqueous phase was discarded following centrifugation and the lower organic phase washed thrice with water and evaporated to dryness. The resulting fatty acid methyl esters (FAMEs) and mycolic acid methyl esters (MAMEs) were dissolved in diethyl ether (CH_2Cl_2) and insoluble residues removed by centrifugation. The clear supernatant was evaporated to dryness and re-dissolved in 200 µl of CH_2Cl_2. Equivalent volumes of the resulting solution of FAMEs and MAMEs were subjected to TLC using silica gel plates (5735 silica gel 60F254; Merck, Darmstadt, Germany), developed in petroleum ether-acetone (95:5).

Autoradiograms were produced by overnight exposure of Kodak X-Omat AR film to the plates to reveal [14C]-labelled FAMEs and MAMEs. Argentation (Ag^{2+}) -TLC was performed by using Ag^{2+} -impregnated TLC plates developed twice in direction 1, hexane-ethyl acetate (19:1, v/v), and then thrice in direction 2, petroleum ether-acetone (17:3, v/v).

6.3 Results

6.3.1 *In silico* analysis of MmpL11 and its neighbouring genes

Genome comparison shows that *MSMEG0241* is homologous to *mmpL11* (Rv0202c) in *M. tuberculosis*. The *M. smegmatis* protein has 69% identity with the *M. tuberculosis* protein in a 969 amino acid overlap, and 70.9% genetic similarity and the genetic region is conserved between mycobacterial species. The *M. smegmatis* protein also has 67.8% similarity in a 1010 amino acid overlap with *Mycobacterium avium* (MAP 3637) MmpL11 protein sequence with a 72.4% genetic match; 65.8% identity in a 1015 amino acid overlap with 55% genetic identity in *Mycobacterium leprae* (ML 2617) and a 69% similarity in a 969 amino acid overlap with *Mycobacterium bovis BCG* (BCG 0239), and 70.9% gene identity. *M. leprae* has a high degree of gene deletions but still possess 5 of the MmpL proteins (Lamichhane et al., 2005), MmpL 11 being one of them. This suggests that the MmpL 11 protein, although non-essential for survival, performs vital functions in cell wall related pathways in mycobacteria. As shown in Fig 6.5 the synteny between MmpL3 and MmpL11 is conserved across all mycobacterial genomes.

The MmpL11 protein also has a high level of structural similarity to MmpL3, which is an essential gene and the both the proteins have similar transmembrane domains. The MmpL3

(*MSMEG0250*) gene has been shown to be involved in mycolate transfer (C.Varela, University of Birmingham, communicated).

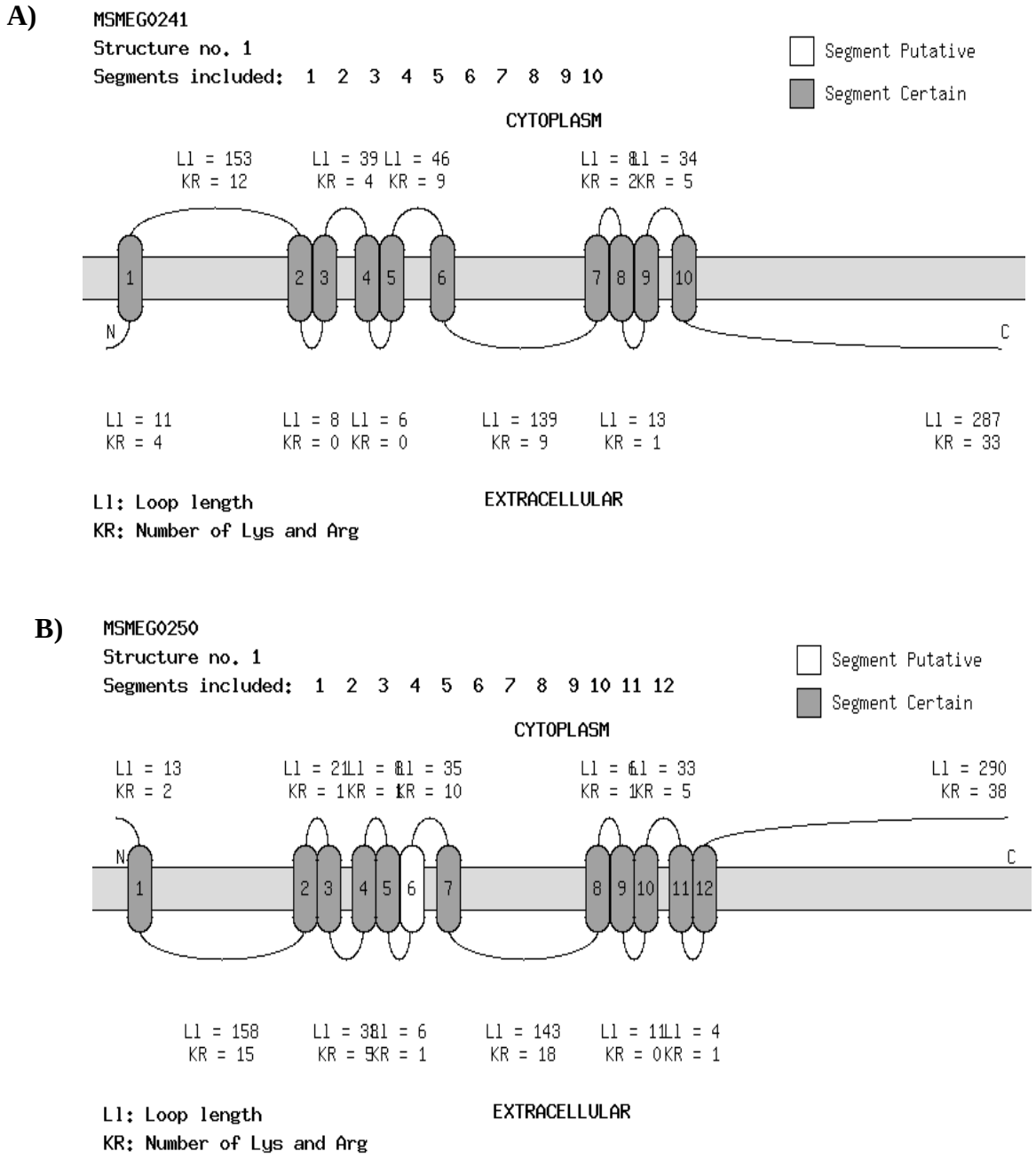
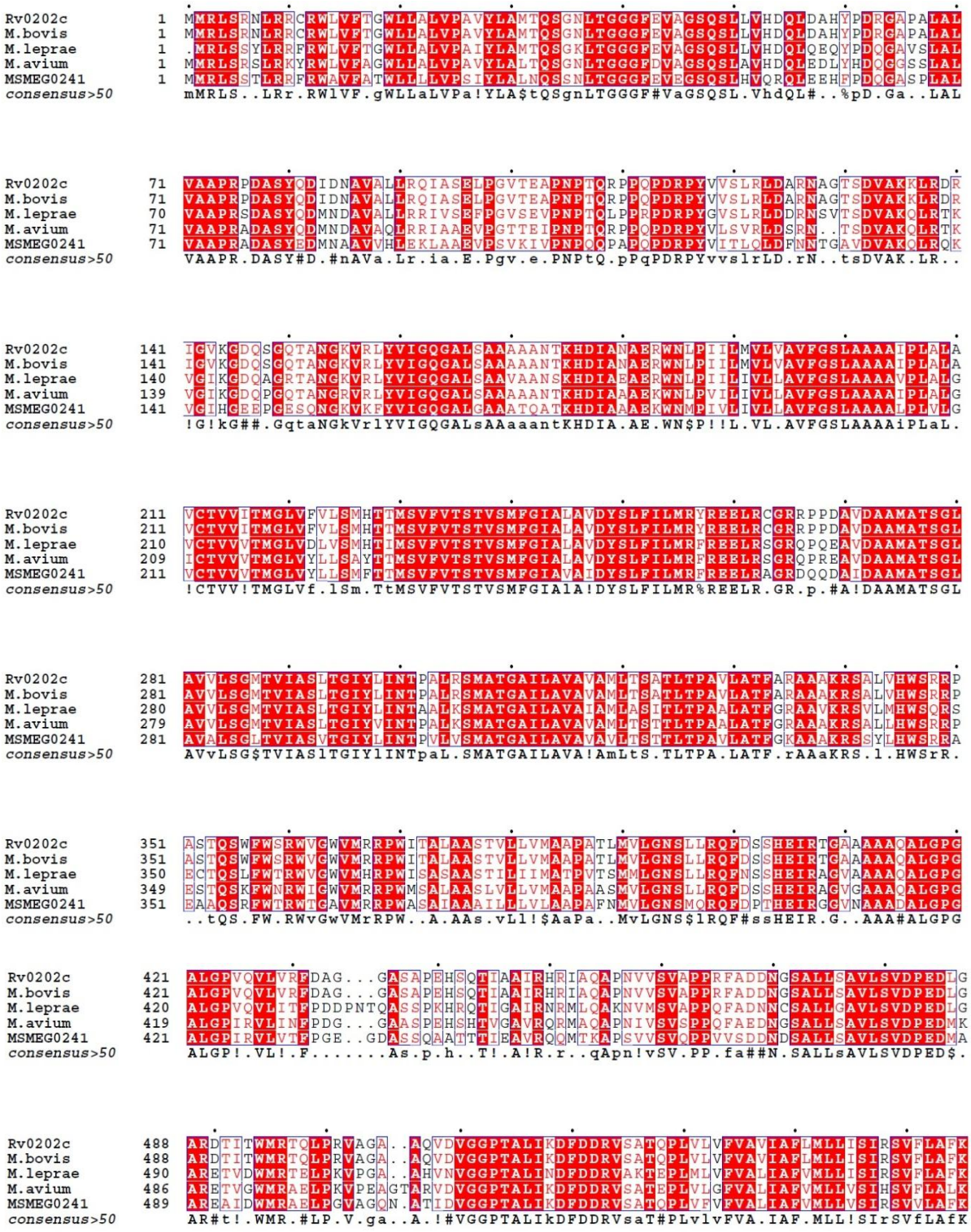


Figure 6.6. Transmembrane domain predictions of MmpL 11 (MSMEG0241) and MmpL 3 (MSMEG0250) from *M. smegmatis*, showing the transmembrane helices, extracellular and cytoplasmic domains, which is similar to the predicted topology of the *M. tuberculosis* proteins.


Rv0202c 1 MMRLSRNLRRCRWLVFTGWLLALVPAVYLAMTQSGNLTGGGFEVAGSQSLLVHDQLDAHYPDRGAPALAL
M.bovis 1 MMRLSRNLRRCRWLVFTGWLLALVPAVYLAMTQSGNLTGGGFEVAGSQSLLVHDQLDAHYPDRGAPALAL
M.leprae 1 .MRLSSYLRRFRWLVFTGWLLALVPAIYLAMTQSGKLTGGGFEVAGSQSLLVHDQLQEQYPDQGAVSLAL
M.avium 1 MMRLSRSLRKYRWLVFAGWLLALVPAVYLALTQSGNLTGGGFDVAGSQSLAVHDQLEDLYHDQGGSSLAL
MSMEG0241 1 MMRLSSTLRRFRWAVFATWLLLLVPSIYLALNQSSNLTGGGFEVEGSQSLHVQRQLEEHFPDQGASPLAL
consensus>50 mMRLS..LRr.RWlVF.gWLLaLVPa!YLA$tQSgnLTGGGF#VaGSQSL.VhdQL#..%pD.Ga..LAL
Rv0202c 71 VAAPRPDASYQDIDNAVALLRQIASELPGVTEAPNPTQRPPQPDRPYVVSLRLDARNAGTSDVAKKLRDR
M.bovis 71 VAAPRPDASYQDIDNAVALLRQIASELPGVTEAPNPTQRPPQPDRPYVVSLRLDARNAGTSDVAKKLRDR
M.leprae 70 VAAPRSDASYQDMNDAVALLRRIVSEFPGVSEVPNPTQLPPRPDRPYGVSLRLDDRNSVTSDVAKQLRTK
M.avium 71 VAAPRADASYQDMNDAVAQLRRIAAEVPGTTEIPNPTQRPPQPDRPYVLSVRLDSRN..TSDVAKQLRTK
MSMEG0241 71 VAAPRADASYEDMNAAVVHLEKLAAEVPSVKIVPNPQQPAPQPDRPYVITLQLDFNNTGAVDVAKQLRQK
consensus>50 VAAPR.DASY#D.#nAVa.Lr.ia.E.Pgv.e.PNPtQ.pPqPDRPYvvslrLD.rN..tsDVAK.LR..
Rv0202c 141 IGVKGDQSGQTANGKVRLYVIGQGALSAAAAANTKHDIANAERWNLPIILMVLVAVFGSLAAAAIPLALA
M.bovis 141 IGVKGDQSGQTANGKVRLYVIGQGALSAAAAANTKHDIANAERWNLPIILMVLVAVFGSLAAAAIPLALA
M.leprae 140 VGIKGDQAGRTANGKVRLYVIGQGALSAAVAANSKHDIAEAERWNLPIILIVLLAVFGSLAAAAVPLALG
M.avium 139 VGIKGDQPGQTANGRVRLYVIGQGALSAAAAANTKHDIAAAEKWNLPVILIVLLAVFGSLAAAAIPLALG
MSMEG0241 141 VGIHGEEPGESQNGKVKFYVIGQGALGAAATQATKHDIAAAEKWNMPIVLIVLLAVFGSLAAAALPLVLG
consensus>50 !G!kG##.GqtaNGkVrlYVIGQGALsAAaaantKHDIA.AE.WN$P!!L.VL.AVFGSLAAAAiPLaL.
Rv0202c 211 VCTVVITMGLVFVLSMHTTMSVFVTSTVSMFGIALAVDYSLFILMRYREELRCGRRPPDAVDAAMATSGL
M.bovis 211 VCTVVITMGLVFVLSMHTTMSVFVTSTVSMFGIALAVDYSLFILMRYREELRCGRRPPDAVDAAMATSGL
M.leprae 210 VCTVVVTMGLVDLVSMHTIMSVFVTSTVSMFGIALAVDYSLFILMRFREELRSGRQPQEAVDAAMATSGL
M.avium 209 ICTVVVTMGLVYLLSAYTTMSVFVTSTVSMFGIALAVDYSLFILMRFREELRSGRQPREAVDAAMATSGL
MSMEG0241 211 VCTVVVTMGLVYLLSMFTTMSVFVTSTVSMFGIAVAIDYSLFILMRFREELRAGRDQQDAIDAAMATSGL
consensus>50 !CTVV!TMGLVf.lSm.TtMSVFVTSTVSMFGIAlA!DYSLFILMR%REELR.GR.p.#A!DAAMATSGL
Rv0202c 281 AVVLSGMTVIASLTGIYLINTPALRSMATGAILAVAVAMLTSATLTPAVLATFARAAAKRSALVHWSRRP
M.bovis 281 AVVLSGMTVIASLTGIYLINTPALRSMATGAILAVAVAMLTSATLTPAVLATFARAAAKRSALVHWSRRP
M.leprae 280 AVVLSGMTVIASLTGIYLINTAALKSMATGAILAVAIAMLASITLTPAALATFGRAAVKRSVLMHWSQRS
M.avium 279 AVVLSGMTVIASLTGIYVINTPALKSMATGAILAVAVAMLTSTTLTPAALATFGRAAAKRSALLHWSRRP
MSMEG0241 281 AVALSGLTVIASVTGIYLINTPVLVSMATGAILAVAVAVLTSTTLTPAVLATFGKAAAKRSSYLHWSRRA
consensus>50 AVvLSG$TVIASlTGIYlINTpaL.SMATGAILAVA!AmLtS.TLTPA.LATF.rAAaKRS.l.HWSrR.
Rv0202c 351 ASTQSWFWSRWVGWVMRRPWITALAASTVLLVMAAPATLMVLGNSLLRQFDSSHEIRTGAAAAAQALGPG
M.bovis 351 ASTQSWFWSRWVGWVMRRPWITALAASTVLLVMAAPATLMVLGNSLLRQFDSSHEIRTGAAAAAQALGPG
M.leprae 350 ECTQSLFWTRWVGWVMHRPWISASAASTILIIMATPVTSMMLGNSLLRQFNSSHEIRAGVAAAAQALGPG
M.avium 349 ESTQSKFWNRWIGWVMRRPWMSALAASLVLLVMAAPAASMVLGNSLLRQFDSSHEIRAGVGAAAQALGPG
MSMEG0241 351 EAAQSRFWTRWTGAVMRRPWASAIAAAILLLVLAAPAFNMVLGNSMQRQFDPTHEIRGGVNAAADALGPG
consensus>50 ..tQS.FW.RWvGwVMrRPW..A.AAs.vLl!$AaPa..MvLGNS$lRQF#ssHEIR.G..AAA#ALGPG
Rv0202c 421 ALGPVQVLVRFDAG...GASAPEHSQTIAAIRHRIAQAPNVVSVAPFRFADDNGSALLSAVLSVDPEDLG
M.bovis 421 ALGPVQVLVRFDAG...GASAPEHSQTIAAIRHRIAQAPNVVSVAPFRFADDNGSALLSAVLSVDPEDLG
M.leprae 420 ALGPVQVLITFPDDPNTQASSPKHRQTIGAIRNRMLQAKNVMSVAPFQFADNNGSALLGAVLSVDPEDLG
M.avium 419 ALGPIRVLINFPDG...GAASPEHSHTVGAVRQRMAQAPNIVSVSPFQFAEDNGSALLSAVLSVDPEDMK
MSMEG0241 421 ALGPIRVLVTFPGE..GDASSQAATTTIEAVRQQMTKAPSVVSVQPFVVSDDNDSALLSAVLSVDPEDMA
consensus>50 ALGP!.VL!.F.......As.p.h..T!.A!R.r..qApn!vSV.PP.fa##N.SALLsAVLSVDPED$.
Rv0202c 488 ARDTITWMRTQLPRVAGA..AQVDVGGPTALIKDFDDRVSATQPLVLVFVAVIAFLMLLISIRSVFLAFK
M.bovis 488 ARDTITWMRTQLPRVAGA..AQVDVGGPTALIKDFDDRVSATQPLVLVFVAVIAFLMLLISIRSVFLAFK
M.leprae 490 ARETVDWMRTELPKVPGA..AHVNVGGPTALINDFDDRVAKTEPLMLVFVALIAFVMLLISIRSVFLAFK
M.avium 486 ARETVGWMRAELPKVPEAGTARVDVGGPTALIKDFDDRVSATEPLVLGFVALIAFVMLLVSIHSVFLALK
MSMEG0241 489 AREAIDWMRAELPGVAGQN.ATIDVGGPTALIKDFDDRVSATQPLVFVFVALIAFVMLLVSIRSVVLAFK
consensus>50 AR#t!.WMR.#LP.V.ga..A.!#VGGPTALIkDFDDRVsaT#PLvlvFVA.IAF.MLL!SIrSVfLAfK

