DISSERTATION

CHARACTERIZATION OF THE MANNOSYLTRANSFERASE RV3779, WHICH FORMS POLYPRENYL PHOSPHOMANNOSE FOR THE BIOSYNTHESIS OF PHOSPHATIDYLINOSITOL MANNOSIDE AND LIPOARABINOMANNAN, AND ELUCIDATION OF THE POLYMERIZATION STAGES OF GALACTAN BIOSYNTHESIS IN MYCOBACTERIA

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY HATAICHANOK SCHERMAN ENTITLED CHARACTERIZATION OF THE MANNOSYLTRANSFERASE RV3779, WHICH FORMS POLYPRENYL PHOSPHOMANNOSE FOR THE BIOSYNTHESIS OF PHOSPHATIDYLINOSITOL MANNOSIDE AND LIPOARABINOMANNAN, AND ELUCIDATION OF THE POLYMERIZATION STAGES OF GALACTAN BIOSYNTHESIS IN MYCOBACTERIA BE ACCEPTED AS FULFILLING IN PART THE REQURIEMENTS FOR THE DEGREE OF DOCTOR OF PHILOSPOHY

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ABSTRACT OF DISSERTATION

CHARACTERIZATION OF THE MANNOSYLTRANSFERASE RV3779, WHICH FORMS POLYPRENYL PHOSPHOMANNOSE FOR THE BIOSYNTHESIS OF PHOSPHATIDYLINOSITOL MANNOSIDE AND LIPOARABINOMANNAN, AND ELUCIDATION OF THE POLYMERIZATION STAGES OF GALACTAN BIOSYNTHESIS IN MYCOBACTERIA

The cell wall complex of mycobacteria, dominated by highly unique structural polysaccharides and a rather impermeable layer of lipids, can give these bacteria the ability to resist the immune response and cause a prolonged and deadly illness. Various lipoglycans and glycolipids in the cell wall complex of mycobacteria, such as lipoarabinomannan (LAM), as well as the synthetic precursors of LAM; lipomannan, and the phosphatidyl *myo*-inositol mannosides, are essential for the normal growth and viability of mycobacteria, although the specifics of their synthesis are still not fully defined. Their synthesis are governed by a myriad of enzymes, including a class of enzymes called glycosyltransferases, which are one of the most diverse and important groups of enzymes in nature. A particular glycosyltransferase, Rv3779, is the primary focus of this study, and from sequence and bioinformatic analysis, we identified it as a putative mannosyltransferase belonging to the GT-C superfamily found to be involved in various aspects of synthesis of the higher forms of the phosphatidyl *myo*-inositol mannosides and the subsequent lipoglycans. Rv3779 is present in a prominent gene cluster involved in cell wall biosynthesis. From the experimental evidence gathered from

construction and analysis of a *Rv3779* knockout mutant of *M. tuberculosis*, the *in vitro* assay of the membrane fractions of an Rv3779-overexpressed strain of *M. smegmatis*, and analysis of the reaction products by thin layer chromatography and mass spectrometry, we have concluded that Rv3779 possesses polyprenyl phosphomannose synthase activity in a homologous manner to Ppm1. Polyprenyl phosphomannose is the key mannosyl donor that is utilized heavily by later enzymes in the extracytoplasmic hypermannosylation of the higher forms of these lipoglycans.

The galactan chain serves as the covalent attachment point for mycolated arabinan and is a central structural polymer in the cell wall complex that is the second focus of this study. The exact stages of the buildup galactan are not fully elucidated, and involve unique polymerization steps. We have utilized various organic extraction and analytical techniques from a cell-free assay utilizing UDP-D-galactofuranose as a donor and the results of these studies suggest that the galactan chain is polymerized one galactofuranosyl residue at a time.

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DEDICATION

I dedicate this dissertation to my husband Michael S. Scherman, my parents Mr. Songsak and Mrs. Noawarat Saringcarinkul and my parents in-law Mr. Francis J. and Mrs. Glenda R. Scherman for their continuing support and love. They have given me such tremendous inspiration and confidence to complete this degree.

Hataichanok Scherman

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ABBREVIATIONS

Ac	acylated (Ac_n where n is number of acyl chains)
AG	arabinogalactan
AIDS	acquired immune deficiency syndrome
Ara*f*	D-arabinofuranose
AraLAM	uncapped lipoarabinomannan
BCG	bacille de Calmette et Guérin
CD14	cluster of differentiation 14 (cell surface marker protein)
CDC	Centers for Disease Control and Prevention
CF	cord-factor
CR	compliment receptor
CpG	cytosine-phosphate-guanine (DNA motif)
DAG	diacylglycerol
DAP	diaminopimelic acid
DC-SIGN	dendritic cell-specific intracellular grabbing nonintegrin
DNA	deoxyribonucleic acid
DOTS	directly observed therapy shortcourse
DPA	decaprenyl-monophosphoryl-D-arabinose (C_{50}-P-Ara*f*)
DPM	decaprenyl-monophosphoryl-D-mannose (C_{50}-P-Man*p*)
dsRNA	double stranded ribonucleic acid
EMB	ethambutol

f	furanose
Gal*f*	D-galactofuranose
Gal*p*	D-galactopyranose
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
GI	gastrointestinal
GDP	guanidine diphosphate
GlcNAc	*N*-acetyl-glucosamine (2-acetamido-2-deoxy-α-D-glucopyranose)
GPL or GL	glycolipid
GT	glycosyltransferase
HAART	highly active antiretroviral therapy
HIV	human immunodeficiency virus
IL-12	interleukin-12
INH	isoniazid
LAM	lipoarabinomannan
LM	lipomannan
LPS	lipopolysaccharide (endotoxin)
MAC	*Mycobacterium avium-intracellular* complex
mAGP	mycolyl-arabinogalactan-peptidoglycan
MALDI-TOF	matrix assisted laser desorption ionization - time of flight
MAME	mycolic acid methyl esters
Man*p*	D-mannopyranose
ManLAM	mannose-capped lipoarabinomannan

ManT	mannosyltransferase
MDR-TB	multi-drug resistant tuberculosis
MDT	multidrug therapy
MGLP	methylglucosyl-containing lipopolysaccharide
MR	mannose receptor
MS	mass spectrometry
MurNAc	*N*-acetylmuramic acid
MurNGlyc	*N*-glycolylmuramic acid
NTM	nontuberculosis mycobacteria
p	pyranose
PAMP	pathogen associated molecular patterns
PCR	polymerase chain reaction
PPD	purified protein derivative
PI	phosphatidyl-*myo*-inositol
PIG	phosphatidylinositol glycan
PILAM	phosphatidyl-*myo*-inositol capped lipoarabinomannan
PIM	phosphatidyl-*myo*-inositol mannoside
PG	peptidoglycan
PMPS	polymethylated polysaccharides
Pol-P	polyprenyl phosphate
PPM	polyprenyl phosphomannose
PRR	pattern recognition receptor
Rha	rhamnose

RIF	rifampin (rifampicin)
SP-A	human surfactant protein A
SRLs	supranational reference laboratories
SL	sulfatides
sSNP	synonymous single-nucleotide polymorphisms
t-	terminal
TB	tuberculosis
TLC	thin layer chromatography
TLR-2	Toll-like 2 receptor
Th1	helper T-cell 1
TNF-α	tumor necrosis factor-α
UDP	uridine-5'-diphosphate
WHO	World Health Organization
XDR-TB	extensively drug resistant tuberculosis

CHAPTER 1

ASPECTS OF MYCOBACTERIAL DISEASE

1.1 THE MYCOBACTERIA

Members of the genus *Mycobacterium* are in the family Mycobacteriaceae of the order Actinomycetales, and are acid-fast, weakly gram-positive, aerobic, rod-shaped bacilli that posses an unique cell wall complex that allows them to survive in hostile environments such as that of the host macrophage. The pathogenic mycobacteria, *Mycobacterium tuberculosis* in particular, are intracellular parasites able to replicate within the macrophage and in host tissues despite the presence of a functioning immune system. *M. tuberculosis* bacilli are rod shaped bacteria with rounded extremities about 2-5 μm long and 0.2-0.3 μm thick.

The genome of *M. tuberculosis* spans approximately 4.4 x 10^6 base pairs in size with around 3,900 open reading frames and contains high guanine-cytosine (GC) content of 65.6% (Ryan, 1992; Cole *et al.*, 1998). Of the more than 70 species of mycobacteria, more than 30 have been isolated from humans, including *M. tuberculosis* (the causative agent of tuberculosis, or TB), *M. avium-intracellulare*, *M. kansasii*, *M. fortuitium*, *M.*

chelonae, and *M. leprae*. Some of these human pathogens are slow growing, such as *M. tuberculosis*, *M. avium-intracellulare*, *M. bovis*, *M. kansasii*, *M. ulcerans*, and *M. marinum*. Typically, slow-growing mycobacteria require 3-8 weeks for growing on specialized media, with mean generation times of 18-24 hours in the case of *M. tuberculosis*, or over 14 days for *M. leprae*. This contrasts markedly with the 3 days growth time of fast growing species such as *M. smegmatis*, *M. fortuitium* and *M. chelonae* (Ryan, 1992). As such, *M. smegmatis* has a mean generation time of only 2-3 hours. Aside from the generally slow growth rate of the bacteria is the fact that they contain a distinguishable cell wall complex.

Various glycolipids, lipoglycans, glycoproteins, and other factors are suspended in the outer portion of the mycobacterial cell wall complex that is hallmarked by an outer layer of mycolic acids covalently attached to the cellular peptidoglycan via a unique macromolecule primarily consisting of the glycosyl residues of D-galactofuransose (D-Gal*f*) and D-arabinofuranose (D-Ara*f*) in a structure based on a model originally proposed by Minnikin (Minnikin, 1982). The layer containing the mycolic acids has not been visualized very well by most methods but lately it was found by cryo-electron tomography to be an asymmetrical bilayer some 8 nanometers thick consisting primarily of the mycolic acids, and to a lesser degree, various extractable lipids and outer membrane associated proteins (Hoffmann *et al.*, 2008).