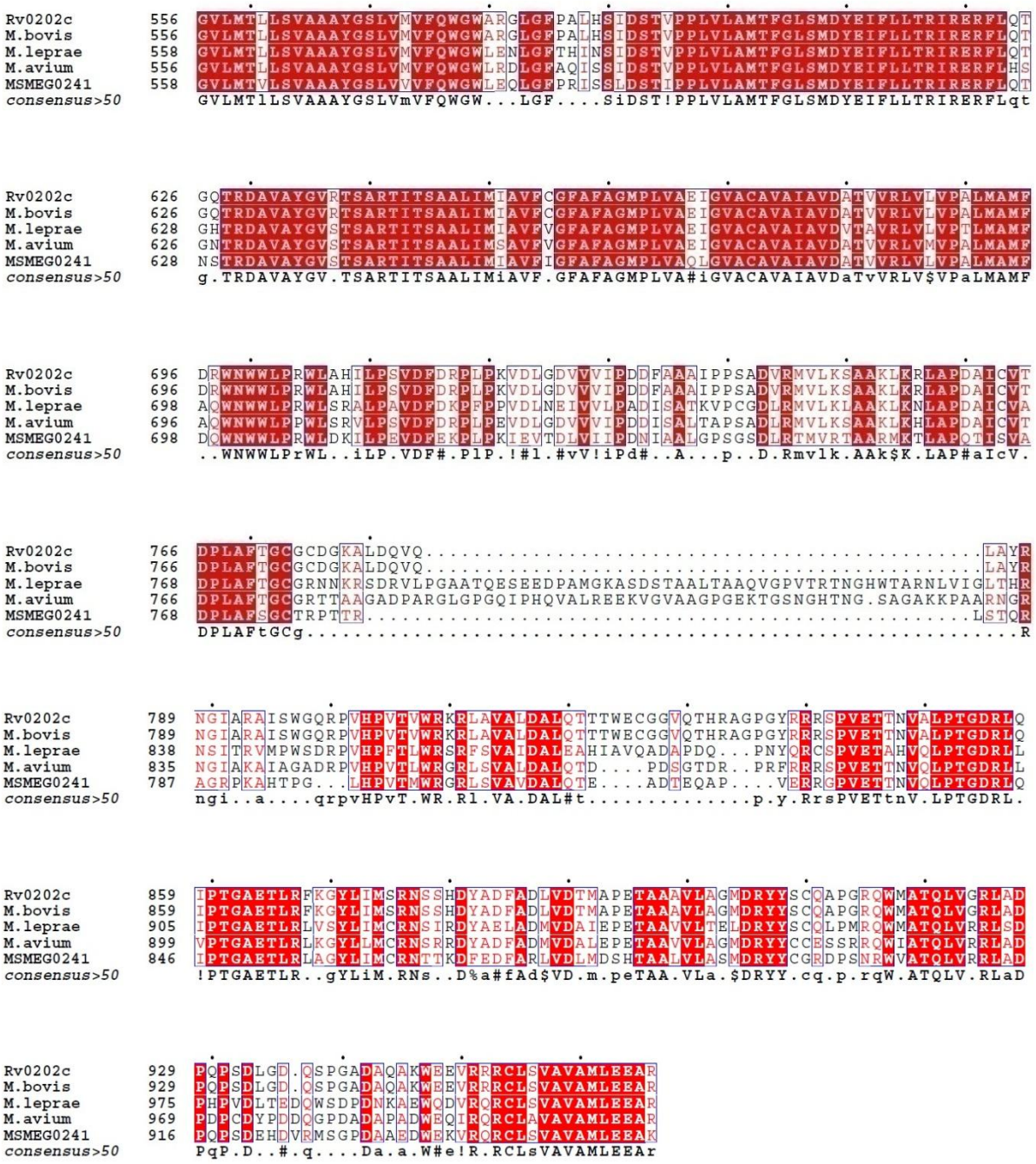


Figure 6.7. Multiple sequence alignment of the *M. tuberculosis* MmpL11 (Rv0202c) protein with MmpL 11 proteins in *M. bovis, M. leprae, M. avium*, and *M. smegmatis* (MSMEG0241). Numbers indicate the amino acid co-ordinates of MSMEG0241. Dots indicate gaps. Red boxes with white letters indicate identical amino acid sequences at the aligned position for all three proteins. White boxes indicate similar or identical residues for two of the three proteins at the aligned position; the bold sequence letters in these boxes indicate identical or similar residues at the aligned position.

6.3.2 Southern blot to confirm knockouts

Southern blot was done as described in Chapter 8 to confirm the replacement of the targeted genes by hyg-SacB. Digestion of the genomic DNA with restriction enzymes *Kpn*I, the wild type would show a 15Kb band and the mutant strain Δ*MSMEG0241* would reveal 2 bands of 5.137Kb and 6.827Kb. Whereas the mutant strain Δ*MSMEG5851* would produce 2 fragments, 7Kb and 11.7Kb in size. Digestion of the wild type and mutant strain Δ*MSMEG3437* genomic DNA with *Nco*I revealed 2 fragments of 2Kb and 5.4Kb in the wild type strain and a 5Kb fragment in the mutant strain. The procedure was performed as suggested by manufacturer guidelines in (DIG High Prime DNA Labelling and Detection Starter Kit II cat no – 11585614910, Roche). This kit uses digoxigenin, a steroid to label DNA probes by random priming. The hybridized probes are then detected uisng anti-digoxigenin-AP (Fab fragments), and subsequently visualised by chemiluminescence.

None of the genes tested were essential for growth under laboratory media. This indicates that either the functions are redundant or they play a minor role in the organism's metabolism.

6.3.3 Characterization of the mutant strains Δ*MSMEG3437* and Δ*MSMEG5851*

6.3.3.1 Effects of deletion of *MSMEG3437* and *MSMEG5851* on colony morphology

Changes in cell wall components, including mycolates affects colony morphology. Previously it has been shown that loss of gene encoding mycolic acid biosynthesis enzymes, e.g., KasB in *M. smegmatis* is responsible for alteration in colony patterns (Bhatt et al., 2007a). In order to study the possible consequences of deletion of the genes *MSMEG3437* and *MSMEG5851* on

M. smegmatis physiology, colony morpholgy was studied. Isolated single colonies of the wild type and mutant strains on TSB agar with and without the presence of tween-80 were compared. The mutants *MSMEG3437* and *MSMEG5851* did not show any variation from the usual crenulated colony morphology exhibited by the wild type *M. smegmatis* strain. colony morphology exhibited by the wild type *M. smegmatis* strain.

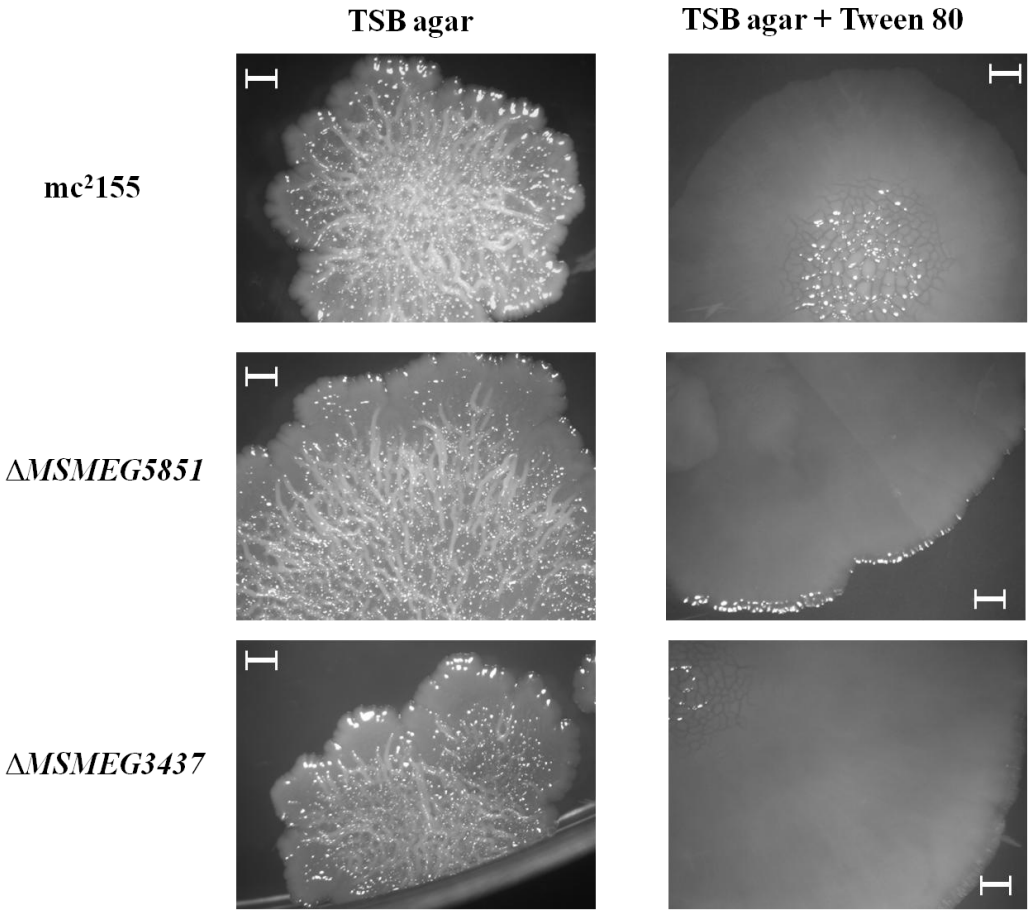


Figure 6.8. Colonies of wild type (mc^{2}155), mutant strains Δ*MSMEG3437* and Δ*MSMEG5851* on TSB-agar and TSB-agar-0.05% Tween-80. Single isolated colonies are shown. Scale bar = 1mm.

6.3.3.2 Analysis of lipid profiles of mutant strains Δ*MSMEG3437* and Δ*MSMEG5851*.

Mutants strains Δ*MSMEG3437* and Δ*MSMEG5851*, predicted mycolyltransferases, did not show any change in colony morphology. Presence or absence of tween did not appear to have any phenotypical effect on the mutants Δ*MSMEG3437* and Δ*MSMEG5851*. To further analyse the effects of deletion of these genes, cell wall lipid analysis was done to detect any alterations in the cell wall components- changes in the free fatty acids and mycolic acid levels /patterns. Bacterial strains were grown in TSB and 7H9 media in the presence and absence of 0.05% Tween 80 for this study. Presence of Tween in media or growth on different media TSB and 7H9 did not affect free fatty acids and free mycolate levels, but interestingly, a spot was observed in the 2D-TLCs of apolar lipids extracted from cultures grown in 7H9 media, both in presence and absence of Tween 80 (Figure 6.9). This accumulation has not been characterised yet but will be analysed in future.

To check if the genes had any role in transferring Myc-PL to trehalose monomycolate (TMM) to synthesise trehalose dimycolate (TDM), the apolar lipids were separated on a 2D-TLC system which separates TMMs and TDMs. This did not show any changes or altered pattern of migration suggesting that either the genes *MSMEG3437* and *MSMEG5851* are not involved in mycolates transfer in TDM biosynthetic pathway, or that these functions are redundant (Figure 6.10).

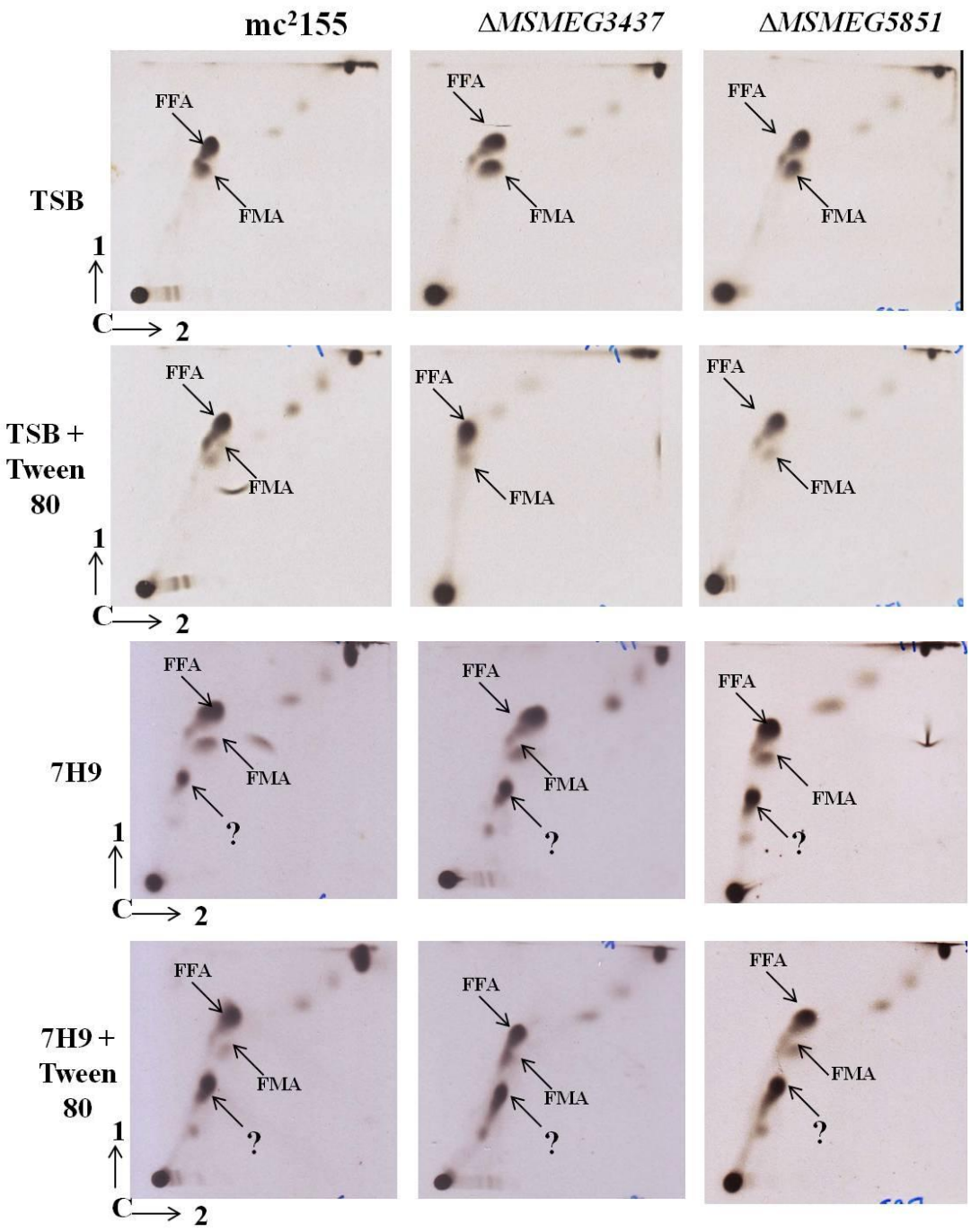


Figure 6.9. 2D TLC autoradiograph of apolar lipids extracted from wild type mc^{2}155 and mutant strains Δ*MSMEG3437* and Δ*MSMEG5851*. The strains were grown on TSB and 7H9 agar +/- 0.05% Tween 80. Lipids were separated in solvent system 'C', direction 1 in chloroform/methanol 96:4, and in direction 2 in Toluene/acetone; 80:20. FFA- free fatty acids, FMA-free mycolic acids; ?, unknown.

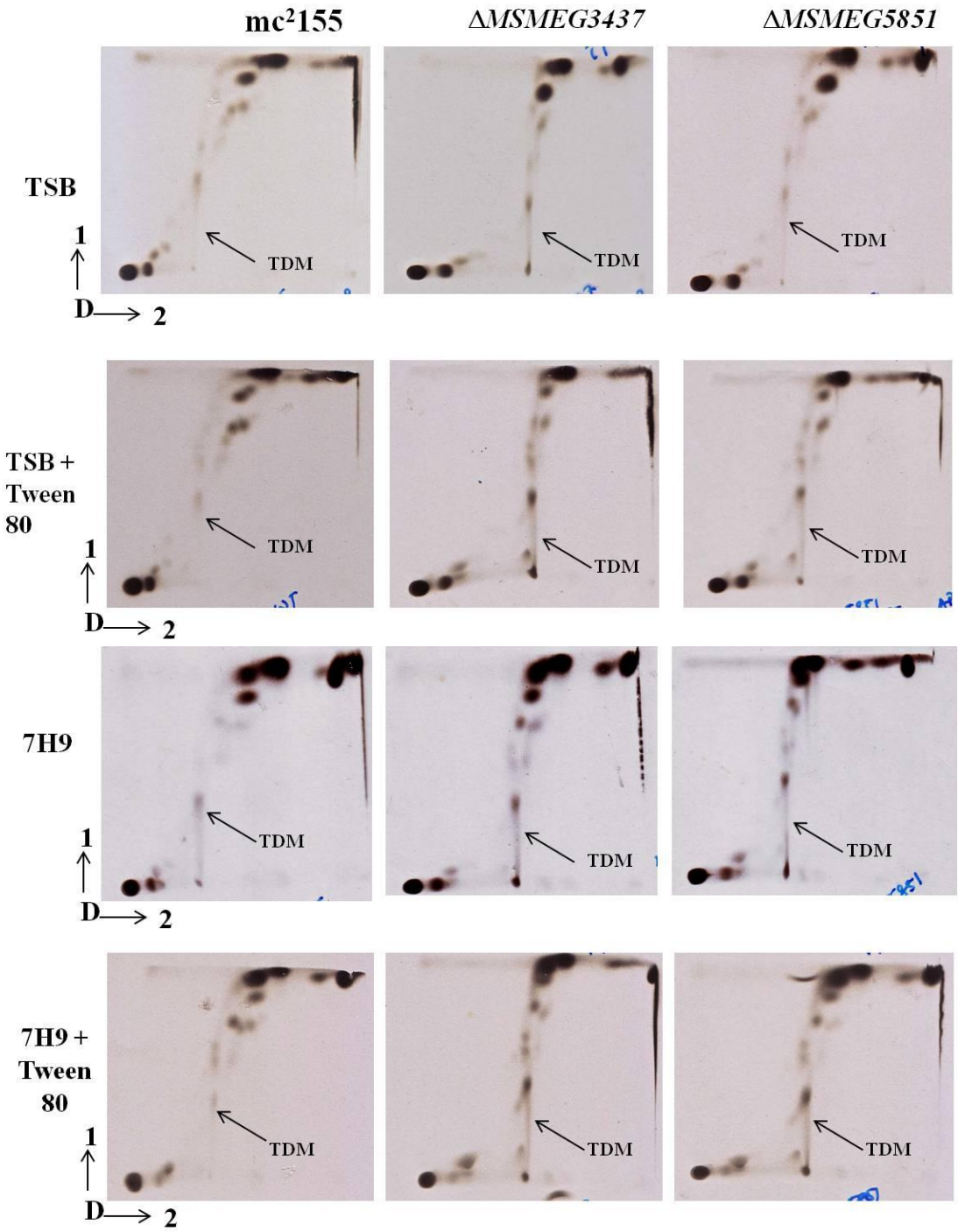


Figure 6.10 2D TLC autoradiographs of apolar lipids extracted from mc²155 and mutant strains Δ*MSMEG3437* and Δ*MSMEG5851*. Lipids were separated in solvent system 'D' direction 1 in chloroform: methanol: water; 100:14:0.8, and direction 2 in chloroform: acetone: methanol: water, 50:60:2.5:3. ?, unknown.

Further to detect any changes in the patterns of cell wall bound lipids in the mutant strains, the delipidated cells were subjected to alkaline hydrolysis and methylation to generate fatty acid methyl esters and mycolic acid methyl esters (FAME's and MAME's) (Chapter 8). *M. smegmatis* produces α, α' and epoxy mycolates. Analysis of the FAME's and MAME's were done by separating the FAME's and MAME's on 1 dimensional TLC (1D-TLC) using the solvents petroleum ether:acetone; 95:5 (v/v). The TLC plates were developed twice in this system and exposed to Kodak X-Omat AR film overnight. Analyses of FAME's and MAME's from the wild type and mutant strains revealed the presence of α, α' and epoxy mycolates. The migration of FAMEs remained unaltered in all the mutant strains suggesting that fatty acid biosynthesis and transport have not been altered by loss of the genes. The profiles of the FAMES and MAMES are shown in Figure 6.11.

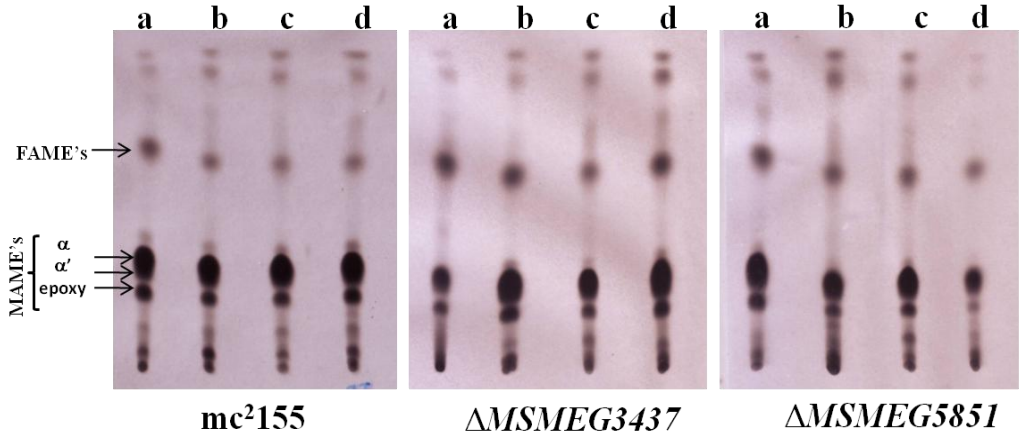


Figure 6.11. 1D-TLC autoradiographs of cell wall bound mycolates (FAMEs and MAMEs) from *M. smegmatis* mc²155, mutant strains Δ*MSMEG3437* and Δ*MSMEG5851*. All strains were grown in different media combinations – a) TSB, b) TSB –Tween 80 c) 7H9 and d) 7H9-Tween 80. Wall bounds fatty acids and mycolates were revealed after two developments in petroleum ether: acetone (95:5).

6.3.4 Characterization of the mutant strains Δ*MSMEG0241*

6.3.4.1 Colony morphology changes due to loss of Δ*MSMEG0241*

In the Δ*MSMEG0241* mutant strain, the colonies were found to be slightly risen and showed reduction in the outer periphery as compared to the wild type. However on the Tween plates, the Δ*MSMEG0241* mutant showed a slower growth and rough, crinkled colony edges as compared to the wild type strain (Figure 6.12).

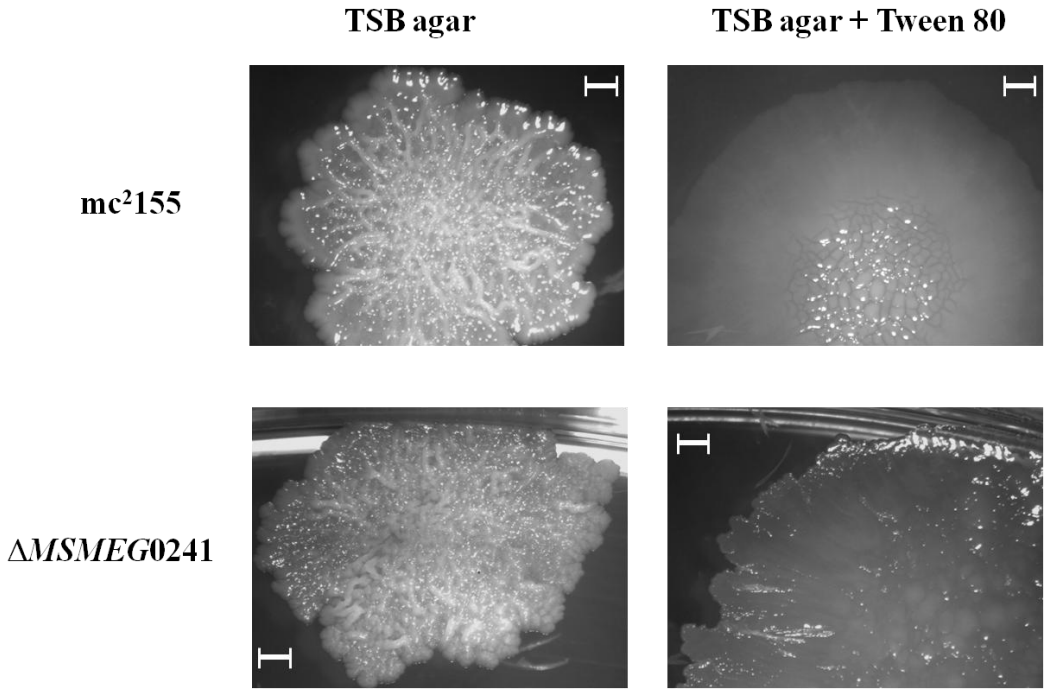


Figure 6.12. Colonies of wild type (mc²155), mutant strains Δ*MSMEG0241* on TSB-agar and TSB-agar-0.05% Tween-80 plates . Single isolated colonies are shown. Scale bar = 1mm.

6.3.4.2 Growth curve of wild type and Δ*MSMEG0241* mutant and Δ*MSMEG0241*-C complemented strain

Growth of wild type mc^{2}155, Δ*MSMEG0241* and Δ*MSMEG0241-C* were compared in TSB medium. Growth of the wild type strain was completed after 48 hours and reached A_{600} of 3.468, but the Δ*MSMEG0241* mutant strain reached to an O.D of 1.08 (Figure 6.13A).

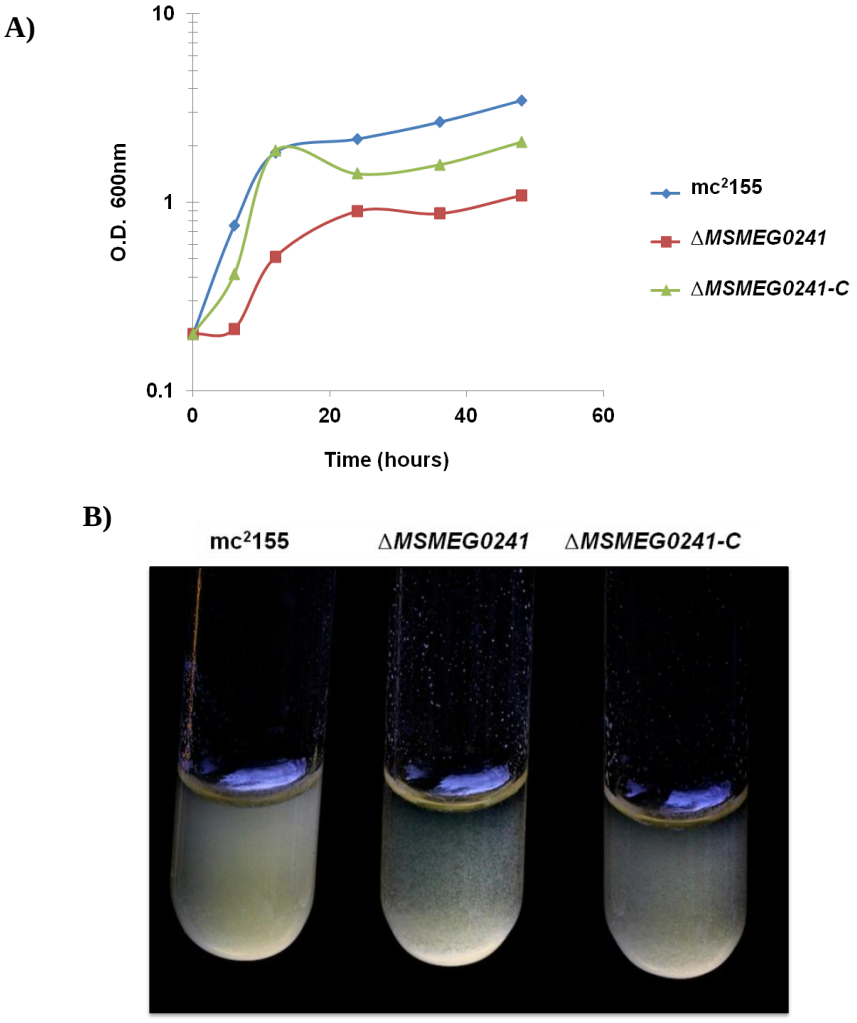


Figure 6.13. Growth analysis of Δ*MSMEG0241*. A) Consequences of deletion of MSMEG0241 on the growth rate of *M. smegmatis*, measured at 6, 12, 24, 36 and 48 hours. B) Growth phenotype in tryptic soy broth medium supplemented with Tween 80 at 24 hours.

However, introduction of plasmid borne wild type copy of the gene *MSMEG0241* to generate a complemented strain Δ*MSMEG0241-C* appears to partially restore the growth rates to wild type phenotype. The complemented strain reached an O.D of 2.094. The mutant strain also showed 'grainy' apearence in liquid medium containing Tween-80, and formed clumps. The complemented strain appears to partially revert to wild type phenotype (Figure 6.13B).

6.3.4.3 Cell wall lipid analysis of Δ*MSMEG0241*

In order to relate the phenotypic changes of the *M. smegmatis* Δ*MSMEG0241* mutant to alterations in the cell wall composition, the Δ*MSMEG0241* mutant strain, complemented strain Δ*MSMEG0241*-C and wild type strain mc^2155 were analysed for changes in cell wall lipids. Bacterial strains were grown in TSB and 7H9 media in the presence and absence of 0.05% Tween 80 for this study. Presence of Tween in media or growth on different media TSB and 7H9 did not affect apolar lipid profiles of free fatty acids and free mycolate. A mild accumulation of free mycolic acids was observed in Δ*MSMEG0241* (Figure 6.14). along with this accumulation, a spot was observed in the 2D-TLCs of apolar lipids extracted from cultures of both wild type and mutant strains grown in 7H9 media, both in presence and absence of Tween 80 (Figure 6.14). This spot has been denoted by '?' in the Figure 6.14. This accumulations seems to disappear in the 2D-TLC profile of the mutant strain grown in 7H9 in presence of Tween 80. The '?' species could possibly be an artefact or it is being actually made by the cells. This would need further characterisations and future work.

To check if the genes had any role in transferring Myc-PL to TMM to synthesise TDM, the apolar lipids were separated on a 2D-TLC system which separates TMMs and TDMs. This did

not show any changes or altered pattern of migration suggesting that *MSMEG0241* is not involved in transfer of mycolates in the TDM biosynthetic pathway (Fig 6.15).