Glycolipids are carbohydrate attached lipids that usually serve as energy sources and/or markers for cellular recognition. Lipoglycans are also glycosylated lipids, however, their degree of glycosylation is much higher, and are typically comprised of

large polysaccharide chains. These are usually structural polymers or immunomodulatory molecules. Mycobacteria therefore posses various specialized glycolipids and lipoglycans that are involved in the modulation of the immune response and pathogenesis. These include the phosphatidyl-*myo*-inositol mannosides (PIMs), lipomannan (LM), and lipoarabinomannan (LAM). In addition to the presence of D-arabinofuranose (D-Ara*f*) in LAM in particular, D-mannopyranose (D-Man*p*) is a major constituent of all of these related glycolipids and lipoglycans. Their synthesis and the identification of the enzymes involved have therefore been the target of much research.

1.2 MYCOBACTERIA THROUGHOUT HISTORY

The genus *Mycobacterium* is believed to herald from the Jurassic period more than 150 million years ago (Hayman, 1984). However, *M. tuberculosis* may have been present in Africa as early as 3 million years ago (Gutierrez *et al.,* 2005). Like the other actimomycetes, the mycobacteria were initially soil dwellers and some species apparently evolved to live in mammals. The domestication of cattle, occurring between 10,000 and 25,000 years ago, would have allowed the passage of a mycobacterial pathogen from domesticated livestock to humans, and in this adaptation to a new host, the bacterium would have evolved to the closely related *M. tuberculosis* (Smith, 2003). Specifically, it had been hypothesized that *M. bovis*, which causes TB in cattle, was the hypothetical evolutionary precursor of *M. tuberculosis,* although modern phylogenetic analysis has shown this not to be the case (Stead, 1997). The genomes of *M. tuberculosis, M. africanum*, *M. microti*, *M. canetti*, and *M. bovis*, have a greater than 99.9% DNA sequence similarity (Brosch *et al.,* 2002), but the existence of synonymous single-

nucleotide polymorphisms (sSNP) allows discrimination between these closely related bacteria, and subsequent sSNP analyses suggest that *M. bovis* evolved about the same time as *M. tuberculosis* (Sreevatsan *et al.*, 1997). Moreover, the distribution of deletions and insertions in the genomes of these mycobacteria species strongly suggests the independent evolution of both *M. tuberculosis* and *M. bovis* from another precursor species, possibly related to *M. canetti* (Brosch *et al.,* 2002; Smith, 2003). Thus, the modern members of mycobacteria, including current strains *M. tuberculosis*, *M. africanum*, *M. bovis*, *M. microti*, and *M. canettii* appear to have diverged from a common ancestor some 15,000-35,000 years ago, and based on phylogenic analysis, the diversity seen in current strains thereby occurred some 200-1,000 years ago (Kapur *et al.*, 1994; Brosch *et al.,* 2002; Hirsh *et al.,* 2004; Gutierrez *et al.,* 2005).

Actual TB disease was documented in Egypt over 5,400 years ago in Egyptian art and effects of this disease are even evident in Egyptian mummies, typically as spinal column abnormalities (Morse *et al.,* 1964; Zimmerman, 1979; Crubezy *et al.,* 1998). Therefore, hard tissues like bone can be preserved for thousands of years, allowing the almost certain identification of individuals with bone TB. The frequency of unearthed mummies and skeletons with apparent tubercular deformities in Egypt suggests that TB was common among that population. The discovery of similar skeletal deformities in various Neolithic sites in Denmark, Italy, and countries in the Middle East also indicates that TB was found throughout the world well over 4,000 years ago. Likewise, the Greeks chronicled TB disease 2,500 years ago, particularly in the Aphorisms of Hippocrates as the most widespread disease of that time. It is likewise mentioned in the Old Testament of the Bible with the Hebrew word "schachepheth", meaning a wasting disease (Daniel,

1997; Daniel and Daniel, 1999; Daniel, 2006). Assyrian clay tablets describe patients coughing blood in the seventh century B.C., and Hippocrates wrote of patients with consumption (the Greek term is "phthisis") in the fifth century B.C. as that of wasting away and likewise coughing up blood in the sputum. He thought the disease was largely inherited, while Aristotle in the 4th century B.C. stressed its contagious nature, as did Galen, the greatest of the Roman physicians, in the 2nd century A.D. (Smith, 2003). In the second half of the 17th century, Italian physicians still maintained that TB was contagious, while doctors and savants in northern countries favored constitutional or hereditary causes of this disease. Such ideas were likely used for political means as well, such as a basis for arguments to bring about new social policy (Smith, 2003). The continual spread of TB throughout Europe and Asia was likely facilitated by the migration of Indo-European cattle herders who were carrying it by virtue of their exposure to cattle infected with the tubercle bacillus (Haas and Hass, 1996).

The rapid growth of large urban centers in the latter half of the second millennium became the epicenter for many TB epidemics starting in the 16th and 17th centuries. TB disease peaked in Europe in the first half of the 19th century, and it is estimated that one-quarter of all Europeans died of TB (Dubos and Dubos, 1952). Improved sanitation, brought about by the urban renewal of Paris in the 1850s, and initiated and directed by Baron Georges Haussmann, caused a significant decline in TB mortality (Chaudun, 2000). European immigrants to the New World brought TB with them, and while the mortality rate never reached the levels found in Europe, large urban centers like Boston and New York experienced TB death rates of 6 to 7 per 1,000 in

1800, however declining to 4 per 1,000 in 1860 to 1870 (Daniel *et al.*, 1994; Smith, 2003).

The cause of infectious disease, including that of TB was beginning to be elucidated by the mid 19th century. In 1865, the French military physician Jean-Antoine Villemin reported that TB could be given to laboratory rabbits by inoculating them with tuberculosis infected tissue from a cadaver. This finding was heavily criticized as being "primitive" by the French medical establishment, notably by Herman Pidoux, who strongly asserted that there had to be more social solutions to the problem of TB, which he and others felt arose in the poorer (working) classes from external causes like mal-nutrition, poor sanitation, and overwork (Smith, 2003). However, Robert Koch just 17 years later conclusively showed that TB was actually caused by a bacterium, thereby discrediting many of Pidoux's arguments (Koch, 1882). In a classic experiment, Edward Trudeau showed that TB could be induced in rabbits with a purified culture of virulent *M. tuberculosis,* but that the environmental conditions in which the animals were maintained greatly influenced the course of the disease (Trudeau, 1887). This observation gave some degree of scientific validity to the treatment of TB with fresh air and good nutrition, and became the basis of the TB sanitarium movement started by European physicians in the mid-1800s. Trudeau likewise implemented such regimes in his TB treatment center that opened in 1884 at Saranac Lake, New York. It was the antibiotic era, beginning with the discovery of streptomycin by Schatz and Waksman in the 1940s, the development and wide-spread use of the *M. bovis* BCG vaccine, along with improved public health practices where a remarkable decline in TB cases in the developed world in the 20th

century was observed. However, that trend has now reversed, yet again, in the 21st century.

1.3 EPIDEMIOLOGY OF TB DISEASE

The increase in incidence of tuberculosis, and rapid emergence of multidrug-resistant TB (MDR-TB) in the worldwide arena, is of particular concern as the disease is infecting greater numbers of people in susceptible populations. The recent emergence of extensively drug resistant (XDR-TB) has further exacerbated the threat this disease poses, not only to susceptible populations, but to healthy individuals as well. Worldwide, one in three people is latently infected with TB and approximately 10% of those infected will have active TB at some point during their lives. Thanks to the improved living conditions, sanitation, nutrition, as well as advances in treatment and medicine, the TB threat was largely under control in many nations for much of the twentieth century. Moreover, the adoption of directly observed therapy shortcourse (DOTS) as an international protocol for the handling of infectious diseases (TB in particular), has shown up to an 80% cure rate for TB patients. DOTS therapy has five core elements and successful implementation greatly reduces the appearance of resistant strains, but requires strict patient monitoring and compliance, making this highly difficult in struggling and poorer nations (WHO Report, 2007). The era of antibiotics heralded the supposed end to the scourge of TB and public confidence soared. Sick individuals felt better during treatment, typically as soon as one-fourth of the time into their treatment, and many stopped taking the necessary drugs to combat the infection. This eventually caused the

rapid emergence of multidrug-resistant strains of *M. tuberculosis* that has complicated matters for once-effective treatment regimes, allowing for the revival of the TB threat.

Today, the TB threat has grown to where it the leading bacterial cause of death worldwide, claiming well over 3 million victims annually. It is estimated that 8.8 million new cases of TB occur every year, with 98% of those occurring in developing countries. The incidence of TB ranges from less than 10 per 100,000 in North America, to between 100-300 per 100,000 in Asia and Western Russia, and to over 300 per 100,000 in Southern and Central Africa. Twenty-two high burden countries identified account for approximately 80% of all new TB cases arising each year (WHO, June 2007). Furthermore, epidemiologists have estimated that over 1.7 billion people worldwide are infected with *M. tuberculosis* (Enarson and Murray, 1996; Brennan, 1997; Rattan *et al.*, 1998). More than 80% of TB patients are in the economically productive ages of 15-49 years (Rattan *et al.*, 1998).

The tuberculosis threat has manifested itself in most areas of the world, appearing in disparate populations, thereby prompting the WHO to declare tuberculosis a global health emergency in 1993 (Brennan, 1997). Today, cases of XDR-TB have been confirmed worldwide, from Asia, Africa, Europe, South America, and even the United States and Mexico. The WHO has increasingly reiterated that threat, prompting the United Nations Secretary General Kofi Annan to declare "the appearance of new drug resistant strains threatens us with a new pandemic" (WHO, November 2006).

Complicated further by the effects of human immunodeficiency virus (HIV) coinfection and the decline of socioeconomic standards in many industrialized nations,

TB is now threatening populations once thought to be rather invulnerable. The presence of MDR-TB and XDR-TB essentially makes successful treatment of TB nearly impossible in HIV patients. MDR-TB is defined by resistance to at least the two most powerful first-line anti TB drugs, isoniazid (INH) and rifampicin (RIF), and is thought to arise as a consequence of sequential accumulations of spontaneous genetic mutations that can confer resistance to an antibiotic (David, 1971; David and Newman, 1971). Typically a bacillary load of 10^9 bacilli will contain several mutant bacilli resistant to any one antitubercular drug. With the mutation being chromosomal, the probability of MDR becomes multiplicative (Rattan *et al.*, 1998). In 2004 alone, 424,203 cases of MDR-TB were estimated to occur worldwide, amounting to 4.3% of all new and previous TB cases and three countries includes China, India, and the Russian Federation accounted for 261,362 cases of MDR-TB which is 62% of the estimated world total (Zignol *et al.*, 2006). XDR-TB raises the bar further and has been recently defined as resistance to any fluoroquinolone and at least 1 of 3 injectable second-line drugs (capreomycin, kanamycin, and amikacin), in addition to being resistant to at least isoniazid and rifampicin, the two most powerful anti-TB drugs (WHO, March 2006).