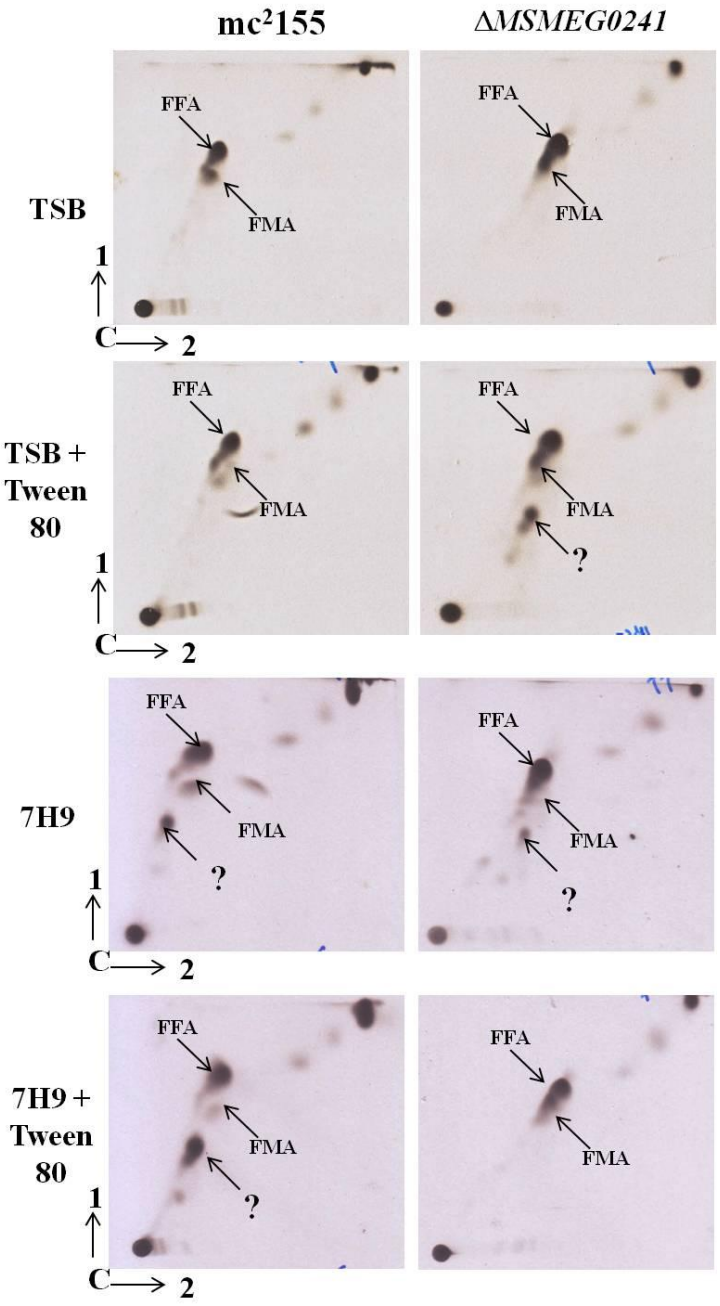


Figure 6.14. 2D TLC autoradiographs of apolar lipids extracted from *M. smegmatis* wild type mc²155 and mutant strains Δ*MSMEG0241*. Lipids were separated in solvent system 'C', direction 1 in chloroform/methanol 96:4, and in direction 2 in Toluene/acetone; 80:20. FFA-free fatty acids, FMA-free mycolic acids.

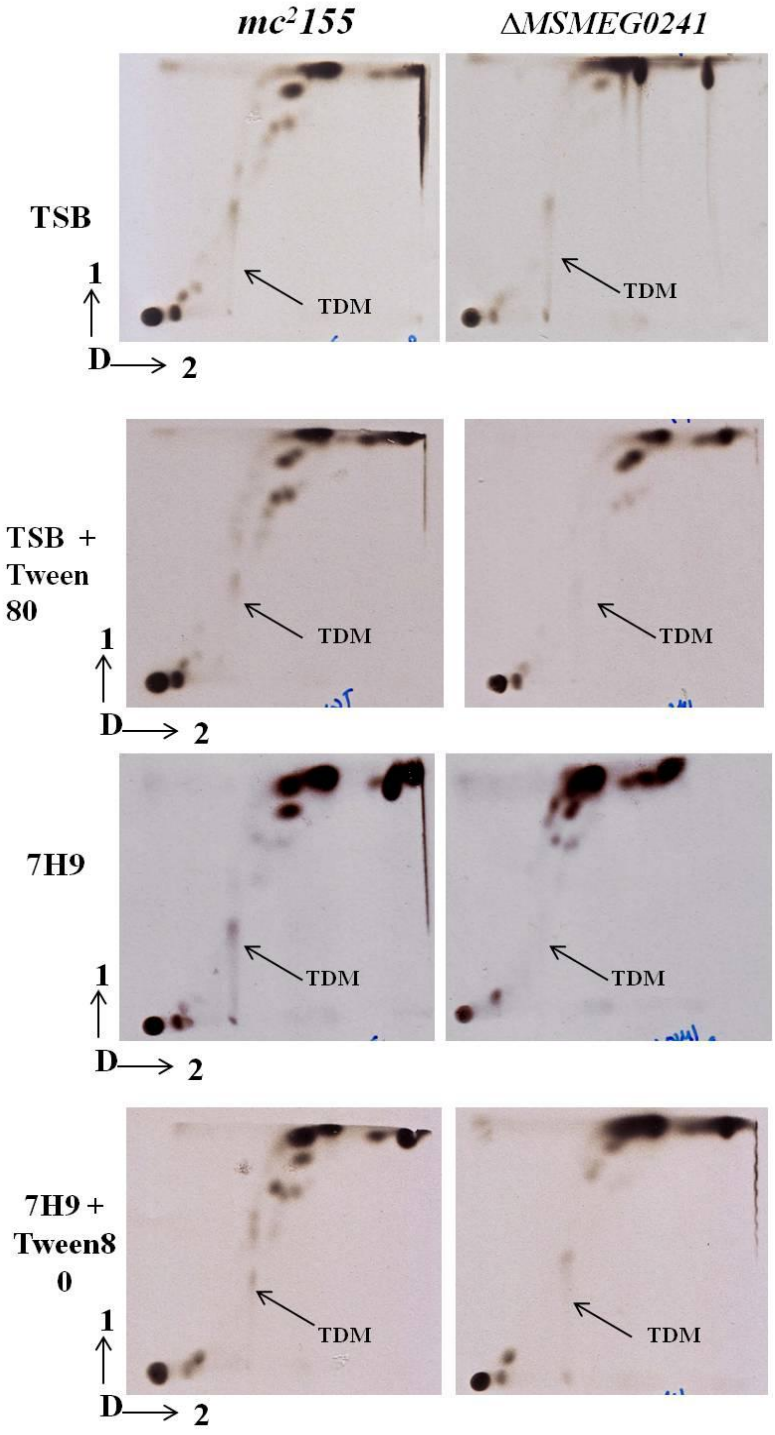


Figure 6.15. 2D TLC autoradiographs of apolar lipids extracted from *M. smegmatis* wild type mc²155 and Δ*MSMEG0241*. Lipids were separated in solvent system 'D', direction 1 in chloroform: methanol: water; 100:14:0.8, and direction 2 in chloroform: acetone: methanol: water, 50:60:2.5:3.

In the mutant Δ*MSMEG0241*, an increased level of mycolic acids was observed when the apolar lipids were separated by 2D TLC as shown in Figure 6.12. To address the cause of the accumulation of mycolates in the mutant strain, first, the surface exposed, non-covalently bound lipids were extracted using petroleum ether, following which apolar and polar lipids were extracted from the remaining cell pellet. The fractions, petroleum ether extractable and apolar lipids from the remaining cell pellet were separated by 2D-TLC and the profiles are shown in Figure 6.16.

The accumulation of free mycolates in and the difference in their migration pattern was consistent in the mutant strain under all four growth media. Majority of the free mycolic acids were found in the cell pellet after surface exposed petroleum fraction was removed indicated an inability of the mycolates in being translocated across the membrane. TLC's analysis reveals the presence of free mycolates in the inside of the cell, which were released on disruption of the cell wall and further solvent extraction. The outer cell wall lipids were comparable between the wild type and mutant strains.

Further to detect any changes in the patterns of cell wall bound lipids in the Δ*MSMEG0241* mutant, the delipidated cells were subjected to alkaline hydrolysis and methylation to FAME's and MAME's as detailed in Chapter 8. Analysis of the FAME's and MAME's from the wild type and mutant strains by 1D-TLC revealed the presence of all the 3 classes of mycolates produced by *M. smegmatis*, α, α' and epoxy mycolates. The migration patterns of FAMEs remained unaltered in the mutant strain suggesting that fatty acid biosynthesis and transport have not been altered by loss of the *MSMEG0241*. The 1D - TLC profiles of the FAMES and MAMES are shown in Figure 6.17.

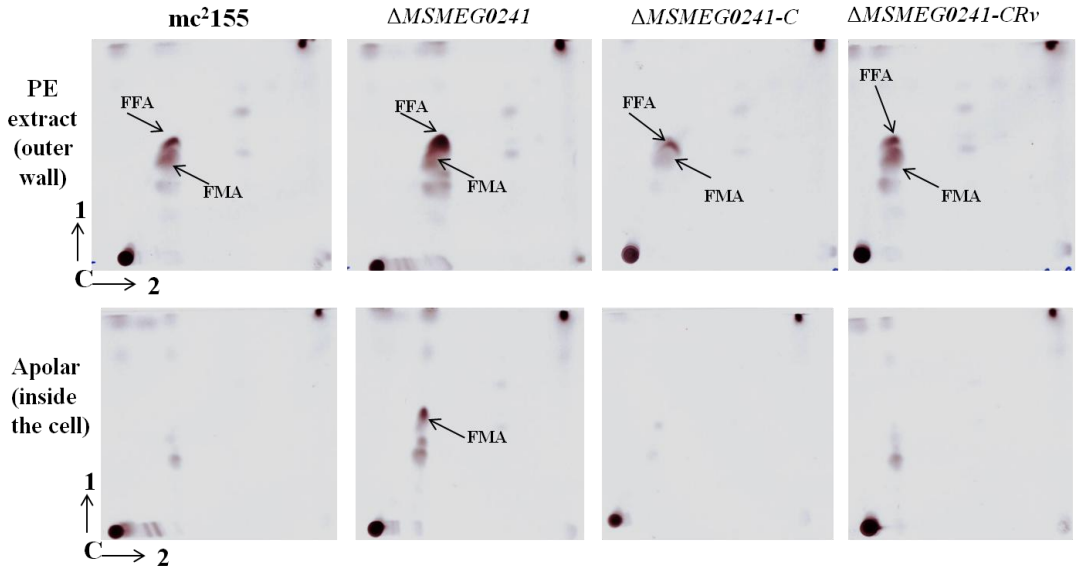


Figure 6.16 2D TLC autoradiographs of apolar lipids extracted from *M. smegmatis* wild type mc²155, Δ*MSMEG0241*, Δ*MSMEG0241*-C and Δ*MSMEG0241*-CRv. Lipids were separated in solvent system 'C', direction 1 in chloroform/methanol 96:4, and in direction 2 in Toluene/acetone; 80:20.

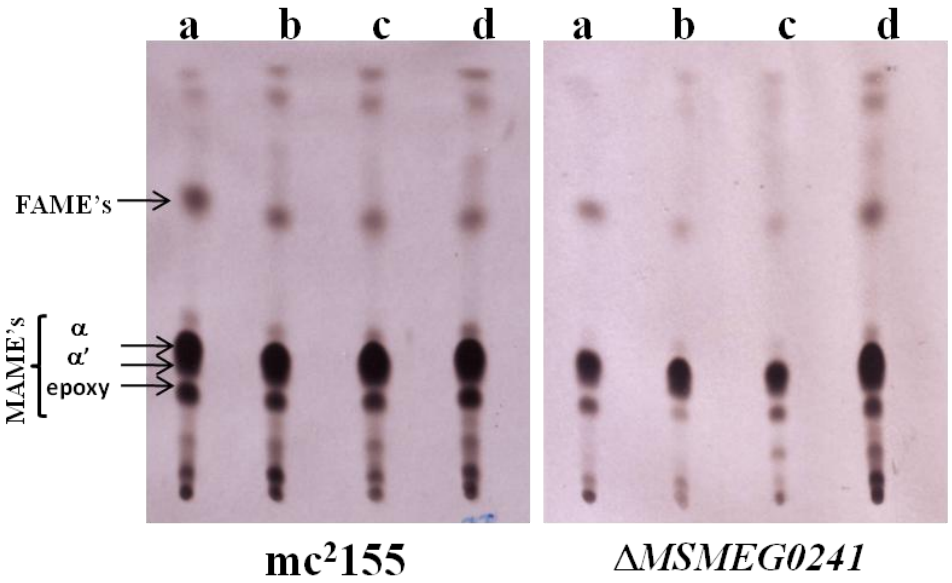


Figure 6.17 1D-TLC autoradiographs of cell wall bound mycolates (FAMEs and MAMEs) from *M. smegmatis* mc²155 and mutant strains Δ*MSMEG0241*. All strains were grown in different media combinations – a) TSB, b) TSB –Tween 80 c) 7H9 and d) 7H9-Tween 80. Wall bounds fatty acids and mycolates were revealed after two developments in petroleum ether: acetone (95:5).

6.3.4.4 Effect of deletion of *MSMEG0241* on biofilm formation

An alteration in colony morphology was observed for the mutant strain Δ*MSMEG0241* and to further study the role of the gene *MSMEG0241*, ability to form biofilms was tested and compared with the wild type and complemented strains. Previous studies have shown that *M. smegmatis* can translocate over the surface of solid growth medium by a sliding mechanism and can also form biofilm on polyvinyl chloride plates (Recht and Kolter, 2001; Recht et al., 2000; Martinez et al., 1999). Mutants of *M. smegmatis* and *Mycobacterium marinum* defective in biosynthesis of glycopeptidolipids, mycolyldiacylglycerol and lipooligosaccharides are defective in biofilm formation (Recht and Kolter, 2001; Chen et al., 2006; Ren et al., 2007). Extracellular non-covalently bound mycolic acids have been reported to be abundant in biofilms in *M. tuberculosis* and *M. smegmatis* (Ojha et al., 2008; Ojha et al., 2005). Involvement of FAS-II enzymes in biofilm maturation and defective biofilm formation by strains mutated in the genes KasA and inhA (Ojha et al., 2005) indicate a role of mycolic acids in biofilm formation.

MmpL11 is reported to be involved in transport of an unknown substrate possibly in the mycolic acid transport pathways (Domenech et al., 2005). To determine the effect of loss of *MSMEG0241* on biofilm formation, the wild type mc^2155, mutant Δ*MSMEG0241* and complemented strain *MSMEG0241-C* strains were allowed to grown on different biofilm media.

The mutant strain displayed less ability to swarm over the media as compared to the wild type strain. The complemented strain appears to have reverted to wild type phenotypes. This data along with the involvement of mycolates in biofilm formation and predicted function of

MSMEG0241 suggest a role of the MmpL11 gene in translocation of the mycolates to the outer cell wall in Mycobacterium.

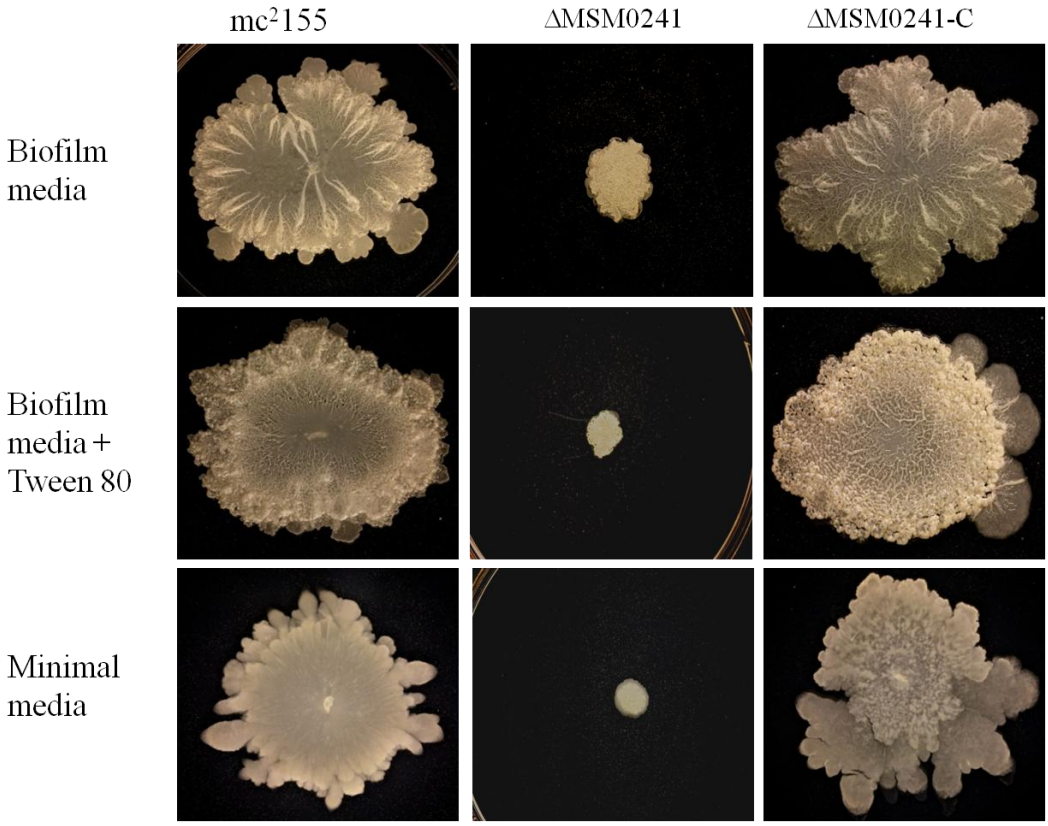


Figure 6.18. Biofilm formations on different media combinations. Comparisons of growth between strain mc²155, Δ*MSMEG0241* and Δ*MSMEG0241*-C, after 5 days of incubation at 37°C under humidified conditions. Actual images of colonies.

6.3.4.5 Analysis of biofilm matrix lipids

Free mycolates have been reported to be involved in biofilm formation in mycobacteria (Ojha et al., 2008). The mutant strain Δ*MSMEG0241* displayed a reduced ability to form biofilms as compared to the wild type strain. Given the involvement of mycolates in the extracellular matrix, lipids extracted from bacteria grown on solid biofilm media were

separated on 2D TLC to relate to change in free mycolate profiles. The wild type, mutant and complemented strains were grown on solid biofilm media (Recht and Kolter, 2001), containing ^{14}C-acetate and incubated at 37°C in a humidified chamber for 5- 7 days. The colony material was scraped and used for extractions of the extracellular non-covalently bound and inner cell wall lipids. The mutant strain shows higher amounts of free fatty acids and reduced levels of mycolic acids in the petroleum ether outer cell wall extract as compared to the apolar lipids (Figure 6.19).

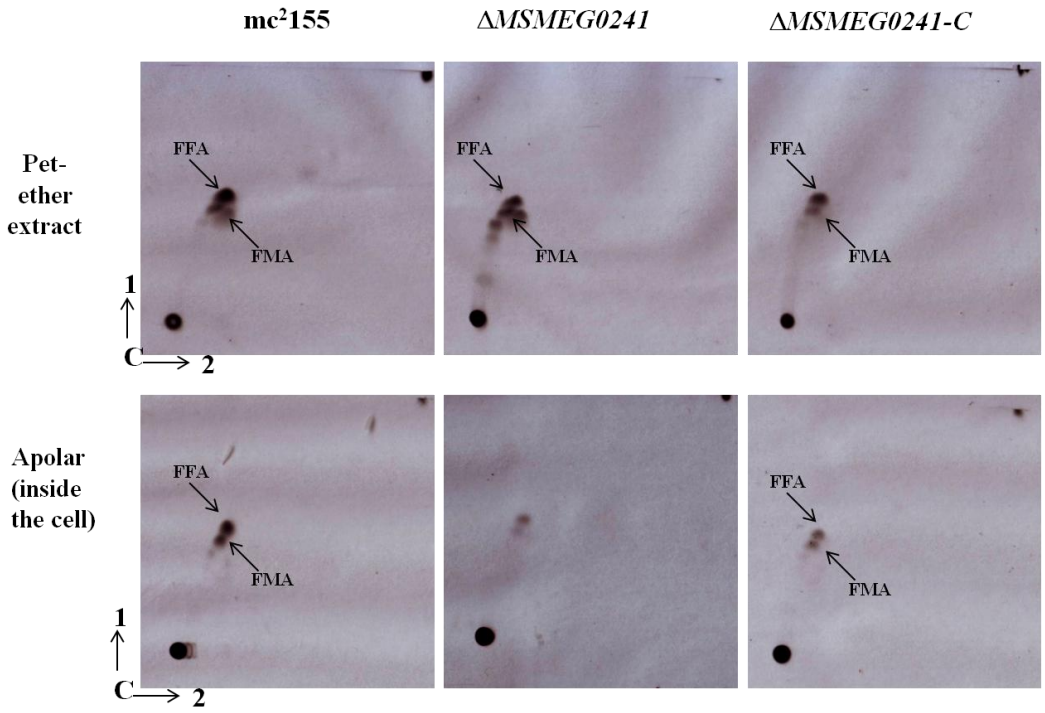


Figure 6.19. 2D TLC of petroleum ether extracts and apolar lipids, extracted from biofilms formed by the wild type strain mc^{2}155, Δ*MSMEG0241* and Δ*MSMEG0241*-C. Lipids were separated in direction 1 in chloroform/methanol 96:4, direction 2 in toluene/acetone 80:20.

MSMEG0241 encodes MmpL11, a putative membrane protein belonging to a family of RND proteins known to be involved in drug resistance and mediates transport of complex

molecules across the membrane. The predicted topology of the MmpL 11 is quite similar to the topology of MmpL 3, which is an essential gene and is involved in transport of mycolates in the final biosynthesis of TDMs (C.Varela communicated, University of Birmingham). These 2 MmpL genes occur in the same gene cluster and the synteny is conserved across all sequenced mycobacterial genomes. Domenech et al. (2005) had observed that an mmpL11 mutant of *M. tuberculosis* was attenuated in the mouse model of infection, but were unable to identify the exported metabolite. We now have preliminary data that suggests a role for MmpL11 in mycolic acid metabolism: a mutant of *M. smegmatis MSMEG0241*, the homologue of mmpL11 had reduced levels of cell envelope-associated free mycolic acids, indicating that while the gene was not essential, its function was related to mycolic acid metabolism. Ojha et al., have suggested that extracellular free mycolic acids are derived from newly-synthesised TDM via the action of exported esterases; our results indicate that MmpL11 may play a role in this process. Free mycolates are required for biofilm formation and interestingly the ΔMSMEG0241 mutant was defective in biofilm formation. From the phenotype of the MmpL11 deletion mutant in *M. smegmatis*, Δ*MSMEG0241* and its probable function, we suggest that *MSMEG0241* maybe involved in shuttling the free mycolic acids to the outer cell wall.

6.4 Discussion

Mycolic acids are key components of the mycobacterial cell wall and enzymes involved in their biosynthesis are the targets of many antituberculosis drugs like INH, ethionamide and thiolactomycin. Mycolic acid biosynthesis has been studied extensively but post biosynthesis processing and transport is not clearly understood, thus obstructing identification of novel

drug targets in the transport of this vital cell wall component. Belisle et al. (1997) demonstrated that the 3 members of the antigen-85 complex, Ag85A (*FbpA*), Ag85B (*FbpB*) and Ag85C (*FbpC*) in *M. tuberculosis* are involved in mediating mycolyltransferase reactions which finally results in forming the mycolyl-arabinogalactan-peptidoglycan of the cell wall (Belisle et al., 1997). Based on these findings and identification of proposed class II mycolyltransferases by Takayama et al. (2005), the homologues of potential mycolyltransferases *Rv1288* and *Rv0519c* in *M. smegmatis*, *MSMEG3437* and *MSMEG5851* were characterised in an attempt to elucidate mycolic acid processing in *M. tuberculosis*.

A group of transport pumps have also been reported to be involved in transport of lipids across the cell wall. The genomes of many mycobacteria including the highly pathogenic *M. tuberculosis* H37Rv contain a number of genes encoding proteins belonging to a family of multidrug resistant pumps known as RND proteins (Domenech et al., 2005). Many of these genes have been reported to be associated with gene cluster involved in biosynthesis of various sulfolipids, glycopeptidolipids, and acylated trehalose and phthiocerol dimycocerosates (Camacho et al., 2001; Domenech et al., 2004). Phylogenetic analysis of the MmpL genes from *M. tuberculosis, M. avium, M. leprae, M. marinum, M. smegmatis* (Figure 6.6) show that some of these genes occur in a closely related cluster, e.g. – *MmpL3* and *MmpL11*, and some are interestingly associated with the pks genes, e.g. –*MmpL8*, *MmpL10* and *MmpL12*. Out of all the MmpL's in mycobacteria only *MmpL* 3 was reported to be essential and is involved in mycolate transport and *MmpL 11* and *MmpL 8* have been reported to play a role in virulence in murine models (Domenech et al., 2005; Converse et al., 2003). MmpL11 has also been identified to be involved in transport of an 'unknown substrate' and in another study, involved in heme transport (Tullius et al., 2011). Tullius et al. (2011) suggested

a role of *MmpL3-MmpL11* gene cluster in heme acquisition. The authors report generation of a strain with deletion in the region *MSMEG0250* in *M. smegmatis.* This strain was deficient in heme uptake. However, we noticed no difference in heme uptake by the Δ*MSMEG0241* strain as compared to the wild type mc^2155. Earlier studies by Domenech et al.(2005) also reported the homologue of *MSMEG0250* in *M. tuberculosis, MmpL3* to be essential for survival and a deletion mutant failed to survive (Domenech et al., 2005). In *M. smegmatis*, the MmpL11 protein is encoded by the gene *MSMEG0241* and shares a very high sequence identity and similar predicted transmembrane topology with MmpL3. Hence, *MSMEG0241* was further characterised to identify if its 'unknown substrate' is a mycolate.

This work was aimed at studying the roles of mycolyltransferases *MSMEG3437, MSMEG5851* and *MSMEG0241* in mycolic acid processing and transport pathway. Mutant strains were generated in *M. smegmatis* mc^2155 using mycobacteriophage mediated specialised transduction (Bardarov et al., 2002). Cell envelope lipid analysis revealed no changes in lipid patterns in the mutant strains Δ*MSMEG3437*, Δ*MSMEG5851*. The strains did not show any difference in growth rates or colony morphology. This indicates that deletion of *MSMEG3437* or *MSMEG5851* does not affect any pathways required for survival of the bacterium and possibly other genes restore the functions/ roles of these genes *in vivo*.

The mutant strain Δ*MSMEG0241* was retarded in growth and had a tendency to form clumps in liquid growth media. The mutant also displayed an inability to form biofilms as compared to the wild type strain. This defect was compensated on introducing a plasmid borne copy of the wild type gene into the mutant strain. The mutant strain also displayed comparable growth phenotypes as the wild type strain with and without the presence of Fe^{2+} and hemin

(heme) in minimal media. Intercellular uptake and availability of heme was determined by spectrophotometric assays for heme, which showed no difference in heme uptake abilities between the wild type and mutant strains, suggesting that the role of *MSMEG0241* in heme transport is superfluous *in vivo*.

Mycolates are readily stripped off from the cells during solvent extractions. In the mutant strain Δ*MSMEG0241* it was observed that the mycolic acids are still contained in the inside of the cell, post petroleum ether extraction of surface exposed lipids which is revealed on methanolic saline disruption of the cell wall and further solvent extractions. This is an indication that the loss of the gene is responsible for blocking a part of the mycolate transport across the cell wall. From our experiments we conclude that the gene MmpL11 is involved in shuttling the free mycolic acids to the outer cell wall. Since there is no complete shutdown of mycolate transport, it is quite possible that the essential gene MmpL3, which is present in the same gene cluster and has similar conserved domain is the primary free mycolic acid transporter in the mycobacterial cell wall.

7

General Discussion

This chapter summarises the findings from the two different research themes covered in this thesis; LOS biosynthesis, and mycolic acid processing and transport in mycobacteria. Areas of future work are also addressed.

At the outset, I used a two pronged approach to identify and characterize genes involved in LOS biosynthesis in *Mycobacterium marinum*: (1) the generation and use of transposon libraries to screen for LOS defective mutant strains, and (2) the generation of targeted mutants of genes with a putative role in LOS biosynthesis The later approach was applied to the glycosyltransferase *MMAR2333* and polyketide synthase *MMAR2340* in *M. marinum* using the mycobacteriophage-based Specialised Transduction technique. Although phage mediated transposon mutagenesis has been reported in *M. marinum* this is the first report of delivery of allelic exchange substrates for targeted gene knockouts in this species. The *MMAR2333* mutant strain was able to produce LOS-I and an intermediate between LOS-I and LOS-II, LOS-II*. Similarity of MMAR2333 to other bacterial DPM-like synthases, 2D-TLC and mass spectrometry analysis suggests that is likely to be involved in the transfer of a nucleotide bound caryophyllose residue to a poyprenol unit to generate the substrate for other GTFs involved in synthesis of the higher LOSs.

The tetraglucose core of LOSs in *M. marinum* possess two different acyl chains. *MMAR2340* encodes a polyketide synthase and is present in the gene cluster identified to be involved in LOS biosynthesis. A second polyketide synthase gene is also present in this cluster –*MMAR2344 (pks5_1)*. This relates to the presence of two different methyl branched acylations in the LOS structure. Biochemical characterization of a *MMAR2340* null mutant revealed that the mutant strain is deficient in production of all the four LOS subtypes

indicating the involvement of *pks5* in synthesis of either one of the methyl branched acyl chains which is further attached to the tetraglucose core by the acyl transferases present in the cluster. Following the synthesis and addition of the first acyl chain, the second acyl chain, possibly synthesised by *pks5_1* is added to acylated core by the acyltransferases.

The frequency of obtaining mutants by Specialised Transduction in *M. marinum* is very low, hence the second approach was focussed on generating a transposon library and screening mutants based on colony morphology changes to further analyse defects in LOS biosynthesis pathway. Additionally we had access to another transposon library of LOS defective strains from an ongoing collaboration with Dr. A. Van der Woude and Prof. W. Bitter, VUMC, Amsterdam, the Netherlands. This library was generated using the wild type strain E11. The strains selected in this study were disrupted in the genes *MMAR2307, MMA2319, MMAR2320, MMAR2327, MMAR2336, MMAR2340, MMAR2341, MMAR2353, MMAR2355, MMAR2356* and *MMAR5170.* From the transposon library created in-house, we were able to isolate a mutant strain disrupted in the gene *MMAR2351*, a glycosyltransferase. 2D-TLC analysis of the above mutant strains revealed various defects in LOS production. Disruptions in MMAR2307 resulted in a strain which accumulated LOS-I, *MMAR2327::aph* and *MMAR2336::aph* strains accumulated an intermediate between LOS-I and LOS-II* and LOS-II respectively, *MMAR2319* and *MMAR2320* disrupted strains accumulated LOS-III and a transposon insertion in the genes *MMAR2340, MMAR2341*, *MMAR2355* and *MMAR2356* was responsible for strains with complete loss of LOSs. Surprisingly, no defect in LOS biosynthesis was observed when the putative GTF genes *MMAR2351* and *MMAR2353* were disrupted.

The mutant strains Δ*MMAR2333*, Δ*MMAR2340, MMAR2327::aph, MMAR2336::aph,* and *MMAR2340::aph* were also assessed for changes in virulence by infecting murine bone marrow derived macrophages. None of the strains were found to be altered in their abilities to survive within macrophages, but strains with total absence of LOS's (deletion/disruption in *pks5*) and a strain accumulating a precursor of LOS-II, LOS-II*(Δ*MMAR2333*) elicited a strong TNF-α release much higher compared to TNF- α levels released on infection with *M. marinum* wild type strains. Interestingly, a LOS-II' accumulating strain *MMAR2336::aph* and LOS-II accumulating strain *MMAR2327::aph* seemed to lower the TNF-α release levels. This relates with reports of inhibition of TNF-α response by LOS-III (Rombouts et al., 2009) and LOS-IV (Rombouts et al., 2010). Our findings suggested that while LOSs did not affect intracellular survival, alterations caused changes in the cytokine response. The cytokine responses also relate with the hypothesis that LOSs act as avirulence factors in the mycobacterial cell wall.

In silico analysis was done to locate the LOS gene cluster in the LOS producing opportunistic pathogen *M. kansasii* and a number of *M. kansasii*-specific GTFs were identified in the cluster reflecting the larger number of LOS species found in *M. kansasii*. A transposon library was generated to screen for strains deficient in LOS biosynthesis. Unfortunately mutant strains obtained from the screen were not in the genetic region involved in LOS biosynthesis in *M. kansasiii* and no further studies were conducted.

Generation of null mutants of the other GTF *MMAR2311*, second polyketide synthase gene *MMAR2344* and transporter *MMAR2342 (mmpl12)* is currently ongoing and will shed more light on the glycosylation and acylations of the LOS core. With the availability of the whole

genome sequences of *M. kansasii* and "*M. canettii"* phages will be generated for delivery of allelic exchange substrates by specialised transduction to generate null mutants of targeted genes in *M.kansasii* and *M. canettii.*

The other theme of this thesis was the study of the processing and transport of mycolic acids in *Mycobacterium smegmatis*. Mycolic acid biosynthesis has been studied in great detail but their processing and transport still remains unknown. I investigated the role of two genes *MSMEG3437* (homologue of *Rv1288*) and *MSMEG5851* (homologue of *Rv0519c*) as the Mycolyl Transferase-II involved in generating an intermediate of mycolic acid processing. The ability to generate null mutants of these genes in *M. smegmatis* showed that the genes were not essential for growth; this was uncharacteristic of genes that play a role in mycolate metabolism which are largely essential genes. Characterisation of the mutant strains revealed no changes in growth, colony morphology or lipid profiles, and together these findings suggested that either the two genes were not Mycolyl Transferase-II candidates, or that the roles they played were redundant.