The stark reality is that TB is becoming more resistant to traditional chemotherapy and disturbing findings by Pablos-Mendez *et al.* in a study of 35 countries and regions over a decade ago, demonstrated that 9.9% of *M. tuberculosis* strains in patients with no prior treatment were resistant to at least one of the four first-line TB drugs. Specifically, 7.3 % were found to be resistant to INH, 6.5 % to streptomycin, 1.8% to RIF, and 1% to ethambutol (EMB). The prevalence of primary multidrug resistance was 1.4%. Among patients with histories of treatment of one month or less, the

prevalence to resistance to any of the four drugs was 36%, and the prevalence of multidrug resistance was 13%. Overall, prevalence was 12.6% for single drug resistance and 2.2% for multidrug resistance (Pablos-Mendez *et al.*, 1998). Contrast these findings with a recent study by Shah *et al.* in 2007, and the picture dramatically worsens, even over just a decade. This study obtained data from a cluster of 14 Supranational Reference Laboratories (SRLs), which are premier TB laboratories on 6 continents that collaborate with various regional reference laboratories and the WHO International Union Project on Anti-TB Drug Resistance to enhance culture and drug susceptibility testing and quality control (Laszlo *et al.*, 2002; Shah *et al.*, 2007). They found from 13 of the SRLs (the 14th being in the Republic of Korea) that 65.5% of isolates from patients were resistant to one more of the first-line TB drugs, and of those, 58.5% were resistant to at least INH and 41.5% were resistant to at least RIF. Single drug resistance was found in 15.4% of patients with 8.1% resistant to INH and 1.8% resistant to RIF. These results were similar to the findings by the SRL in the Republic of Korea. MDR-TB was found in 29.4% of patient isolates from the 13 SRLs and 10.9% from Korea. Resistance to all four first-line TB drugs was found in 18.6% of patient isolates from the 13 SRLs and 2% from the Republic of Korea SRL. Second-line drug susceptibility was also analyzed, and resistance to aminoglycosides was detected in 8.7% of isolates and to fluoroquinolones was 5.3% (similarly for Korea). From all SRLs, resistance to one or more class of second-line drugs was observed in 43.8% of MDR TB patients. MDR TB patients with resistance to 3 or more classes of second-line TB drugs were classified as XDR TB and 9.9% of MDR TB patients meet this classification (Shah *et al.*, 2007). These findings are parallel to observations by the Global XDR-TB Task Force, as 6.6% of all analyzed

isolates were considered XDR TB, and among these patients, combination resistance patterns included 3.4% with resistance to aminoglycosides, capreomycin, and fluroroquinolones, 3.4% with resistance to aminoglycosides, fluoroquinolones, and thioamides, and 3.8% resistant to fluoroquinolones, thioamides, and para-aminosalicyclic acid. Moreover, 48.1% of all XDR TB isolates were resistant to all 4 first-line drugs, therefore bringing the total to 7 or more drugs that the isolate was resistant to (WHO, February 2007; WHO report, 2007). Hence, MDR-TB has become a major concern, since resistance to RIF and INH require the use of even more toxic drugs, which can be harmful to weakened, elderly, pediatric, or immune-compromised people. XDR-TB has taken that to an all new level that is likely to affect populations once thought more resistant. It would not be an overstatement to say that MDR-TB, in its various forms, is a potential global crisis waiting to happen.

HIV infection can curtail the immune response that is necessary for repression of latent tuberculosis, as the antigen presenting capacity is greatly reduced and $CD4^+$ T cell depletion is more evident. The synergy between HIV and tuberculosis is thought to be the largest single factor for the temporary reemergence of this disease in the United States. Coinfection greatly increases the risk that dormant *M. tuberculosis* will develop into the active disease and extrapulmonary tuberculosis. The first link between tuberculosis and HIV was seen in the United States from a study in New York. It is estimated that the risk of developing tuberculosis in people with coinfection of *M. tuberculosis* and HIV is 8% per year, compared to 8% per lifetime risk for a person with *M. tuberculosis* alone (Hopewell, 1992; Brennan, 1997). The reactivation of TB in HIV-positive TB patients occurs at a rate of 37% within the first 6 months, in marked contrast

to 2-5% in the first 2 years in HIV-negative patients (Daley *et al.*, 1992). The recent deployment of highly active antiretroviral therapy (HAART), particularly in developing nations, has led to improved treatment of acquired immune deficiency syndrome (AIDS) (WHO progress report, April 2007). This treatment is hampered however, by coinfection with tuberculosis, underscoring the need to treat TB in AIDS patients. In some parts of Africa, up to 75% of AIDS patients are coinfected with TB. Clearly, *M. tuberculosis* poses a dangerous threat which must be dealt with in earnest.

1.4 OTHER MYCOBACTERIAL DISEASES

Mycobacterium avium-intracellular complex (MAC) consists of two species of mycobacteria; *M. avium* and *M. intracellulare*. MAC is ubiquitous in distribution and has been isolated from freshwater and saltwater sources worldwide. The common environmental sources of MAC include warm water distribution systems, aerosolized water, and aerosols of debris such as house dust, soil, dander from various farm animals, birds, human skin debris, and cigarette tobacco, among many sources (Reed *et al.*, 2006). Even hospital water supplies have been implicated in the transmission of respiratory MAC (Tobin-D'Angelo *et al.,* 2004). The modes of transmission include inhalation through the respiratory tract and ingestion. There, MAC can invade and cross the mucosal epithelium of the respiratory and gastrointestinal (GI) tract. They subsequently infect the macrophages and monocytes present and spread down into the sub mucosal tissue where they are carried to the local lymph nodes to possibly initiate bacteremia (Griffith, 1998). In immunocompromised hosts, such as patients with AIDS, they are then subsequently spread via hematogenous routes to the liver, spleen, bone marrow, and other

sites. In AIDS patients, infection of the GI or respiratory tract has been associated with an increased risk of developing MAC associated bacteremia (von Reyn *et al.,* 1996).

MAC infection in humans is usually of minor concern, since only a small minority of immunocompetent people develop MAC lung disease. Such MAC infection in HIV negative patients is typically due to underlying lung disease or lymphadenitis in children (Reed *et al.*, 2006). According to the American Thoracic Society (ATS) criteria, diagnosis of lung disease requires pulmonary symptoms with consistent radiographic features, exclusion of other diagnosis (such as tuberculosis), and appropriate microbiological findings (Griffith, *et al.,* 2007 and ATS, 1997). Pulmonary MAC infection has also been associated with the pulmonary infection and bronchiectasis in elderly women without a pre-existing lung disease. Interestingly, this may be a result of voluntary cough suppression (mannerisms such as social modesty or politeness) that results in the stagnation of respiratory tract secretions and allows for the growth of the organisms. This particular type of infection is referred to as Lady Windermere syndrome (Reich and Johnson, 1992).

Disseminated opportunistic pulmonary infections by MAC can occur in HIV infected (and other immunocompromised) patients who have entered the AIDS stage of their illness, usually after latent TB has developed (due mainly to lowering levels of $CD4^+$ cells, at levels below 50 cells per microliter). MAC is the most common cause of infection by mycobacteria other than *M. tuberculosis* in patients with AIDS, and more than 95% of MAC infections in patients with AIDS are caused by *M avium*, while 40% of

infections in immunocompetent patients are caused by *M. intracellulare* (Reed *et al.*, 2006).

Leprosy is an ancient disease that evokes strong emotions stemming from fear and ignorance. A cumulative total of the millions of people who have suffered its chronic course of incurable disfigurement and physical disabilities can never be calculated. Leprosy is a chronic infectious disease spread primarily by aerosolized droplets from the nose and mouth of infected individuals. The disease is caused by *Mycobacterium leprae,* which infect macrophages and Schwann cells in the respiratory tract and then invade the bloodstream, ultimately leading to serious skin lesions and peripheral nerve degeneration. Granulomas frequently result in patients with mycobacterial infections, including leprosy (Janeway *et al.*, 2005). Leprosy mainly affects the skin, the peripheral nerves, mucosa of the upper respiratory tract, and even the eyes.

Leprosy can manifest in two different clinical forms; tuberculoid and lepromatous. Tuberculoid leprosy (also known as paucibacilear Hansen's disease) is the more mild form of the disease, in which proliferation and destruction of the bacteria are to a large extent contained by the cell-mediated immune response. Patients exhibit on average one to five skin macules in the early stages of paucibacilear Hansen's infection which is rarely fatal. Lepromatous leprosy (also known as multibacilear Hansen's disease) however, is more severe as the humoral immune responses predominate and are largely ineffective at containing the infection. *M. leprae* can replicate freely inside macrophages and progress to bring about extensive tissue damage. Patients typically

exhibit numerous skin lesions, nodules, and plaques, a thickened dermis, and congestion of the nasal mucosa (CDC, 2005).

The disease has an average incubation time of 5 years and symptoms can take up to 20 years to appear. Most patients infected with the disease for over fifteen years also show some sort of ocular damage such as cataracts, conjunctivitis, and complications of the lacrimal system to blindness (Wani *et al.*, 2005). Left untreated for long periods of time, leprosy leads to the characteristic and horrific deformities associated with "lepers", including permanent skin, limb, and nerve degradation. When *M. leprae* was discovered by the Norwegian physician Gerhard Henrik Armauer Hansen in 1873, it was the first bacterium to be identified as causing disease in man (WHO, October 2005). Leprosy was renamed "Hansen's disease" in his honor and in part to deflect the growing negative stigma associated with the term "leprosy".

The prevalence of leprosy has dropped worldwide from 5.2 million cases in 1985 to 805,000 in 1995, and to 286,000 cases by the end of 2004. It has been eliminated from 113 out of 122 countries, and over the past 20 years more than 14 million people have been cured. Such a dramatic decrease in the prevalence of this disease is due in part to a resolution passed in 1991 by the World Health Assembly to eliminate leprosy as a public health threat by 2000. This target was achieved and the prevalence rate dropped from 21.1 per 10,000 people to less than one in 10,000, a 90% drop. Effective multidrug therapy (MDT) was the key element outlined by the Assembly to affect this favorable change (WHO, October 2005).

Treatment for leprosy appeared in the late 1940s with the introduction of dapsone, and its derivatives. Leprosy bacilli resistant to dapsone gradually appeared and became widespread. The current treatment strategy for leprosy is an MDT approach. This strategy combines RIF, Dapsone, and Clofazimine, as use of the three drugs in combination dramatically prevents the development of resistance. While monotherapy can result in drug resistance and therefore be a problematic treatment option, there have not been many reports of resistance to multidrug therapy (Ishii, 2003). Three more drugs have recently been shown to have bactericidal effects against *M. leprae*: ofloxacin, minocycline, and clarithromycin. However, due to cost-effectiveness and success using the traditional drugs, the World Health Organization's multidrug therapy recommendations have not changed.

Other infections or diseases caused by mycobacteria include various skin infections associated with *M. chelonae*, *M. abscessus*, *M. fortuitum*, *M marinum*, and *M. ulcerans. M. chelonae* and *M. abscess* are nontuberculosis mycobacteria (NTM) found in the respiratory and digestive tracts of healthy individuals, natural and processed water sources, sewage, and even tap water (Zhibang *et al.* 2002). Various clinical syndromes due to an established *M. chelonae* infection (which typically arise out of trauma or surgical site infection) include localized cutaneous disease, lung disease, joint infections, endocarditis, and ocular diseases (such as keratitis or corneal ulcers) (Hadjiliadis *et al.*, 1999). Dissemination of NTM, usually in the form of skin and soft tissue lesions, almost always occurs in the setting of immunosuppression, and in patients without HIV infection is primarily carried out by cell-mediated immune defects (Hadjiliadis *et al.*, 1999 and Chetchotisakd *et al.*, 2007). Clarithromycin (biaxin) in combination with another select

antibiotic for 4 months is the drug of choice when treating serious *M. chelonae* infections, as it arrests RNA dependent protein synthesis (Chetchotisakd *et al.,* 2007).

The other TB-causing mycobacteria include *M. bovis*, *M. africanum*, and *M. microti*, however, *M. bovis*, *M. africanum* rarely cause disease in immunocompetent people. *M. microti* is not usually pathogenic, but it is possible that the prevalence of *M. microti* infections has been underestimated (Niemann *et al.*, 2000). However, *M. tuberculosis* TB is garnering world attention, as it is among the leading killers of humans, second to heart disease, and the greatest killer of all the infectious bacterial diseases.

1.5 MECHANICS OF TB INFECTION

Tuberculosis has many manifestations, affecting bone, the central nervous system, and many other organs; however, it is primarily a pulmonary disease. The progression of TB can have several outcomes, determined largely by the health and response of the host immune system. The efficacy of this response is affected by intrinsic factors such as the genetics of the host immune system, as well as extrinsic factors, such as the nutritional and physiological state of the host. Moreover, the pathogen itself may dictate the progression of disease as some strains of *M. tuberculosis* are reportedly more virulent than others, and can be defined by increased transmissibility as well as being associated with higher morbidity and mortality in infected individuals (Smith, 2003). *M. tuberculosis*, among the other mycobacteria, utilize their unique cell wall structure in the modulation of the immune response. Such host-pathogen relationships are characterized by the complex interplay between host defense mechanisms and attempts to circumvent them by the pathogen. *M. tuberculosis* targets the alveolar macrophages, where they

invade and multiply inside them despite a harsh environment that is fatal to many other microbes. Here, they persist, despite the presence of a healthy immune system, and are protected from many of the immune mechanisms.

1.5A STAGE 1 OF INFECTION

Stage 1 of a TB infection is initiated by the deposition of *M. tuberculosis* containing aerosol droplets onto lung alveolar surfaces. Tuberculosis infection can be initiated by ingesting, inoculating, or inhaling virulent mycobacteria. When people with active TB cough or sneeze, they can expel aerosols with droplets 0.5 – 5 μm in diameter, and a single sneeze can release up to 40,000 of these droplets (Cole and Cook, 1998). The infectious dose for TB is very low so each droplet alone can possibly transmit the disease, and it actually takes as low as a single bacterium to start an infection (Nicas *et al.,* 2005). The larger aerosol droplets containing *M. tuberculosis* are efficiently excluded from the lower respiratory tract, since most land on the ciliated epithelium of the airways and are then carried up to the trachea be expelled or swallowed (and destroyed by the stomach acids). It seems evident that virulent mycobacteria must reach the alveolar surface to begin infection and if the bacilli are inhibited or killed by the alveolar macrophage that subsequently ingests it, infection is aborted. Otherwise, the ingested bacilli multiply and kill the macrophage, initiating infection (Iademarco and Castro, 2003). Mycobacteria are ingested by alveolar macrophages via conventional receptor-mediated phagocytosis, where cellular pseudopodia move circumferentially around the bacilli and then fuse, thereby leaving the bacilli in a membrane lined vacuole, the phagosome (Piessens and Nardell, 2000). TB bacilli must also overcome the cell-mediated immune response that

detects signals originating from infected cells. An analysis of *M. tuberculosis*-specific responses as compared to *Staphylococcus aureus* and *Escherichia coli* has revealed inhibition of IL-12 production by infected macrophages, suggesting one more way in which this organism survives host defenses (Nau *et al.,* 2002). This indicates that *M. tuberculosis* possesses molecules able to modulate the host immune response.

M. tuberculosis has developed multiple strategies to enhance its entry and ensuing intracellular survival in mononuclear phagocytes and monocytes by engaging a defined set of phagocytic receptors. This has the effect of initiating specific signaling pathways and allows the modulation of several immunobiological processes during and after phagocytosis (Fenton *et al.,* 2005; Kang *et al.,* 2005). *M. tuberculosis* activates the classical complement pathway, in addition to the alternative complement pathway, in human serum to be effectively phagocytized by alveolar macrophages and monocytes (Ferguson *et al.,* 2004). The classical pathway of activation of the complement system is a group of proteins that mediate specific antibody responses and is initiated by antigen bound antibodies binding to the C1 component (Janeway *et al.,* 2005). The alternative pathway of activation of the complement system is a humoral component of the immune response that can operate without antibody participation, and involves opsonization of the pathogen (Sahu and Lambris, 2001). It is initiated by the hydrolysis of C3. Figure 1.5.1 illustrates the general classical and alternative complement pathways of the immune response.

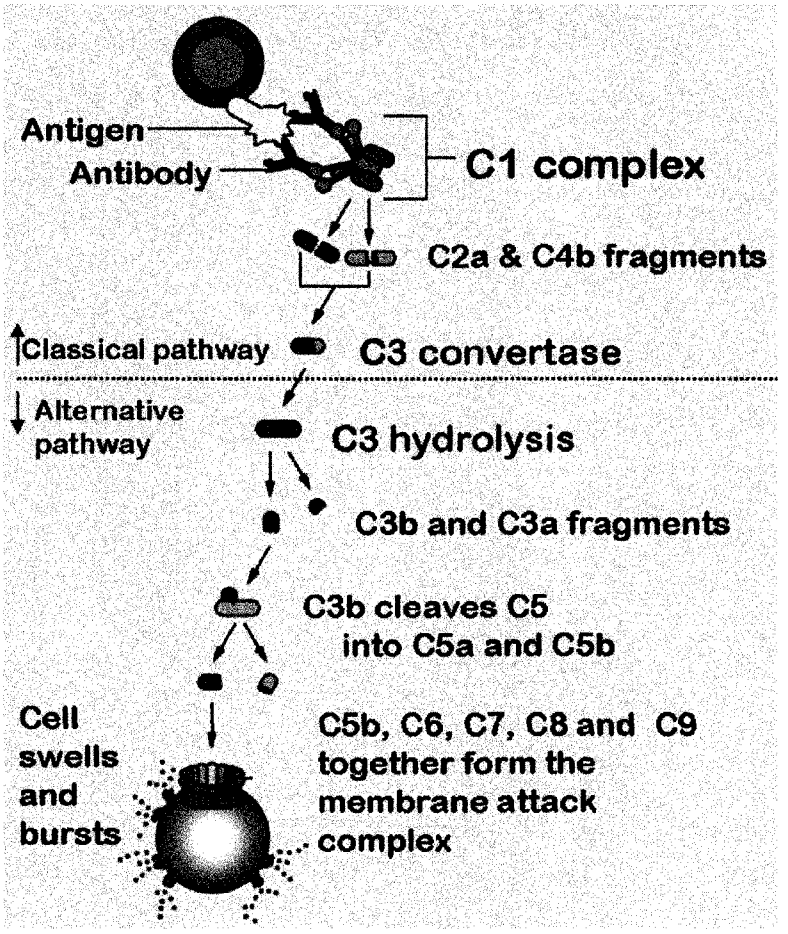

Figure 1.5.1. The classical and alternative compliment pathways. (Figure credit: NIH Publication No. 03-5423, Spetember 4, 2006)

In the process of opsonization, antigens are bound by antibody and/or complement molecules to facilitate phagocytosis, and is the primary method *M. tuberculosis* uses to invade phagocytic cells. Alveolar and other mononuclear phagocytes posses a multitude of receptors involved in phagocytosis of pathogens and particulate matter. If the bacilli are coated with IgG antibody, they can be ingested via Fc receptors on the macrophages, but if no host antibody is present, the receptors that mediate

phagocytosis by monocytes and macrophages in humans are complement receptors CR1, CR3, CR4, and the receptors for mannose (Ernst, 1998; Hu *et al.*, 2000). Additional receptors for *M. tuberculosis* likely exist, but their precise role in the phagocytosis of *M. tuberculosis* is not clear (Ferguson *et al.*, 2004). Complement receptors (CR) bind with complement protein C3 deposited on *M. tuberculosis* when the bacterial surface glycolipid trehalose dimycolate (cord factor) activates the alternate complement pathway, or when pathogenic mycobacteria recruit C2a directly to form a C3 convertase, resulting in increased ligation of CR (Ramanathan *et al.*, 1980; Schorey *et al.*, 1997). Nonopsonic binding of *M. tuberculosis* to CR3 is also important in phagocytosis as well. It seems evident that during the innate immune response, complement opsonization of *M. tuberculosis* plays a very important role during the first encounter of the bacterium (when the initial inoculum of bacteria is low) with the human host, and the survival of the bacilli in the lungs is enhanced by a relatively small increase in phagocytosis by the alveolar macrophages (Ferguson *et al.*, 2004).