In contrast to the above genes, I obtained a phenotype for the *M. smegmatis mmpl11* gene (*MSMEG0241*) which also identified as a probable candidate involved in transport of mature mycolates. Whilst the gene was non-essential, the mutant strain exhibited retarded growth, and was unable to form biofilms. Furthermore, 2D-TLC of cell envelope lipids reveals that the gene *MSMEG0241* is involved in shuttling the free mycolic acids to the outer cell wall. Ongoing work in our laboratory has now established that the neigbouring essential gene *mmpl3*, which is present in the same cluster as *mmpl11* is the primary transporter of mycolates in the mycobacterial cell wall, and together these data suggest a

co-ordination of the two processes. Protein-protein interaction studies will potentially shed more light on the complexes involved in the transport of mycolic acid intermediates.

8

General materials and methods

8.1 Media preparations

8.1.1 Luria-Bertani (LB) broth

25g of LB (Tryptone-Yeast extract-NaCl 2:1:2 w/w/w) is dissolved in 1 litre of distilled water and sterilised by autoclaving.

8.1.2 LB agar

37g of LB-agar mix (Tryptone-Yeast extract: NaCl: Agar 2:1:2:3 w/w/w/w) was dissolved in 1 litre of distilled water and autoclaved. Antibiotics were added to molten agar after the cooling it down to approximately 55°C, mixed thoroughly before pouring into petri dishes (25ml) aseptically. .

8.1.3 Tryptic Soy Broth (TSB)

30g of TSB mix (Pancreatic casein digest-Papaic soybean digest-dextrose-NaCl-K_2HPO_4 34:6:5:10:5 w/w/w/w) is dissolved in 1 litre of distilled water and sterilised by autoclaving.

8.1.4 Tryptic Soy Agar (TSA)

TSA is prepared by mixing 30g of TSB powder and 15 grams of agar in 1 litre of distilled water and autoclaved. Antibiotics were added to molten agar after the cooling it down to approximately 55^0C, mixed thoroughly before pouring into petri dishes (25ml) aseptically.

8.1.5 Middlebrooks 7H9 broth

4.7g of the 7H9 broth mix was dissolved in 900ml distilled water. To this 2.5ml of glycerol was added and filter sterilised. 100ml ADC (BD) was added aseptically and the solution stored

at 4°C. 10% OADC (BD) was used when growing *M. marinum* and *M. kansasii* as it was observed that these two species thrived better when supplemented with OADC. *M. smegmatis* was grown with the usual 10% ADC supplementation.

8.1.6 Middlebrooks 7H10 broth

7H10 broth was made up according to media composition of Middlebrook's 7H10 agar, without the addition of agar and filter sterilised. The broth was supplemented with 10% OADC (BD).

8.1.7 Middlebrooks 7H10 agar

19g of the 7H10 broth mix was dissolved in 900ml distilled water. To this 2.5ml of glycerol was added and filter sterilised. 100ml OADC (BD) was added aseptically and the solution stored at 4^0C.

8.1.8 Middlebrooks 7H11 agar

4.7g of the 7H11 broth mix was dissolved in 900ml distilled water. To this 2.5ml of glycerol was added and filter sterilised. 100ml OADC (BD) was added aseptically and the solution stored at 4^0C.

8.1.9 7H9 Basal agar

2.35g of 7H9 broth mix was dissolved in 500ml distilled water and 1.25 ml glycerol was added. 7.5g of agar (Bacto Agar – BD) was added and the solution was autoclaved. Once the temperature was ~ 55^0C, 20 ml plates are poured aseptically.

8.1.10 7H9 Soft agar

2.35g of 7H9 broth mix was dissolved in 500ml distilled water and 1.25 ml glycerol was added. 3.75 grams of agar (Bacto Agar – BD) was added and the solution was autoclaved. Once the temperature was ~ 55^0C. the tubes were ready for overlay.

8.1.11 Antibiotic and Supplements

Table 8.1 List of antibiotics and supplements

Additives	Stock Concentration	Storage
Hygromycin B	50mg/ml	4 ^{0}C, protected from light
Kanamycin	25mg/ml	-20^{0}C , filter sterilised
Chloramphenicol	30mg/ml	-20^{0}C , filter sterilised
Gentamicin	50mg/ml	4 ^{0}C, protected from light
Oleic acid-albumin-dextrose-catalase(OADC) and ADC	10%	4 ^{0}C
Tween 80	10%	Room temperature, protect from light

8.2 Molecular biology techniques

8.2.1 DNA electrophoresis

DNA fragments are usually separated by electrophoretic means. Agarose gel electrophoresis have been used in this work for separation of DNA. Different percentage of agarose gels (0.6% - 1%) were prepared by dissolving / melting Molecular biology grade agarose (Bioline) in Tris acetate EDTA (TAE) buffer. DNA samples loaded onto the agarose gel in wells were separated under a horizontal electric field (110 – 140 V, 400mA) and DNA was visualised by

staining the gel with ethidium bromide and viewing under UV light (Bio-Rad Gel Doc systems).

8.2.2 Polymerase Chain Reaction (PCR)

PCR mix using Phusion polymerase

Components	Condition A	Condition B	Condition C	Condition D
Forward primer	1µl			
Reverse primer	1µl			
Template DNA	0.5µl			
dNTP	1µl			
Polymerase	1µl			
GC/HF buffer	2µl			
Milli Q water	13.5µl	9.5µl	11.5µl	8.5µl
DMSO	-	4µl	-	4µl
Magnesium sulfate	-	-	1µl	1µl

8.2.3 Digestion of DNA

20 µl single or double digestion reactions were set up depending on experimental requirements. 5 µl of purified DNA (plasmid / genomic DNA/ PCR product) was mixed with 2 µl of digest buffer , 1 µl of restriction enzyme, 2 µl of 10X BSA (wherever required) and the solution id made upto 20 µl with distilled water. This is incubated for 30 -120 minutes at 37°C, unless special temperatures are required for activity of ceratin enzymes, e.g. BssHII, 55°C.

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8.2.4 Ligation

Digested DNA fragments with compatible ends can be ligated using T4 DNA ligase. A 20 µl ligation reaction is set up using 1 µl of T4 DNA ligase enzyme, 2 µl of 10X T4 ligase buffer, required concentrations of DNA fragments and distilled water. This is incubated at 16^0C for 12-16 hours and transformed into competent E.coli cells to amplify and purify plasmids.

8.3 Preparation of chemically competent *E. coli* cells

A single colony of *E. coli* (TOP-10 strain) was inoculated into 2.5 ml LB medium and incubated at 37°C overnight. The overnight culture was then used to inoculate 250 ml LB medium with 20 mM MgSO4. Cells were grown to an O.D 600 of 0.4-0.6 and then harvested by centrifugation at 4500*g* for 10 min at 4°C. Cell pellets were gently resuspended in 1/4th volume of ice-cold TFB1 (30 mM potassium acetate, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 100 mM $RbCl_2$, 15% glycerol, filter-sterilised and stored at 4°C) and resuspended cells were incubated on ice for 5 min. Cells were harvested by centrifugation as described previously and were then gently resuspended in 1/25 of the volume of ice-cold TFB2 (10 mM MOPS or PIPES, pH 6.5, 75 mM $CaCl_2$, 10 mM $RbCl_2$, 15% glycerol, filter-sterilised and stored at 4°C). Cells were incubated on ice for 15-60 min and then stored as 100µl aliquots at -70°C.

8.3.1 Transformation of *E. coli* competent cells

E. coli competent cells were thawed on ice and then the ligation reaction (5µl) was added to the cells. Cells and the ligation reaction were mixed gently and then incubated on ice for 30 min. Cells were transformed by heat shock at 42°C for 90 seconds followed by which the cells were placed on ice to cool. LB medium (1ml) was added into the tube and incubated at 37°C

for about 45 min with shaking. The transformed cells were then plated onto appropriate selection plates.

8.3.2 Plasmid extraction

Plasmid DNA purification kits by QIAGEN were used for all of this work. Bacteria containing the gene of interest borne on a plasmid are grown with appropriate selection markers in a shaking incubator. The cells are harvested and lysed with buffers P1& P2 which contains RNase and sodium dodecyl sulfate respectively. This allows precipitation of DNA and proteins from the cells. Neutralization was done using buffer N3 (acetate and guanidine hydrochloride). This precipitates large DNA and facilitates binding of plasmid DNA to the spin column.The mix is centrifuged and the supernatant is loaded onto the provided spin columns, which retains the plasmid DNA. The spin column was washed with PE buffer (containing ethanol) and plasmid DNA is eluted with distilled water or 10 mM Tris-HCl pH8.0.

8.4 Generation of knockout phage for null mutant creation using Specialised Transduction.

Approximately 1 kb sequences of the upstream and downstream regions of the 'target gene (*geneX*)' were PCR-amplified from *M. marinum* 1218R or *M. smegmatis* mc^2155 genomic DNA using appropriate primer pairs. The figure 8.1 shows the sequential procedures involved in generation of the knockout phage. The PCR products were purified and the primer incorporated *Van*91I sites were digested with *Van*91I, following which the digested PCR fragments were cloned into *Van*91I-digested p0004S to generate the allelic exchange plasmid

p∆*geneX*. One positive plasmid was *Pac*I digested and ligated to *Pac*I digested phAE159 DNA. The ligation mix was then packaged into empty λ-phage heads and transduced into *E. coli* HB101. Cells containing phasmid DNA were selected for on LB agar containing hygromycin at 37°C. Packaging of p∆*geneX* into phAE159 was confirmed by *Pac*I digestion. The positive phasmids were transformed by electroporation into *M. smegmatis* at 1800V and recovered at 30°C for ~4 hours in TSB. The recovered cells were then harvested and resuspended in 200 µl of MP buffer. This was mixed with 200 µl of freshly growing *M. smegmatis* and 5 ml molten soft agar (50°C) and poured on 7H9 basal agar plates and incubated at 30°C for 2 -3 days and allowed to form plaques. The plates were soaked in minimum amount of MP buffer for 5-6 hours and the solution containing phages was filtered and stored at 4°C. This generated the recombinant phage - ph∆*geneX* designed to replace the 'targeted gene' with *hyg*.

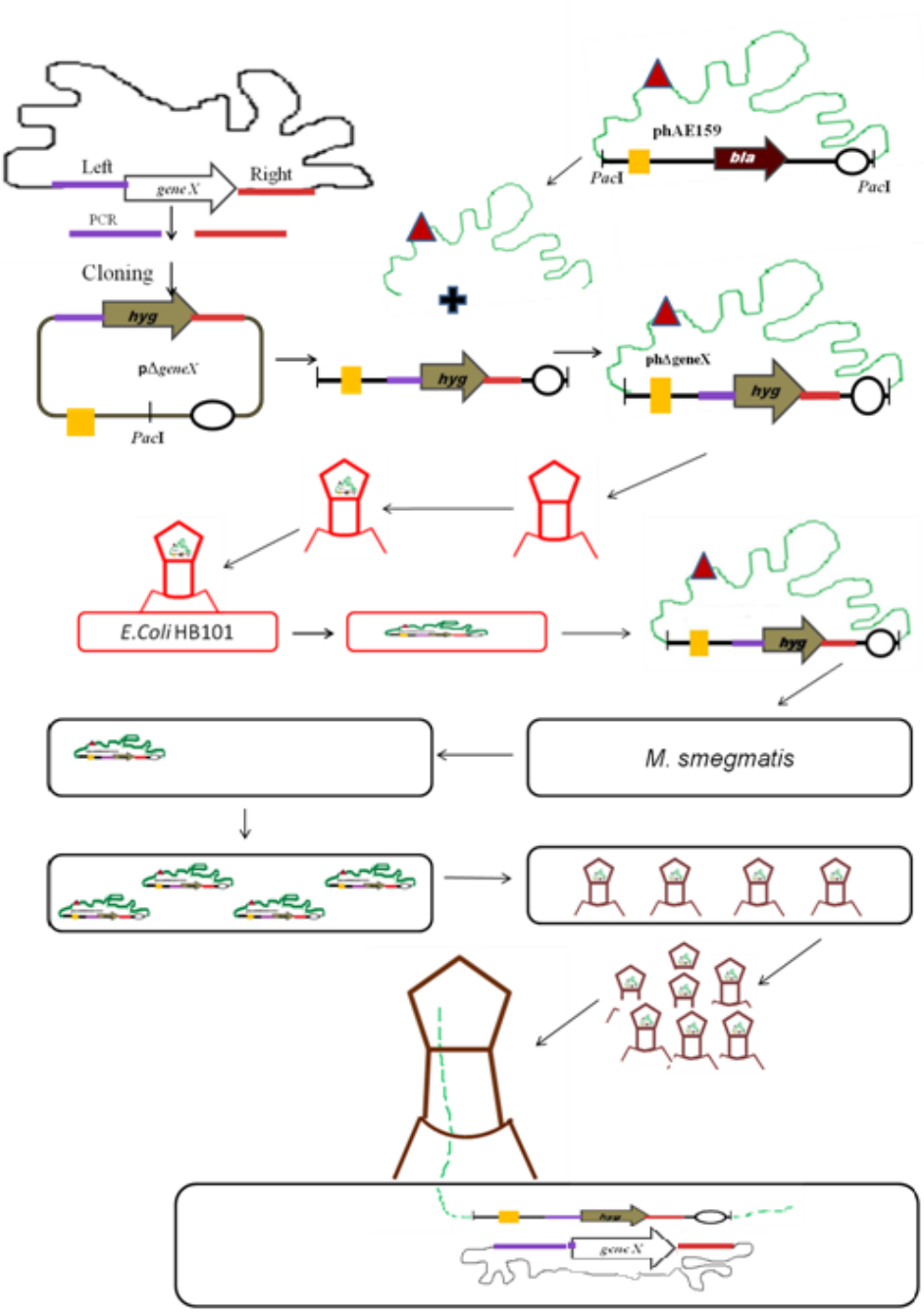

Figure 8.1 Schematic representations of the events during generation of the knockout plasmid leading to the recombinant phage which is used to replace the targeted gene by specialised transduction, adapted from (Bhatt and Jacobs, 2009).

Specialised transduction of *M. marinum* 1218R was performed as described previously for other mycobacteria (Bardarov et al., 2002). *M. marinum* or *M. smegmatis* cultures were grown in 50ml of 7H9+10% OADC or TSB respectively with 0.05% Tween 80 to an OD_{600} of about 0.8 and harvested by centrifugation at 4500xg for 10 minutes. The cell pellet was washed twice with 50ml of MP buffer and resuspended in 2ml of MP buffer and 2ml of high titre (10^{-10} pfu /ml) phage lysate was mixed with the cells. A control was set up where 0.5 ml of resuspended cells was mixed with 0.5ml of MP buffer. The mix was incubated overnight at 37°C followed by harvesting and recovered with 10ml 7H9+10% OADC with Tween-80 at 37°C for 4-5 hours incase of *M. smegmatis* and overnight for *M. marinum* and *M. kansasii*. This was plated onto 7H10+10% OADC-agar plates with hygromycin B and plates were incubated at 37°C for 1-4 weeks depending on the strain of Mycobacteria. Hygromycin resistant colonies obtained after transduction were inoculated in 10ml 7H9+10% OADC - Tween 80 with hygromycin B for genomic DNA extraction and further characterization. Allelic exchange of *geneX* with a hygromycin resistance cassette in hygromycin resistant transductants was confirmed Southern blot. One such transductant was chosen for subsequent experiments.

8.5 Genomic DNA extraction

Bacterial cells were harvested from a 10 ml culture by centrifugation at 4500*g* for 10 minutes. The cell pellet were resuspended in 450 µl glucose Tris-EDTA (GTE) solution (25 mM Tris-HCl pH 8.0, 10 mM EDTA, 50 mM glucose) with 10 mg/ml lysozyme and incubated at 37°C overnight. To this 10% sodium dodecyl sulfate (100 µl) and 50 µl of 10 mg/ml Proteinase K (Sigma, cat. P4914) were added, and the mixture was incubated at 55°C for 3 – 4 hrs. 5M

NaCl (200 µl) and preheated CTAB solution (160 µl) were added and the mixture incubated at 65°C for 10 min.

Chloroform: isoamyl alcohol (24:1) extraction was performed twice to exclude protein before DNA was precipitated with 560µl of isopropanol (0.7 volume of total mixture). The DNA pellet was washed with 70% ethanol and air-dried before dissolving in Tris buffer (10 mM Tris-Cl, pH 8.5) or water.

8.6 Southern blotting

The agarose gel was depurinated in 0.25M HCl for 10minutes, followed by denaturation (1.5 M NaCl, 0.5 M NaOH) for 10 -15 minutes and finally neutralised for 15 – 20 minutes (0.5 M Tris-HCl pH 7.2, 1 M NaCl). The DNA on the gel was then transferred onto a nylon membrane (Nylon Membrane, positively charged, No-11209299001, Roche) by capillary transfer with 20X SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0), overnight. DNA was fixed to the membrane by UV cross-linking. After cross-linking, the membrane was rinsed with distilled water and used for hybridization, labelling and detection. The procedure was performed as described in DIG High Prime DNA Labelling and Detection Starter Kit II (cat no – 11585614910, Roche). This kit uses digoxigenin, a steroid to label DNA probes by random priming. The hybridized probes are then immunodetected by anti-digoxigenin-AP (Fab fragments), which are visualised by chemiluminescence.