Mannose receptors (MR) on macrophages can interact with the terminal mannose residues on mannose capped LAM (ManLAM) molecules, present within and on the cell envelope of *M. tuberculosis*. The terminal mannose caps of *M. tuberculosis* ManLAM, engage the MR during phagocytosis by macrophages and also Dendritic Cell-Specific Intracellular adhesion molecule 3 Grabbing Non-integrin (DC-SIGN) (Geijtenbeek *et al.*, 2003; Maeda *et al.*, 2003; McGreal *et al.*, 2005). DC-SIGN is a C-type lectin receptor present on both macrophages and dendritic cells. It activates $CD4^+$ T cells, and on macrophages, it aids in the recognition of pathogen haptens and binds to mannose-type carbohydrates, a class of pathogen associated molecular patterns (PAMPs).

PAMPs are commonly found on viruses (such as dsRNA or cytosine-phosphate-guanine (CpG) DNA motifs), bacteria (such as LPS, flagellin, and peptidoglycan), and fungi. They are recognized also by toll-like receptors and other pattern recognition receptors (PRRs) (Geijtenbeek *et al.,* 2002; van Kooyk and Geijtenbeek, 2003). It is this binding interaction that initiates phagocytosis (McGreal *et al.,* 2005). *M. smegmatis*, an avirulent species of mycobacteria, has phosphatidyl-*myo*-inositol capped LAM (PILAM) that does not engage the MR or DC-SIGN, but rather toll-like receptor 2 (TLR-2), thereby causing a proinflammatory response (Schlessinger *et al.,* 1994; Madea *et al.,* 2003). Therefore, MR-mediated phagocytosis by primary human macrophages is modulated by ManLAM and the *M. tuberculosis* bacilli are directed to a phagosomal compartment that has limited fusion with lysosomes (Kang *et al.,* 2005).

Once inside the macrophage, the bacilli have to contend with the wide array of mechanisms that mononuclear phagocytes use to kill intracellular organisms. The bacteria are encompassed in the phagosome where they are subjected to killing processes. Phagocytosed particles within macrophages are normally routed to acidic lysosomal compartments for destruction, and mycobacteria avoid this by use of sulfatides in their cell envelopes to inhibit fusion of the lysosome with the phagosome, by inhibiting fusion of phagocytic vacuoles with destructive vesicles containing the proton-ATPase, by scavenging O_2 with LAM, and by inducing detoxifying enzymes with catalase, superoxide dismutase, and protective heat shock proteins (O'Brien *et al.,* 1996). While *M. tuberculosis* isolates differ in their susceptibility to hydrogen peroxide and reactive nitrogen intermediates, the state of differentiation and activation of blood derived alveolar macrophages affects their ability to control the growth of the pathogens

(Piessens and Nardell, 2000). The time between phagocytosis and fusion of the lysosome with the phagosome is rapid, so the *M. tuberculosis* has to interrupt this process for its survival and initiation of a successful infection (Aderem and Underhill, 1999). Inside the phagosome, *M. tuberculosis* uses ManLAM to regulate phagosome maturation. ManLAM has been shown to regulate phagosome maturation in murine macrophages and a human monocytic cell line (Chua *et al.*, 2004; Hmama *et al.*, 2004). ManLAM also blocks the increase in Ca^{2+} in the cytosol of the macrophage which inhibits the interaction of the phosphatidylinositol-3 kinase (PI3K), hVPS34, with calmodulin. This step is necessary for the production of PI-3-phosphate, which is required in the recruitment of the Rab5 effector early endosomal antigen 1 to phagosomes. This antigen is needed for the delivery of lysosomal components from the trans-golgi network to the phagosome and regulates fusion of vesicles of the endosomal-lysosomal pathway with the phagosome (Fratti *et al.*, 2003; Kang *et al.*, 2005).

LAM likewise interacts with endotoxin receptor CD14 (soluble form), and sulfatides from *M. tuberculosis* can bind scavenger receptors (Pugin *et al.*, 1994; Yu *et al.*, 1998). The expression of CR4 and mannose receptors increase and the abundance of CR3 decrease as monocytes mature into tissues and alveolar macrophages. Likewise, human surfactant protein A (SP-A), which regulates the level of lung surfactant, also enhances phagocytosis of *M. tuberculosis* bacilli (Piessens and Nardell, 2000; Hu *et al.*, 2000). In summary, all of these alternate mechanisms can therefore initiate and enhance phagocytosis, thus allowing *M. tuberculosis* to replicate and initiate infection.

1.5B STAGE 2 OF INFECTION

When the innate bactericidal activity of alveolar macrophages fails to destroy the initial few *M. tuberculosis* bacilli for any reason, they can then multiply and rupture the macrophage. This begins stage 2 of TB infection. The released bacilli are taken up by other macrophages and monocytes are attracted to the area and begin to transform into immature macrophages. These immature macrophages are less able to destroy or control the growth of the bacilli and thereby allow the pathogen to replicate logarithmically over the next few weeks after infection. Successive cycles of pathogen growth and macrophage rupture leads to the formation of a primary lesion, consisting of concentric layers of immature macrophages containing mycobacteria (Herrmann and Lagrange, 2005). Some mycobacteria are transported to draining lymph nodes and various metastatic sites by the circulatory system where the infection and lytic process can be repeated (Iademarco and Castro, 2003). The initial lesion and inflamed lymph nodes form the primary complex.

1.5C STAGE 3 OF INFECTION

In stage 3 of TB infection, after about 3 weeks, the growth of the bacilli cease as the host develops acquired immunity and begins the formation of tuberculosis granulomas, the host's way of containing the infecting mass. The granuloma is formed and maintained by cell-mediated immunity (CMI), with the presence of activated macrophages around the solid caseous tuberculosis foci. A Ghon focus (named for Anton Ghon, a Czech pathologist) is a primary lung lesion or a small area of granulomatous inflammation only detectable by chest X-ray if it calcifies or grows substantially in the

lung of a previously uninfected individual (Herrmann and Lagrange, 2005). Granulomas are sites of local inflammation characterized by a central aggregation of infected macrophages surrounded by T cells and are useful to isolate pathogens and subject them to some degree of regulatory control from the surrounding T cells (Janeway *et al.*, 2005). Delayed-type hypersensitivity is the cytotoxic immune process that kills non-activated immature macrophages, thereby preventing further replication of the bacilli (Dannenberg, 1991). The CMI process involves the killing of *M. tuberculosis* by macrophages that have been activated by cytokines from Th1-like, $CD4^+CD8^-$ T-helper cells. Phagosomal maturation and activation is initiated by activation with interferon-γ (IFN- γ), however, *M. tuberculosis* is able to live within the macrophage by blocking the phagosome at an early step of maturation and by preventing the fusion of phagosome with the lysosome (Russell, 2001). IFN-γ also stimulates anti-mycobacterial mechanisms in macrophages and is a key cytokine for host defense against TB infection. The ability to control the pathogen requires activated macrophages, and several T cell populations. Conventional $CD4^+$ and $CD8^+$T cells and unconventional T cells, such as those that recognize non-peptidic antigens in the context of CD1 molecules are likewise employed (Kaufman, 2001). Proinflammatory cytokines, such as IL-18, IL-12, and tumor necrosis factor α (TNF-α) are produced upon macrophage activation. IL-12 and IL-18 cytokines promote differentiation of naïve $CD4^+$ T cells into Th1 IFN-γ producing cells, while TNF-α participates by regulating the formation of the granuloma. Indeed, individuals unable to produce or respond to INF-γ demonstrate enhanced susceptibility to infection by *M. bovis* BCG or other opportunistic mycobacteria (Doffinger *et al.,* 2000; Ottenhoff *et al.,* 2002).

In a typical tuberculosis granuloma, activated "mature" macrophages accumulate around the caseous lesion to prevent its further expansion. Caseous granulomas in the lung are microscopic or barely visible during the first 4-5 weeks of infection. At this point, lung lesions in resistant individuals contain few bacilli, some lymphocytes, many activated macrophages, Langerhan's cells (multinucleated giant cells), and little necrosis. In more susceptible individuals, there may be relatively more bacilli and more necrosis (Grosset, 2003). In most infected persons, the immune response maintains the bacillary population in reactivatable sites in a steady-state dormant level that can persist for decades. The continual release of small amount of mycobacterial antigens from caseated granulomas maintains both TB reactivity and protective immunity, in separate phenomena (Orme and Collins, 1984). As such, in most immunocompetent persons, primary tuberculosis is a subclinical infection, or a mild and transient illness that does not pass the third stage in this pathogenic scheme.

1.5D STAGE 4 OF INFECTION

Stage 4, from months to years later, is typified by the reactivation and transmission of dormant TB. The end stage of caseation, liquefaction, is triggered by somewhat unknown means, although stress, exogenous reinfection, treatment with steroids or chemotherapeutic drugs, HIV infection, malnutrition, and advanced age, among others, play a role. Mycobacterial toxins and host factors such as Langerhan's and epitheloid cells in granulomas expressing mRNA for TGF-β may also play a role (Dannenberg and Sugimoto, 1976; Toossi *et al.,* 1995). Liquefaction results from continual hydrolysis of protein, lipid, and nucleic acid components of caseated host tissue

by hydrolytic enzymes from the host tissue and the mycobacteria bacilli. Once the liquefied caseous lesion discharges its necrotic, highly antigenic, infectious liquid into a nearby bronchus, a cavity is formed. This becomes an evident pathological presentation in radiographic chest X-rays as a tuberculosis pneumonitis. Such cavitation of the lesion is responsible for the transmission of human TB.

Several cytokines and other host factors can deactivate macrophages for the promotion of mycobacterial growth, such as IL-2, IL-4, IL-7, IL-12, TNF-α, and IFN (Wallis and Ellner, 1994). The TB bacilli can then replicate wildly in the liquefied necrotic tissue and cavity, largely free from the host immune mechanisms in those areas. Here the patient begins to suffer from systemic symptoms such as fever, remarkable weight loss, coughing, and anexoria that are associated with cavitary disease. Given the baseline mutation rate of mycobacteria, this environment also favors the emergence of drug-resistant tubercle bacilli as well.

1.6 DIAGNOSIS AND TREATMENT OF TUBERCULOSIS

"Koch's Lymph" or "old tuberculin" was developed by and announced in 1890 by Robert Koch at the World Congress of Medicine in Berlin as a cure and preventative measure against TB and was later refined into the tuberculin Purified Protein Derivative (PPD) skin test, a diagnostic method still widely employed (Sbarbaro and Iseman, 1997). An intracutaneous injection of 5 tuberculin units (0.1 ml) of PPD is administered typically on the forearm and the development of induration is observed after 48-72 hours. Tuberculin PPD-S1 is typically used in the United States and since 1951 has been used to standardize commercial PPD regents used in special tuberculin surveys (Villarino, *et al.*

2000). A positive reaction is due to the cell-mediated immune response, and is indicated by an induration at the injection site, a palpable raised hardened area across the forearm, perpendicular to the long axis of the arm. This induration is measured in millimeter (mm) (however any surrounding reddened erythema is not), and >10 mm is considered positive. Patient risk factors are taken into account when defining what level of induration is considered positive. High risk factors, such as being HIV positive, previously exposed to TB, chest X-rays showing nodulation and/or fibrotic changes, or otherwise immunosuppressed patients, are considered positive at 5 mm or more. Injection drug users, diabetics, residents or employees of high-risk or congregate settings, and children under 4 years of age are considered positive at over 10 mm of induraiton. People with no know risk factors for TB are considered positive at 15 mm or more of induration. An increase of 10 mm or more over a two-year period is considered seroconverted, regardless of age. However, this method is limited by the fact that many individuals are vaccinated with *M. bovis* BCG and the cross-reactivity with PPD-S1 can give false positives (Chaturvedi and Cockcroft, 1992). Moreover, in cases of anergy, there is a little, if any response to the PPD administration, due to the patient being highly immunocompromised, such as that of an AIDS patient, thereby lessening the value of Mantoux testing.

The definitive diagnosis of TB is typically first carried out with an acid-fast staining technique applied to sputum samples to look for the acid-fast bacilli of *M. tuberculosis*. Two methods are typically employed; the carbolfuchsin methods (Ziehl-Neelsen and Kinyoun) and a fluorochrome method using aquamine-rhodamine dye (Koch and Cote, 1965). Such staining and microscopy as a preliminary diagnostic tool for

mycobacterial infection is highly effective. It is relatively easy to do, even in the field, and allows for rapid and quantitative identification of bacilli. As few as $1x10^5$ bacteria per ml of sputum can be identified. However, this method cannot differentiate the species of mycobacteria (Rickman and Moyer, 1980).

The rapid radiometric culture system or BACTEC (Becton-Dickinson) has been developed for isolation of mycobacteria in culture using an enriched Middlebrook 7H12 medium containing ^{14}C-labeled palmitic acid. Mycobacterial growth is determined by the utilization of ^{14}C with release of $^{14}CO_2$ by the multiplying mycobacteria and is detected in an ionic chamber with electronic detector in the BACTEC instrument. Unlike in conventional culture methods where growth of bacterial colonies is detected only by the naked eye, the BACTEC instrument is able to detect low levels of bacterial growth too low to produce colonies in the conventional media. However, the faster results obtained with the BACTEC system cannot be overemphasized. It does not, however, free personnel from the unnecessary hours of tedious work processing specimens prior to inoculation to the culture media. There are some shortcomings with BACTEC, such as needle stick risks to personnel while using syringe needles in aspirating BACTEC vials. Another disadvantage of the BACTEC system is the release of radioactive $^{14}CO_2$. Although the gas is still confined within the vial and not harmful in small amounts, disposal is still a problem. For a developing country, cost is an important factor to consider with the use of the BACTEC radiometric system and there is an additional cost burden on the patient. If however, this is applied to drug susceptibility testing for relapse and re-treatment cases, the cost is minimal since results are obtained within a week's time, unlike the typical three or more weeks by conventional drug susceptibility tests for

mycobacteria (Roberts *et al.*, 1983; Hawkins, 1986). Several studies have already been published on the reliability and comparability of results using BACTEC versus conventional drug susceptibility testing with *M. tuberculosis* (Siddiqi *et al.*, 1981; Siddiqi *et al.*, 1985; Tarrand and Gröschel, 1985; Woodley, 1986). The BACTEC 460 system (Becton Dickinson Biosciences, Sparks, MD) radiometric assay is the reference method used for testing of susceptibility *M. tuberculosis* isolates to the first line anti-tuberculosis drugs such as pyrazinamide although it is falling out of use. It is being replaced by the newer liquid culture based methods that can utilize somewhat more acidified culture media, such as the non-radiometric BACTEC MGIT 960 (Becton Dickinson Biosciences), the nitrate reductase assay, and the Bac/ALERT 3D (bioMerieux Inc., Durham, NC) (Jureen and Werngren, 2008). These methods are more adept at determining pyrazinamide resistance of *M. tuberculosis* isolates, though the low pH necessary (~pH 6.0) makes such testing difficult. The MGIT 960 method is commonly used for drug sensitivity testing in many developing nations having adequate laboratory facilities (Martin *et al.*, 2008).

The polymerase chain reaction (PCR) allowed detection of specific *Mycobacterium* species rapidly and definitively in a wide range of tissues and clinical samples. Various genes have been used as diagnostic targets, such as the IS6110 repeat sequence for example, to detect *M. tuberculosis* in clinical samples (Eisenach *et al.*, 1990). The Centers for Disease Control and Prevention (CDC) in the United States now recommends the use of PCR testing on sputum samples for rapid identification of TB in clinical samples in pulmonary TB diagnosis.

With proper drug selection and patient compliance, TB cases have actually declined significantly in prevalence in most industrialized nations. A short-course treatment regime such as DOTS is the standard method for treating tuberculosis. DOTS is an adopted regime worldwide in many nations and is comprised of 5 core components; 1) political and economic support, 2) improved case detection, 3) standardized treatment and supervision, 4) a reliable drug supply system, and 5) an oversight system with periodic evaluation and measurement to gauge therapy effectiveness. Most TB patients are cured under the DOTS regime and more than 26 million TB patients worldwide have been treated under DOTS (WHO Report, 2007). However, in much of the impoverished world, adherence to DOTS is not at all prevalent. The decline of TB came to an end with coinfection with HIV, and the emergence of MDR strains of *M. tuberculosis*, which rapidly allowed the disease to reemerge even more dangerous than before. Such resistance was caused primarily by improper prescriptions and patient noncompliance to treatment practicum (Snider and Roper, 1992; Rattan *et al.*, 1998). DOTS programs became feasible for high-load and impoverished countries with the development of trial-validated intermittent short course chemotherapy regimes developed by the International Union Against Tuberculosis and Lung Disease (IUATLD) and the WHO. These regimes recommend directly supervised treatment consisting of a 2 month daily regimen of rifampin (RIF), isoniazid (INH), pyrazinamide (PZA), and ethambutol (EMB), or streptomycin (SM), followed by a 4 month continuation with either daily or intermittent INH and RIF for newly diagnosed patients (Girling *et al.*, 1988; Gbayisomore *et al.*, 2000).

Typically, TB is properly treated with an initial and intensive 2 month chemotherapeutic regime comprising of multiple antibiotics. RIF, INH, PZA, and EMB, or streptomycin, are employed to ensure that bacterial resistance to a single drug does not emerge (Combs *et al.*, 1990). The drugs that act as cell wall biosynthetic inhibitors include D-cycloserine, INH, EMB, and ETH. The drugs that inhibit nucleic acid synthesis are RIF and the various quinolones. The drug that disrupts cell membrane metabolic function is PZA (Zhang *et al.*, 2003), and all of these comprise the first and second line anti-tubercular drugs (Zhang, 2005).

Over the next 4 months, only RIF and INH are administered to eliminate any persistent tubercle bacilli. RIF and INH are by far the most potent of the antitubercular drugs and alone kill more than 99% of the tubercle bacilli within the first 2 months of therapy. PZA acts best on semi-dormant bacilli that are not affected by other antitubercular drugs. This regime, requiring strict patient compliance and physician control, reduces chemotherapy from 18 months to 6 months, and can assure elimination of any drug-resistant bacilli.

Isoniazid was discovered in 1952 as the first oral mycobacterial drug and one of the most efficient and the most widely used antituberculosis drugs (Youatt, 1969). Early work demonstrated that INH inhibits synthesis of mycolic acids in *M. tuberculosis* (Takayama *et al.*, 1972; Takayama, 1974). INH is a prodrug which requires activation by the mycobacterial catalase-peroxidase (KatG) enzyme into the active form as an electrophilic inhibitor (Zhang *et al.*, 1992). Two enzymes involved in the elongation cycle of the fatty acid biosynthesis, an enoyl-acyl carrier protein reductase (InhA) and β-

ketoacyl-acyl carrier protein synthase, are believed to be targets of the activated inhibitor(s) (Banerjee *et al.,* 1994; Mdluli *et al.,* 1998). A purified InhA-inhibitor complex is generated in KatG-catalyzed INH activation and has no enoyl reductase activity, as the inhibitor binds noncovalently to InhA (Lei *et al.,* 2000).

Rifampin (Rifampicin) is the most important of the anti-mycobacterial agents for treatment of tuberculosis, leprosy, and mycobacterial infections in patients with AIDS (Tillotson, 1996). Discovered in 1957, RIF inhibits protein synthesis by binding to the β-subunit of bacterial DNA-dependent RNA polymerase to prevent initiation of transcription (Levin and Hatfull, 1993; Cole *et al.*, 1998). Treatment with RIF results in the impairment of cellular replication, transcription, and DNA repair by the inhibition of DNA gyrase (topoisomerase II) and chromosomal segregation during replication is disrupted by the inhibition of topoisomerase IV (Musser, 1995; Onodera *et al.*, 2001). Therefore, RIF is responsible for shortening the duration of therapy, despite the emergence of RIF-resistant mycobacteria. Such RIF-resistant mutants typically arise from mutations in the β-subunit of the ribosomal polymerase gene *rpoB* during the course of therapy for active TB (Williams *et al.*, 1998).