8.7 Preparation of mycobacterial electrocompetent cells

Mid-log phase mycobacterial cells were washed with 1 volume (original volume of culture) of 10% glycerol (filter-sterilized, pre-cooled to 4°C) twice and harvested by centrifugation at 4°C at 4500*g*. Cells were resuspended in 1/10 volume of 10% glycerol and aliquoted (200 µl) for immediate use or can be stored at -70°C.

8.7.1 Electroporation of mycobacteria

200 µl electrocompetent cells were thawed on ice and 3µl - 5µl of plasmid dissolved in water was added to the cells. The mix was left on ice for 15 minutes and then placed into a chilled 1 mm electroporation cuvette. Electroporation was done using an Eppendorf Electroporator (model no - 2510) at 1800V. TSB (1ml) (for *M. marinum*, 7H9 was used) was added and cells were allowed to recover at 37°C (for *M. marinum* at 30°C) for at least one generation time before plating on TSB agar (*M. smegmatis*) or 7H10 agar (*M. marinum*) agar with appropriate antibiotics.

8.8 Radioactive labeling of lipids

Mid-log phase mycobacterial cultures (10ml) were labelled with 1µCi/ml of [^{14}C] acetic acid-sodium salt (50mCi/ml, Amersham Pharmacia Biotech) and incubated for 12 hours in case of *M. smegmatis* and 24 hours for *M. marinum*. The [^{14}C]-labelled cells were harvested by centrifugation and washed with PBS before freeze-drying.

8.9 Lipid extraction

[^{14}C]-labeled polar and non-polar lipids were extracted using the methods described by Dobson et al., (1985). The dried [^{14}C]-labelled cells were mixed with 2 ml of CH_3OH/0.3% NaCl (100:10, v/v) and 2 ml of petroleum ether (b.p.60-80°C) for 30 min. The mixture was centrifuged at 4000*g* for 5 minutes and the upper layer removed and stored in a different tube. Petroleum ether (2 ml) was added to the lower fraction and allowed to mix on a rotator for another 30 minutes. This was centrifuged and the upper layer was removed .The combined upper layers containing the non-polar lipids were dried under liquid nitrogen and were further analysed.

Polar lipids were further extracted by adding 2.3 ml of $CHCl_3$/CH_3OH/0.3 % NaCl (90:100:30, v/v/v) to the lower aqueous layer. The solution was mixed for 1 hour and centrifuged and the supernatant removed and stored in a fresh tube. The remaining pellet was extracted twice by the addition of 0.75 ml of $CHCl_3$/CH_3OH/0·3% NaCl (50: 100: 40, v/v/v) and mixing for 30 min each time before centrifugation and removal of supernatant. The defatted cells were stored for further analysis. To the pooled supernatants, 1.3 ml of $CHCl_3$ and 1.3 ml of 0.3 % NaCl was added and mixed for 10 min, centrifuged and the lower layer recovered and dried. The dried apolar and polar lipids were dissolved in 200 µl of $CHCl_3$/CH_3OH (2:1, v/v); and 5 µl was dried in a scintillation vial and then mixed with 5 ml scintillation fluid and radioactivity incorporated was measured in terms of counts per minutes.

8.9.1 Fatty acid methyl esters (FAMEs) and Mycolic acid methyl esters (MAMEs) extraction from defatted cells and whole cells

The [^{14}C]-labeled cells or de-fatted cells were subjected to alkaline hydrolysis using 5% aqueous tetrabutylammonium hydroxide at 100°C overnight. CH_2Cl_2 (4 ml), CH_3I (300 µl) and water (2 ml) was added to the reaction mixture followed by mixing for 30 min. The upper aqueous phase was discarded, and the lower organic phase was washed twice with water and evaporated to dryness. MAMEs were redissolved in diethyl-ether, and the solution was evaporated to dryness. The final residue was either weighed in case of cold samples or dissolved in CH_2Cl_2 (200 µl) and 5 µl of the resulting solution was used for liquid scintillation counting.

8.9.2 Large scale lipid extraction and analysis

Polar and apolar lipids were extracted as described by Dobson et al.,(1985). Briefly, 6 g of dry *M. marinum* cells were treated in 220 ml of methanolic saline (20 ml 0.3% NaCl and 200 ml CH_3OH) and 220 ml of petroleum ether for 2 h (Dobson et al., 1985). The suspension was centrifuged and the upper layer containing apolar lipids was separated. The step was repeated twice. The two upper petroleum ether fractions were combined and dried. For polar lipids, 260 ml $CHCl_3$/CH_3OH/0.3% NaCl (9:10:3, v/v/v) was added to the lower aqueous phase and stirred for 4 h. The mixture was filtered and the filter cake re-extracted twice with 85 ml of $CHCl_3$/CH_3OH/0.3% NaCl (5:10:4, v/v/v). Equal amounts of $CHCl_3$ and 0.3% NaCl (145 ml each) were added to the combined filtrates and stirred for 1 h. The mixture was allowed to settle, and the lower layer containing the polar lipids recovered and dried. The polar lipid extract was examined by two dimensional thin-layer chromatography (2D-TLC) on aluminum

backed plates of silica gel 60 F_{254} (Merck 5554), using $CHCl_3/CH_3OH/H_2O$ (65:25:4, v/v/v) in the first direction and $CHCl_3/CH_3COOH/CH_3OH/H_2O$ (40:25:3:6, v/v/v/v) in the second direction. The thin-layer chromatographic plates sprayed with the appropriate staining solution to detect the presence of lipids, glycolipids or phospholipids as described earlier.

8.9.3 Thin layer chromatography (TLC) analysis for lipids

Equivalent amounts (25,000 to 50,000 cpm) of each sample were spotted on TLC plates (5554 silica gel 60F524; Merck) for further 1D- or 2D-TLC analysis using following solvent systems. Incase of cold samples, 50 -100ug of apolar or polar lipids were spotted on TLC plates for 1D /2D separation and analysis. Cold samples were stained with MPA , alpha-naphthol or phosphate stains and charred where required to reveal the positions of the lipids on the TLC plates. [^{14}C]-labelled lipids were revealed by 24-72hrs exposure to Kodak X-Omat AR film.

8.9.3.1 Solvent systems for 2D TLC analysis.

Apolar lipids are separated by systems A,B,C,D. Polar lipids are separated using systems D and E. TLC plates should be air dried well before every solvent run.

System A
Direction 1
Thrice with petroleum ether (b.p. 60-80°C)/ ethyl acetate - 98:2 (v/v)
Direction 2
Once with petroleum ether (b.p. 60-80°C)/acetone - 92:8 (v/v)

System B
Direction 1
Thrice with petroleum ether (b.p. 60-80°C)/acetone - 92:8 (v/v)
Direction 2
Once with toluene/acetone - 95:5 (v/v)

System C
Direction 1
Once with chloroform/methanol - 96: 4 (v/v)
Direction 2
Once with toluene/acetone - 80:20(v/v)

System D
Direction 1
Once with chloroform/ methanol /water - 100:14:0.8 (v/v/v)
Direction 2
Once with chloroform/acetone/ ethanol /water - 50:60:2.5:3 (v/vv/v)

System E
Direction 1
Once in chloroform/ methanol /water - 60:30:6 (v/v/v)
Direction 2
Once in chloroform/acetic acid/ methanol /water - 40:25:3:6 (v/vv/v)

Note : TLC plates are activated by baking at 100^0C for one hour before application of samples for running in system E. One hour drying between runs is also required for system E.

8.9.3.2 TLC analysis for Fatty Acid Methyl Esters (FAMEs) and Mycolic Acid Methyl Esters (MAMEs)

The FAMEs/MAMEs mixture (25,000 cpm) was subjected to 1-D TLC (5554 silica gel 60F524; Merck), developed in petroleum ether (b.p. 60°C-80°C):acetone (95:5, v/v).[^{14}C]-labeled FAMEs/MAMEs were revealed by overnight exposure to Kodak X-Omat AR film.

8.9.3.3 Argentation TLC

Silica gel TLC plates were coated with 10% silver nitrate solution. Coating is done in a way that only the run in direction 2 will encounter silver coating. The plates were air-dried followed by heating for about 2 hours in an oven. The plates are then used immediately or can be stored away from light and under airtight conditions for a few days. These plates are

developed twice in hexane: ethyl acetate /95:5; (direction 1), followed by thrice in petroleum ether: diethyl ether / 85:15; (direction Ag 2).

8.9.4 Derivatization of sugar residues in LOS-U and analysis by MALDI-MS

Per-*O*-methylation of LOS-U was done by adding 0.5ml of anhydrous dimethyl sulfoxide to 2mg of the purified LOS-U, followed 100-200µl of 4.8M dimethyl sulfinyl carbanion (dimsyl ion). The reaction was incubated at room temperature with stirring for 1 h in a reducing environment. 100µl methyl iodide was then added to the reaction mix and the incubation was continued for another hour. The above step was repeated and the final reaction mixture was quenched with ~1ml of water. The sample was then dried and the resulting per-*O*-methylated sample was diluted in DMSO/H_2O (1:1 v/v) and applied to a Sep-PakC18 cartridge (Alltech) and eluted with 100% acetonitrile followed by 100% ethanol. The eluate was dried and the per-*O*-methylated sample was dissolved in chloroform.

8.10 MALDI-TOF-MS analysis

MALDI-MS analysis was performed by Dr. P. Ashton and Mr N.G. May, School of Chemistry, University of Birmingham.The matrix used was 2,5-dihydroxybenzoic acid at a concentration of 10 µg/µl, in a mixture of water/ethanol (1:1, v/v), 0.1% trifluoroacetic acid. Analyses were performed on Voyager DE-STR MALDI-TOF instrument (PerSeptive Biosystems, Framingham, MA) using linear mode detection. The mass spectra were mass assigned using external calibration.

8.11 NMR spectroscopy

NMR spectroscopy was performed by Dr. N. Spencer, School of Chemistry, University of Birmingham. NMR spectra of purified LOS-III, isolated from *MMAR2320::aph* were recorded on a Bruker DMX-500 equipped, with a double resonance (1H/X)-BBi z-gradient probe head.

8.12 Chemicals, reagents and enzymes

All chemicals and solvents were purchased from BD, Sigma-Aldrich (Dorset, UK), Bio- Rad (Ca, USA) and Fisher Chemicals (UK) unless otherwise stated, and were of AnalR grade, Molecular Biology or equivalent. Enzymes were obtained from New England Biolabs (NEB), Fermentas, Invitrogen, and Finzymes.

8.13 Extraction and infection of bone marrow derived macrophages

Lower body parts of Balb/c mice were obtained from Dr. D. A. Lammas (Institute of Biomedical Research, University of Birmingham).Tissue was removed and legs were dissected away from the body. The pelvic and femoral bones were cleaned and separated at the knee joint. The bone was cut off at each end and bone marrow was expelled with 5 ml bone marrow medium (Dulbecco's modified Eagle's medium (DMEM- Invitrogen) , supplemented with 4.5 g/L glucose, L-glutamine, pyruvate and 10% heat-inactivated fetal bovine serum (FBS), from both ends by using syringe. Cells were collected in to a 30ml screw top universal tube and harvested by centrifuging at 1500 rpm, 5 minutes. Cells were resuspended gently in 10 ml fresh DMEM and gently aspirated to break down the cell aggregates. Cell suspension was adjusted to 10^6cells/ml and seeded into 12 well plates with 10^6cells/well. Cells were incubated 5-7 days at 37^0C under 10% (v/v) CO_2 and were fed every 2-3 days. The medium

was changed on day 6. The bone marrow derived macrophages were activated, using recombinant mouse INF-γ (Invitrogen) at a concentration of 100 units/ml.

9

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Appendix

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Identification of a Glycosyltransferase from *Mycobacterium marinum* Involved in Addition of a Caryophyllose Moiety in Lipooligosaccharides[▽]†

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Deletion of *Mycobacterium marinum MMAR2333* resulted in the loss of three of four subclasses of lipooligosaccharides (LOSs). The mutant was unable to extend an intermediate (LOS-II*) by addition of caryophyllose. These data and the predicted domain structure suggest that MMAR2333 is a glycosyltransferase involved in the generation of a lipid-linked caryophyllose donor.

The cell envelope of members of the genus *Mycobacterium*, including the tubercle bacillus *Mycobacterium tuberculosis*, consists of a characteristic lipid-rich cell wall that constitutes an effective permeability barrier, imparting resistance to many therapeutic agents (3), and contributes to virulence (10). This distinct cell envelope consists of a covalently linked mycolyl-arabinogalactan-peptidoglycan (mAGP) complex, which in turn intercalates with various noncovalently bound complex lipids that vary between different mycobacterial species and within strains of the same species. These include the highly polar lipooligosaccharides (LOSs), which are produced by a number of mycobacteria, including the opportunistic pathogen *Mycobacterium kansasii*, the poikilotherm pathogen *Mycobacterium marinum*, and the *M. tuberculosis* complex strain *Mycobacterium canetti* (4, 11, 16). Studies on LOS-deficient *M. kansasii* strains, *M. marinum* LOS mutants, and purified LOSs also suggest a role in virulence (6, 19, 20). The vast majority of biochemical and genetic information on LOS biosynthesis comes from studies on *M. marinum*, which produces four types of LOSs (4), LOS-I, LOS-II, LOS-III, and LOS-IV, each containing a common acylated glycan core consisting of four glucose residues and one methylated rhamnose (Fig. 1). A D-Xyl*p* (xylopyranase) residue is present in LOS-II, LOS-III, and LOS-IV in addition to the glycan core. LOS-II contains a further caryophyllose sugar [3,6-dideoxy-4-*C*-(D-*altro*-1,3,4,5-tetrahydroxyhexyl)-D-*xylo*-hexopyranose; previously referred to as sugar X] (20), while LOS-III and LOS-IV contain two caryophyllose residues, with LOS-IV containing a novel N-acetylated dideoxy galactose (4,6-dideoxy-Gal*p* replaced by a 3-hydroxy-3-methylated-pyrrolidone cycle; previously referred to as the "YZ" component) (21). Whether LOSs from other mycobacteria such as *M. canetti* also contain caryophyllose, the YZ sugar, or related components remains to be determined. Additionally, Rombouts et al. (20) identified further subclasses of LOS-II, LOS-III, and LOS-IV in *M. marinum* which contained a hydroxylated equivalent of caryophyllose. Transposon mutagenesis screens and studies on a natural strain with altered LOS patterns have revealed a genetic locus associated with LOS biosynthesis (4, 19, 20). Given the sugar-rich composition of LOSs, numerous glycosyltransferases potentially play a role in LOS biosynthesis pathways. Until now, *losA* (*MMAR2313*) was the only glycosyltransferase-encoding gene shown to be involved in LOS biosynthesis in *M. marinum*; a *losA* transposon mutant failed to synthesize LOS-IV, suggesting that LosA was likely involved in the transfer of the terminal N-acetylated dideoxy galactose residue to LOS-III, resulting in the formation of LOS-IV (4). Also present in this gene cluster is *MMAR2333*, encoding a putative glycosyltransferase. Initially annotated as *wcaA* (a glycosyltransferase gene involved in the biosynthesis of the exopolysaccharide colanic acid in enteric bacteria [23]), the encoded protein is predicted to contain two transmembrane domains located near the C terminus (Fig. 2), suggesting that the enzyme is membrane anchored, an attribute of many mycobacterial glycosyltransferases involved in the biosynthesis of cell wall-associated glycolipids and carbohydrate polymers. Additionally, the MMAR2333 sequence revealed domains characteristic of eukaryotic dolichol phosphate mannose (DPM) synthases (Fig. 2), members of the GT-2 family of glycosyltransferases that catalyze the transfer of sugars to dolichol phosphate by using nucleotide sugars as substrates. In bacteria, homologues of DPM synthases use polyprenol phosphate rather than dolichol phosphate, and one such enzyme from *M. tuberculosis* is Ppm1, which catalyzes the generation of polyprenol monophosphomannose from GDP-mannose and polyprenolphosphate for subsequent use as substrates for biosynthesis of lipomannan and lipoarabinomannan (15). Surprisingly, the best matches obtained from a BLAST

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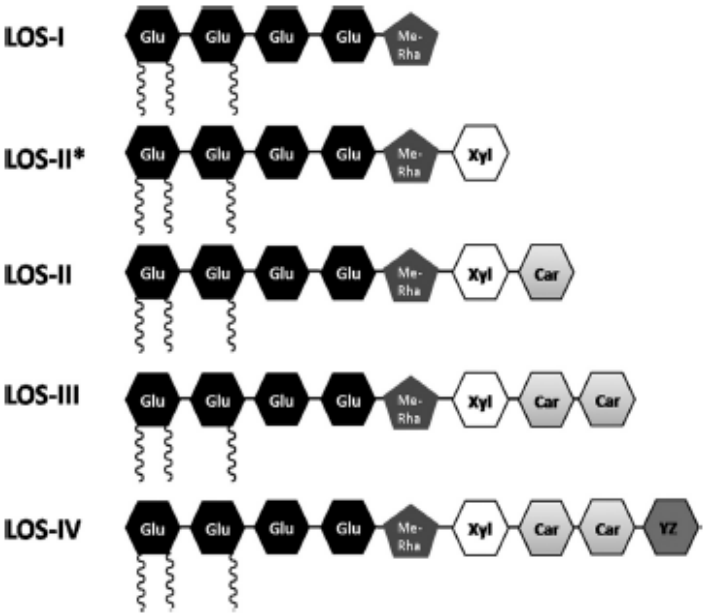

FIG. 1. Schematic representation of four subclasses of LOSs and one LOS intermediate (LOS-II*) produced by *M. marinum*. Glu, glucose; Me-Rha, methyl rhamnose; Xyl, xylose; Car, caryophyllose; YZ, pyrrolidone-substituted dideoxy galactose. Acyl chains are shown as black coils.

search using the MMAR2333 amino acid sequence as the query were putative glycosyltransferases from cyanobacteria, which showed a higher homology to MMAR2333 than glycosyltransferases from other LOS-producing mycobacteria (the best match is shown in Fig. 2). Given the proximity of *MMAR2333* to genes involved in LOS biosynthesis and its similarity to DPM synthases, it was likely that the *MMAR2333*-encoded glycosyltransferase was involved in the generation of a lipid-bound sugar moiety, utilized subsequently as a donor for the addition of one or more sugar residues found in *M. marinum* LOSs.