Takayama and Kilburn first shown that ethambutol (EMB) inhibits the incorporation of radiolabeled isotope from [^{14}C]glucose into cell wall arabinan immediately upon adding EMB to young cultures (Takayama and Kilburn, 1989). The effects of EMB also applied to the arabinan of both arabinogalactan (AG) and lipoarabinomannan (LAM), however the inhibition of isotope incorporation into AG was immediate, whereas the incorporation into LAM was not until after 1 hr of exposure

(Mikusova *et al.*, 1995). This implies that specific arabinosyltransferases involved in AG biosynthesis are the target of EMB as well as in LAM biosynthesis, and three arabinosyltransferases have been identified, *embC*, *embB*, and *embA* (Belanger *et al.*, 1996; Telenti *et al.*, 1997).

The prodrug pyrazinamide does not have a well-defined mode of action, though it is activated by *M. tuberculosis* pyrazinamidase (PZase, coded by *PncA*) at acidic pH, converting it to pyrazinoic acid. PZA is thought to disrupt the cell membrane energy potential and membrane transport (Zhang *et al.*, 2003). PZA is effective in that it shortens chemotherapy from 9-12 months to 6 months and is effective against persisting tubercle bacilli (Zhang *et al.*, 2008). The acidic conditions required for optimal PZA activity occurs *in vivo* when inflammatory cells produce lactic acid (Zhang and Mitchison, 2003, Zhang *et al.*, 2008). Acidic pH is inhibitory to the growth of M. tuberculosis *in vitro*, thereby regular drug susceptibility testing on solid media of PZA is ineffective (Pureen, *et al.*, 2008). Other testing methods such as the BACTEC assays, as previously described, are better suited to PZA susceptibility testing as is the nitrate reductase assay using nicotinamide resistances as the marker for pyrazinamide resistance at neutral pH (Martin, et al. 2008). PZase is a monomeric enzyme utilizing Mn^{2+} or Fe^{2+} with similar hydrolytic activity for PZA and nicotinamide with an optimal pH of 7.0 at 40°C (Zhang *et al.*, 2008). PZase is encoded by *pncA* and mutations in this gene result in lost or reduced PZase activity. Such mutations are thought to confer PZA resistance in *M. tuberculosis* (Hirano *et al.*, 1997; Jureen *et al.*, 2008; Zhang *et al.*, 2008).

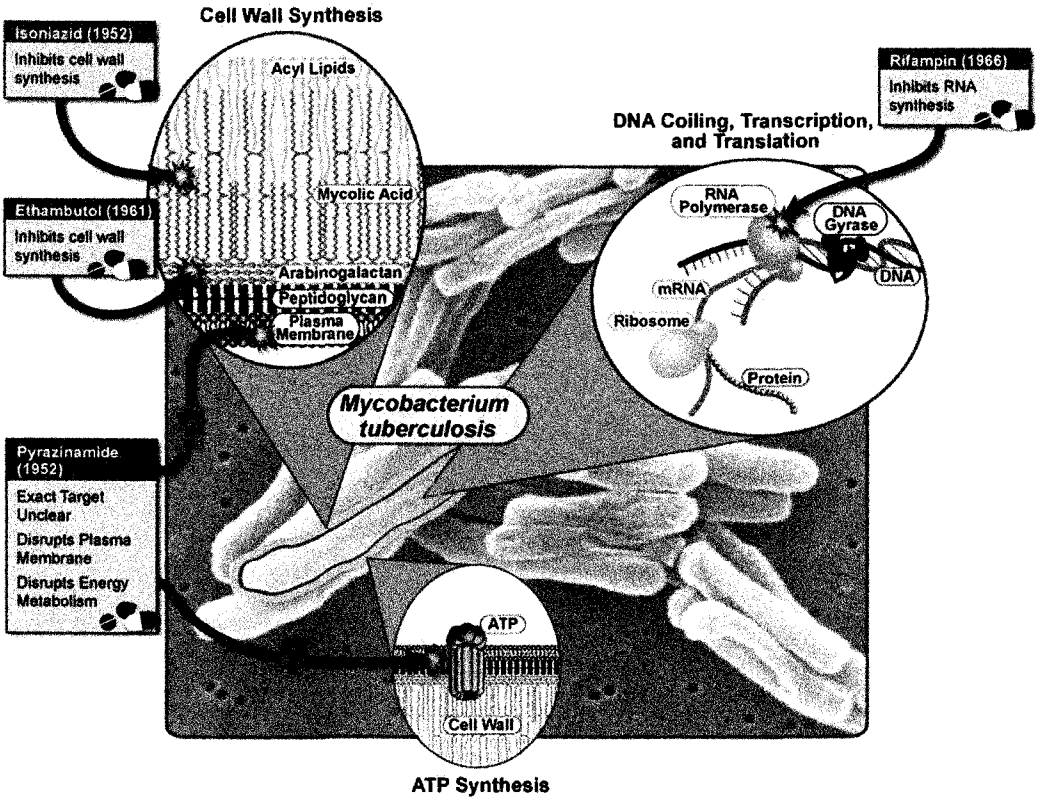

Figure 1.6.1. First-line treatment of tuberculosis (TB) for drug-sensitive TB. (Picture from the National Institute of Allergy and Infectious Disease (NIAID), National Institute of Health (NIH) website http://www3.niaid.nih.gov/topics/tuberculosis/WhatIsTB/ScientificIllustrations/firstLineIllustration.htm)

1.7 CONCLUSION

The mycobacteria are a special class of prokaryotes, some of which have evolved as efficient intracellular parasites able to replicate inside a host macrophage. They are complex bacteria with highly unique and complex cell wall envelopes that confer dangerous pathogenicity. Various unique lipoglycans and glycolipids are

interspersed in this cell wall complex that serve to modulate and help the bacterium evade the host immune response. The mycobacteria, *M. tuberculosis* and *M. leprae* in particular, have been with humanity for many thousands of years and likely predate mankind altogether. Both TB and leprosy are well chronicled throughout ancient history. TB has now become serious worldwide threat. This pathogen has developed resistance to multiple antibiotics despite aggressive treatment regimes like DOTS. Coupled with HIV disease, TB is infecting people in both rich and poor nations alike, and is poised to become a worldwide crisis in the 21st century. Traditional antibiotic therapy is failing in many cases, and the need for novel drugs is of paramount importance.

Moreover, other mycobacterial diseases also plague humans and animals alike, such as *Mycobacterium avium*-intracellular complex and leprosy. The TB and leprosy pathogens have evolved a complex arsenal to invade the host macrophage, enhancing phagocytosis, and subsequent phagosome packaging. These bacilli can alter the environment of the phagosome to their liking and prevent the fusion of destructive lysosomes. Fortunately, the diagnosis of TB is becoming more effective from the time of Dr. Koch, but the need for efficient, easy, and inexpensive diagnosis and treatment is critical to combat the disease in the developing world. The four main first line drugs that fight TB are effective, but the pathogen is evolving multiple drug resistance at an alarming rate.

Study of the cell wall structure of mycobacteria is therefore necessary in developing new antibiotics, as disruption of it or its synthesis is likely the best way to kill this pathogen. A myriad of enzymes is involved in cell wall synthesis and

polyprenylphosphates play an active role. The glycosyltransferases, many of which use polyprenylphosphates in the build up of cell wall components, are among the most important enzymes in nature and likewise are central to cell wall synthesis. Glycosyltransferases are key enzymes involved in the synthesis of the AG, PIMs, LM, and LAM, among many other molecules. This aspect of cell wall synthesis may open the door for exciting new chemotherapeutics.

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CHAPTER 2

STRUCTURAL ASPECTS AND BIOCHEMISTRY OF MYCOBACTERIAL CELL WALL

2.1 MYCOBACTERIA CELL WALL STRUCTURE

The cell wall complex of mycobacteria confers immunomodulatory ability and pathogenesis to the bacterium as well as protection from the immune response and various chemotherapeutics. The greatest hope in eliminating the TB threat and that of the other insidious mycobacterial related illnesses lies most probably in disruption of the cell wall complex, either by disrupting some of the steps in its synthesis or by disrupting its structure. A thorough understanding of the mycobacteria cellular structure is essential in developing future arsenals of chemotherapeutics and treatments. There has been a tremendous effort to identify novel biochemical targets for new drugs to attack, bolstered in part by the availability of a complete sequence of the *M. tuberculosis* genome. The targeting of metabolic pathways and biosynthetic/assembly pathways are a major thrust in current research as the hopes that affecting dormant TB may lie with these targets (Mikusova *et al.*, 1995). The cell wall complex of mycobacteria, and its synthetic pathways is just such a target group with enormous possibilities.

The variety of lipids and lipoglycans in the cell wall complex of mycobacteria is responsible in large part for the low permeability of the cell envelope and as a selective diffusion barrier for solutes and most of the known antibiotics and chemotherapeutic agents (Hoffner and Svenson, 1991; McNeil and Brennan, 1991; Nikaido and Jarlier, 1991; Rastogi, 1991; Lisowska *et al.*, 1996; Draper, 1998). Hydrophilic compounds typically cross such a barrier through water filled channels, formed by porin proteins, such as those recognized in *M. chelonae* (Trias *et al.*, 1992) and *M. smegmatis* (Trais and Benz, 1994). However these porins are relatively rare in the mycobacterial cell wall complex, thereby reducing levels of transport. The mechanism of permeation of hydrophobic compounds is less understood. A simplified representation of the core cell wall complex is shown in Figure 2.1.1