Generation of an *M. marinum MMAR2333* null mutant. To determine whether *MMAR2333* played a role in LOS biosynthesis in *M. marinum*, we first generated an allelic exchange plasmid, p*ΔMMAR2333*, which consisted of PCR-amplified flanks upstream and downstream of *MMAR2333* cloned on either side of a hygromycin resistance cassette (*hyg*) in the vector p004S (17). p*ΔMMAR2333* was then packaged in the temperature-sensitive mycobacteriophage phAE159 to generate a recombinant phage, ph*ΔMMAR2333*, designed for replacement of *MMAR2333* with *hyg* by using specialized transduction, a highly efficient phage delivery-based knockout method for mycobacteria (2). Hygromycin-resistant (75-μg/ml) colonies obtained after transduction of *M. marinum* 1218R (ATCC 927; referred to as the wild type) using protocols described by Larsen et al. (17) were confirmed by Southern blot analysis, and one such strain, the *ΔMMAR2333* mutant, was used for further analysis. The mutant strain exhibited an altered colony morphology with a slightly "rough" appearance (Fig. 3), suggesting potential alterations in the cell wall. The colony morphology was restored to that of the parental type upon introduction of a plasmid-borne copy of *MMAR2333* into *ΔMMAR2333*, indicating that the phenotype observed in the mutant was due solely to the loss of *MMAR2333* function (Fig. 3; *ΔMMAR2333*-C contains a cloned copy of *MMAR2333* in the mycobacterial replicative plasmid pMV261 [24] delivered by electroporation). This study also demonstrated the utility of specialized transduction to generate null mutants of *M. marinum*. While phage delivery methods have been used to generate transposon mutants in *M. marinum* (1, 19), this is the first report of the use of phages for delivering allelic exchange substrates for targeted gene knockouts in this species. This tool should facilitate the generation of targeted mutants in *M. marinum*, which has relied so far on laborious two-step selection methods based on *sacB* counterselection (8, 14, 18).

Altered LOS profiles in the *ΔMMAR2333* mutant. To assess the effects of the loss of *MMAR2333* function on cell wall lipid composition, cultures of wild-type, mutant, and complemented strains grown at 30°C in 7H10 broth (Middlebrook 7H10 minus agar) were first pulsed with 1 μCi/ml [^{14}C]acetate (57 mCi/mmol) to label lipids. Labeled polar and apolar lipids were extracted and analyzed by two-dimensional thin-layer chromatography (2D-TLC) using five solvent systems (A to E) as described by Dobson et al. (12). Differences in the lipid profiles of the wild-type and *ΔMMAR2333* strains were visible only in TLCs run in solvent system E, which is designed to separate phospholipids and LOSs (Fig. 4). While LOS-I was present in the *ΔMMAR2333* mutant, LOS-II, LOS-III, and LOS-IV were missing and the strain accumulated instead a ^{14}C-labeled species that migrated to a position between that of LOS-I and LOS-II (Fig. 4). Complete LOS biosynthesis was restored in the complemented strain, indicating that the changes observed in the mutant were due solely to the loss of *MMAR2333*. Staining of solvent system E TLC plates of lipids extracted

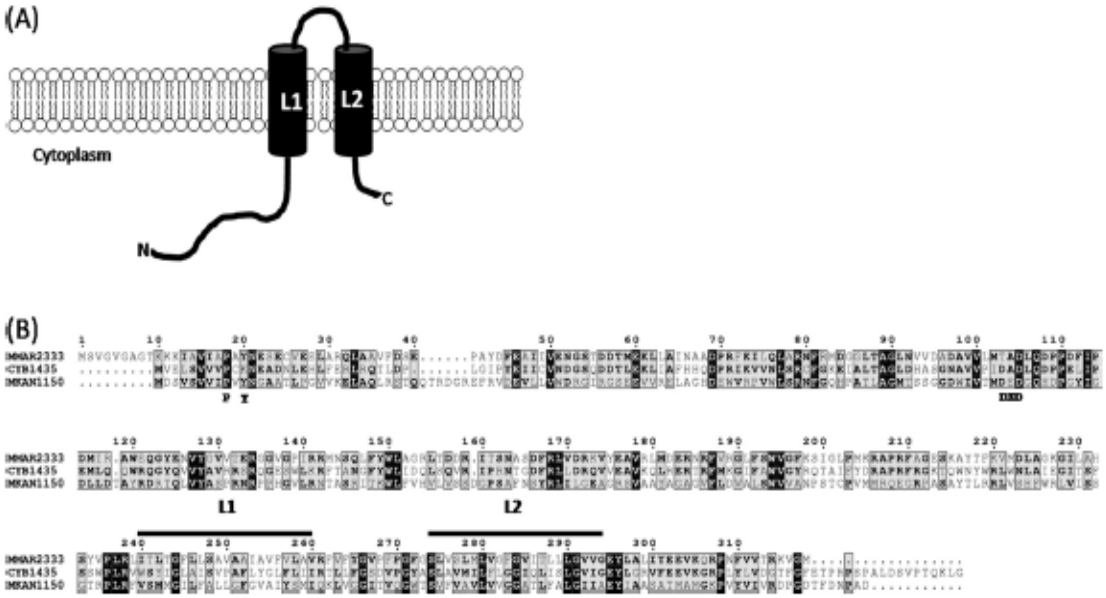

FIG. 2. (A) Predicted topology of MMAR2333. The predicted transmembrane domains are denoted by L1 and L2. N, N terminus; C, C terminus. (B) Alignment of the MMAR2333 amino acid sequence with that from the putative *Synechococcus* sp. glycosyltransferase (CYB1435) and the *Mycobacterium kansasii* homologue MKAN1150. Characteristic sugar binding residues are indicated below the alignment. The sequences spanning the transmembrane domains of MMAR2333, depicted as L1 and L2 in panel A, are indicated by bars above the sequences. Numbers are amino acid coordinates of MMAR2333. Black boxes show identity for all three proteins. Grey boxes indicate similar or identical residues for two of the three proteins; bold type indicates identical or similar residues.

from the mutant strain with α-naphthol revealed that the new accumulating lipid species was a glycolipid (data not shown). As the appearance of this glycolipid was paralleled by the disappearance of LOS-II, -III, and -IV, it was quite likely that the accumulated glycolipid was a LOS intermediate, and we thus termed this unidentified lipid LOS-U (LOS-unknown).

Characterization of LOS-U. Another LOS biosynthesis intermediate, LOS-II*, was also reported to migrate to an intermediate position between LOS-I and LOS-II on 2D-TLC plates (19). Isolated from *M. marinum* MRS1178, a transposon mutant of *MMAR2332*, LOS-II* is a precursor of LOS-II and contains D-Xyl*p* attached to the glycan core but lacks the caryophyllose found in LOS-II. When mixed samples of ^{14}C-labeled lipids from the MRS1178 and Δ*MMAR2333* strains were separated on the same 2D-TLC plate, LOS-II* and LOS-U migrated to the same position, appearing as one spot (data not shown), suggesting that LOS-U and LOS-II* were likely the same glycolipid species. In order to ascertain this by determining the chemical nature of LOS-U, we first purified LOS-U using a combination of column chromatography and preparative TLC, and per-O-methylated LOS-U was analyzed by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) as described earlier for other LOS subclasses (4). A prominent signal was obtained at *m/z* ~1,219.9 $[M + Na]^+$ (Fig. 5), which was within the same range (*m/z* 1,219 ± 1) as that obtained for LOS-II* (*m/z* 1,219.4) (19). These data indicated that LOS-U, the intermediate we isolated from the Δ*MMAR2333* strain, was the LOS-II precursor LOS-II*, and like the *MMAR2332* transposon mutant, the *MMAR2333* deletion mutant also accumulated LOS-II*. In other words, the addition of the first caryophyllose residue to the D-Xyl*p* glycan core did not occur in the Δ*MMAR2333* mutant. Given the similarity of MMAR2333 to DPM-like glycosyltransferases, these results suggest that MMAR2333 was likely involved in the cytoplasmic transfer of a nucleotide-bound caryophyllose residue (or its precursor) to a polyprenol phosphate or other lipid-based unit for subsequent use as a sugar donor by another glycosyltransferase to extend LOS-II* to LOS-II. Additionally, the identical LOS patterns of the *MMAR2332* transposon mutant and the Δ*MMAR2333* strain on 2D-TLC plates made it likely that *MMAR2332*, which encodes a putative protein homologous to thiamine pyrophosphate (TPP)-requiring carboxylases, was involved in the biosynthesis of the unique caryophyllose sugar.

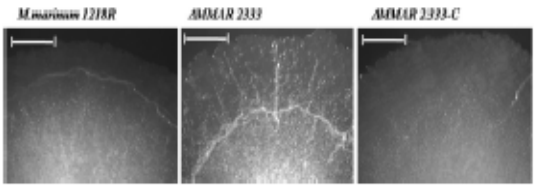

FIG. 3. Effect of deletion of *MMAR2333* on colony morphology. Colonies of *M. marinum* wild-type (1218R), mutant (Δ*MMAR2333*), and complemented (Δ*MMAR2333*-C) strains on 7H10 agar plates. Bar, 1 mm.

Effects of *MMAR2333* deletion on virulence. Transposon-mediated disruption of *MMAR2332*, which also led to the ac-

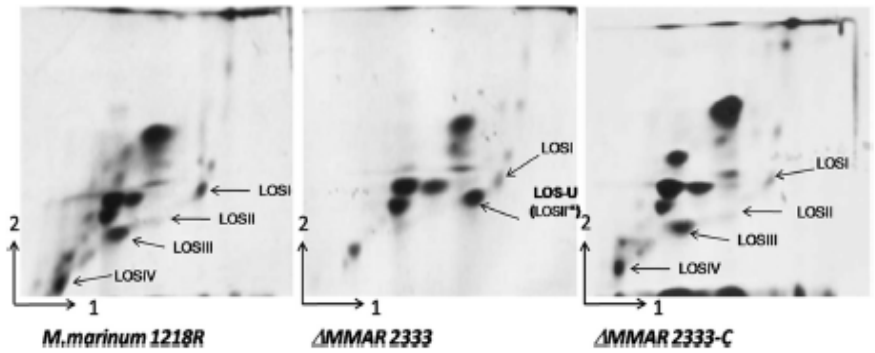

FIG. 4. 2D-TLC analysis of ^{14}C-labeled polar lipids from *M. marinum* strains. Polar lipids were extracted from wild-type (1218R), mutant (Δ*MMAR2333*), and complemented (Δ*MMAR2333*-C) strains, separated using solvent system E (direction 1, $CHCl_3$:CH_3OH:H_2O [60:30:6]; direction 2, $CHCl_3$:CH_3COOH:CH_3OH:H_2O [40:25:3:6]), and visualized on X-ray films by autoradiography. LOSI, LOS-I.

cumulation of LOS-II*, did not alter the ability to survive inside cultured macrophages (19). Similarly, we did not observe any differences in the ability of the Δ*MMAR2333* strain to survive in murine bone marrow-derived macrophages (data not shown). Additionally, we did not observe any differences in bacterial loads between zebrafish embryos infected with wild-type and mutant strains (see the supplemental material), suggesting that the loss of LOS-II, LOS-III, and LOS-IV and the parallel accumulation of LOS-II* in the mutant strain did not affect the survival of the mutant strain in the above-described models of infection.

Later stages of LOS biosynthesis may occur in an extracytoplasmic environment. The similarity of MMAR2333 to bacterial DPM-like synthases suggested that the glycosyltransferase was not directly involved in the transfer of caryophyllose to LOS-II* but instead was likely to catalyze the transfer of nucleotide-bound caryophyllose to a lipid (polyprenol) carrier. Alternately, MMAR2333 could catalyze the formation of a polyprenol-bound precursor of caryophyllose, which is subsequently modified to caryophyllose. Efforts to confirm this by *in vitro* enzyme assays are currently limited by the unavailability of nucleotide-bound caryophyllose substrates. In addition to *MMAR2333*, two other genes, *MMAR2311* and *MMAR2313* (*losA*), also encode DPM-like glycosyltransferases. The presence of this class of glycosyltransferases in the LOS biosynthesis cluster suggests that some glycosyltransferases may be involved in the generation of lipid-bound sugar substrates which are "flipped" to the extracytoplasmic side of the membrane to be subsequently used by extracytoplasmic glycosyltransferases to extend the oligosaccharide moiety of LOSs. Indeed, the *losA* mutant is devoid of LOS-IV, suggesting that LosA could likely be involved in the generation of a lipid-bound YZ sugar which is subsequently transferred by an extracellular glycosyltransferase to LOS-III. This affords a model for LOS biosynthesis wherein acylated hexasaccharide comprising LOS-II* is synthesized intracellularly and transported across the membrane.

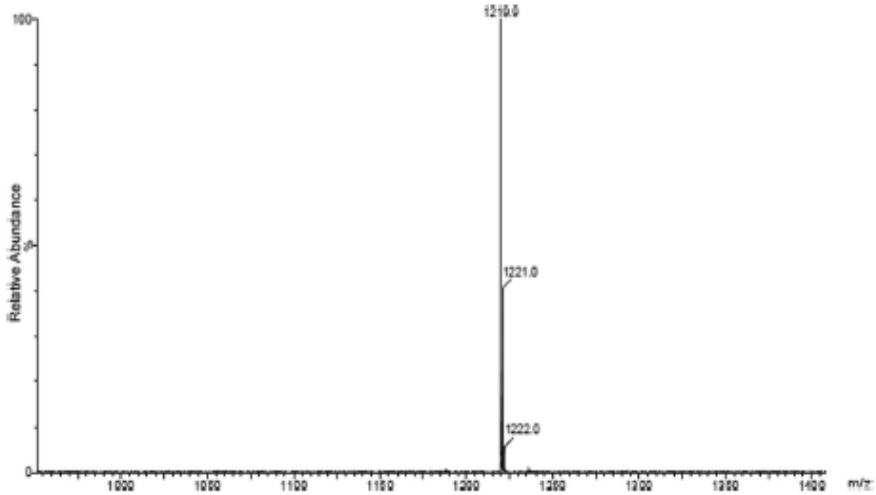

FIG. 5. Estimation of the molecular size of LOS-U by MALDI-MS analysis of per-O-methylated LOS-U. The sample was prepared and processed as described by Ren et al. (19).

This process could be initiated by *MMAR2342*, which encodes a transmembrane protein belonging to a group of larger mycobacterial proteins, termed MmpL proteins, which are involved in the transport of mycobacterial glycolipids or their intermediates (5, 7, 9, 13, 22). On the extracytoplasmic side, LOS-II* would then be extended by specific glycosyltransferases that use lipid-bound sugars (caryophyllose or the YZ sugar) as sugar donors to yield LOS-II, LOS-III, and LOS-IV. In an alternative model, LOS-I could be transported by the MmpL protein, with xylose being the first sugar added on the extracytoplasmic side, a process that would require the generation of a lipid-bound xylose substrate. MMAR2311, the third putative DPM-like glycosyltransferase in the LOS cluster, is a potential candidate for this function. In summary, later stages of LOS biosynthesis may involve a distinct set of glycosyltransferases that catalyzes the formation of lipid-bound sugar donors and another set that extends the LOSs. The generation of mutants of *MMAR2311* and other putative glycosyltransferase genes in the LOS cluster will shed more light on the biosynthesis of these carbohydrate-rich mycobacterial glycolipids.

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