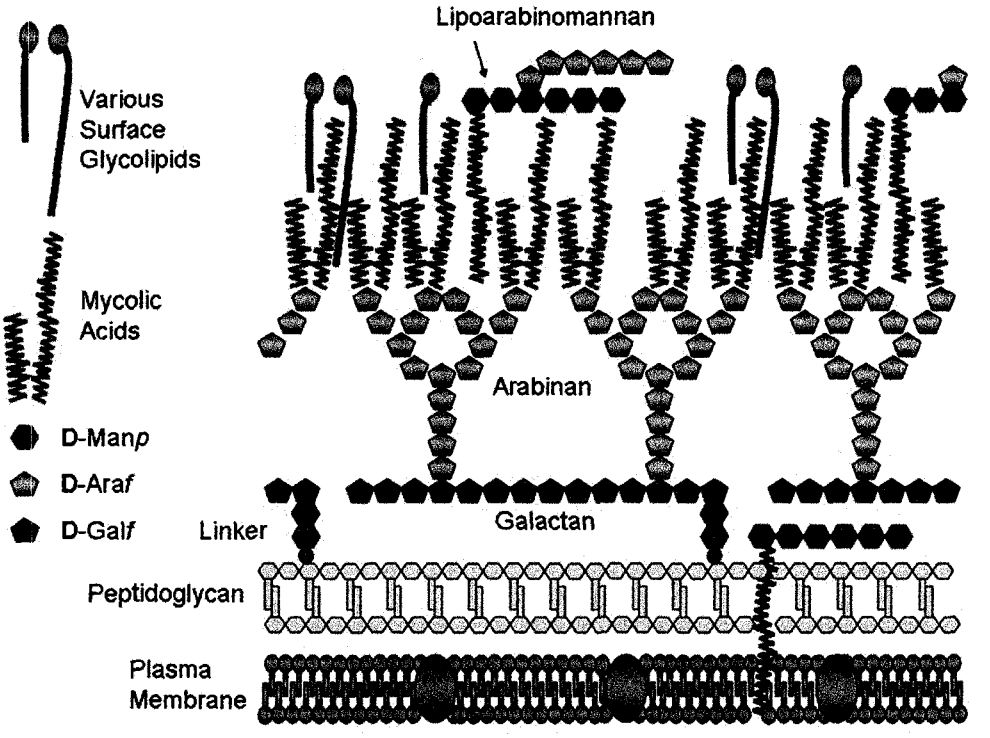

Figure 2.1.1. A representation of the cell wall complex of mycobacteria. The cell wall core is composed of a covalently linked complex of mycolic acids, to D-arabinan and D-galactan (together, arabinogalactan), attached to peptidoglycan via an α-L-Rha$_p$-(1→3)-α-D-GlcNAc linkage unit. This is often referred to as the mAGP complex. The outer monolayer contains a mixture of phospholipids and various members of several classes of lipoglycans. LAM and its relatives are thought to be present in the outer monolayer, as well as well as anchored in the plasma membrane.

2.2. CELL WALL COMPLEX

The cell wall complex of mycobacteria has been implicated in the virulence and durability of the bacilli since the time the ultrastructure of the cell wall complex of mycobacteria was first described by Imaeda *et al.* (Imaeda *et al.*, 1968). Electron microscopic studies show the cross section of the cell wall of mycobacteria as alternating zones of electron density and transparency. This deviates from typical Gram positive

bacteria and it was theorized that a substantial layer of lipids surrounds the cell, thus a chemical model was proposed by Minnikin and later by Daffe *et al.* that supports this contention (Minnikin, 1982; Daffe *et al.*, 1990). As such, the *M. tuberculosis* cell is surrounded by a complex and unique envelope that is mainly composed of long chain fatty acids (the mycolic acids), various glycolipids, lipoglycans, and polysaccharides (Brennan and Nikaido, 1995). This is in agreement with data showing that 30% of *M. tuberculosis* genes are devoted to lipid synthesis or metabolism (Cole *et al.,* 1998). Much of the inner half of this lipid layer is composed of assorted extracellular polysaccharides and species-specific lipoglycans. The outer layer, the outer "electron dense" layer (in terms of transmission electron microscopy), is composed of esterified mycolic acids from which mycobacteria derive their name, and to which their acid fastness can be attributed (Smith *et al.*, 1957; McNeil *et al.*, 1991).

Middlebrook *et al.* observed that virulent strains of *M. tuberculosis* could be distinguished from avirulent strains (and other saprophytic mycobacteria) by an ability to absorb the cationic phenazine dye, Neutral Red (Middlebrook *et al.,* 1947). This suggested that cell wall components may be involved, and subsequent research has shown the presence of two families of trehalose based lipids; the trehalose mycolates (cord factor) and the sulfatides; both play a role in pathogenesis (Bloch, 1950; Middlebrook *et al.,* 1959). A significant correlation between the absence and presence of sulfatides and the degree of virulence in the guinea pig over a broad sampling of *M. tuberculosis* cell wall isolates was observed, and thus, the biological activity of sulfatides was proposed to be an antagonist of the fusion of lysosomes and phagosomes (Goren, 1977; Grange *at el.,* 1978; Goren *et al.,* 1982). Cord factor is able to induce cytokine

mediated events such as systemic toxicity, inhibition of Ca^{2+}-induced fusion between phospholipid vesicles and migration of leukocytes, granulomagenic activity, anti-tumor activity, and macrophage release of chemotactic factors (Besra, 1998). As such, the presence of the 2,4,6-trimethyltetracos-2-enoic acids and the C_{16} and C_{18} straight chain heavily acylated trehaloses in the cell envelope gives passive protection for virulent *M. tuberculosis* by providing a resistive barrier to intracellular digestion during the bacilli's stay in the macrophage (Daffe *et al.,* 1988).

The mycolyl-arabinogalactan-peptidoglycan (mAGP) complex is a macromolecule that is the predominant entity in the cell wall complex of all mycobacteria. It is a suspected immunogen, although its roles appear to be primarily structural and as that of a protective barrier. This macromolecule is composed of the mycolic acids, arabinogalactan (AG), and peptidoglycan (PG) and is built up from polyprenyl phosphate (Pol-P). The mycolic acids are high molecular weight α-alkyl β-hydroxy fatty acids (C_{70}-C_{90}) in which the main chain contains additional functional groups such as keto, methoxy, and cyclopropyl rings as well as double bonds (Minnikin, 1982). These are grouped in strongly hydrophobic clusters of four that are attached to the nonreducing ends (C-5 position) of hexaarabinoside moieties of the arabinan chains. These fatty acids succumb to strong hydrophobic Van der Waals packing interactions which allow them to form a thick and rather impermeable layer with the fatty acid chains running perpendicular to the plasma membrane out from the underlying arabinogalactan.

The underlying macromolecule, AG, consists of polymerized arabinofuranose and galactofuranose, and serves as the attachment point for the mycolic acids.

Arabinogalactan is a combination of branched arabinan and galactan chains. The arabinan chain is composed of D-arabinofuranosyl (Ara*f*) residues in branched chains of 5-linked β-D-Ara*f* with branching introduced as 3,5-linked-α-D-Ara*f* units with the terminal nonreducing segment of arabinose mostly comprised of pentaarabinosyl motifs. This arabinan is covalently linked to a semi-linear galactan chain, which consists of approximately 30 alternating 5- and 6- linked-β-D-galactofuranosyl (Gal*f*) residues (Daffe *et al.,* 1990; Besra *et al.,* 1995).

The biosynthesis of AG begins on polyprenyl phosphate (Pol-P), typically in the C_{50} form (decaprenylphosphate or DP). This is followed by the addition of GlcNAc-1-P and rhamnose, forming a unique linker unit that will eventually anchor the rest of the macromolecule to peptidoglycan (Figure 2.2.1). This linker unit is comprised of an L-rhamnosyl residue linked to an *N*-acetylglucosamine residue. The action of WbbL, rhamnosyltransferase, therefore plays a central role in the synthesis of AG, while the similarly important action of *N*-acetylglucosamine (GlcNAc) transferase (Rv1302) has been overlooked by many researchers (Ma *et al.,* 2002; Mills *et al.,* 2004). GlfT (Rv3808c) polymerizes polyprenyl-P-P-GlcNAc-rhamnose with some 30 Gal*f* residues to form the galactan backbone. The presence of differing types of glycosyl linkages within the galactan chain suggests two or more galactosyltransferases may be involved in its synthesis. Two galactofuranosyl transferases, Rv3808c (GlfT) and Rv3782, are responsible for the polymerization of galactan (Belanova *et al.*, 2008). Rv3782 bifunctional transferase enzyme capable of adding beta-(1→4) and beta-(1→5) linked Gal*f* residues to GL-2 and is therefore involved in the initial steps of galactan formation. Its product, decaprenyl-P-P-GlcNAc-Rha-Gal*f*-Gal*f,* serves as the substrate for the

continual polymerization of galactan (to completion), catalyzed by GlfT (Belanova *et al.*, 2008; Mikusova *et al.*, 2006). GlfT is reported to be a bifunctional transferase enzyme capable of adding alternating beta-(1→5) and beta-(1→6) linked Gal*f* residues (Mikusova *et al.*, 2000; Kremer *et al.*, 2001; Alderwick *et al.*, 2007; Alderwick *et al.*, 2008).

The Ara*f* residues, rather unique to mycobacteria and some plants, originate from the pentose phosphate pathway-hexose monophosphate shunt (Scherman *et al.*, 1995; Scherman *et al.*, 1996). These are added by the arabinosyltransferases AftB (Rv3805c), EmbA (Rv3794), and EmbB (Rv3795) among others, in branched chains to the galactan core in α(1→5), α(1→3), and β(1→2) linkages and form the predominate carbohydrate mass of AG (Escuyer *et al.*, 2001). EmbA and EmbB are thought to be a heterodimer involved in the formation of the crucial terminal hexaarabinofuranoside motif ([Araβ(1→2)Araα(1→5)] [Araβ(1→2)Araα(1→3)]Araα(1→5)Araα1→ (Ara_6)) that serves as the attachment point for the mycolic acids to AG (Amin *et al.*, 2008). At some point, the Ara$f_{\sim 70}$-Galf_{30}-Rha-GlcNAc-P-P-polyprenyl complex is then transglycosylated to peptidoglycan (Alderwick *et al.*, 2007). The GlcNAc residue of the linker is thereby linked to peptidoglycan via a phosphodiester bond between the C-1 position of the N-acetylglucosamine residue and the C-6 position of the muramic acid residue of peptidoglycan (Brennan *et al.*, 1990; Brennan and Besra, 1997). The hexaarabinofuranoside terminal motif is then mycolated to varying degrees (McNeil *et al.*, 1994). As such, the structure and synthesis of the AG layer, as well as that of the mAGP complex, have been the focus of many studies, especially in terms of pathogenicity and cellular protection from host challenges (Crick *et al.*, 2000). As such,

the synthesis of mAGP is governed by a large number of enzymes and is illustrated in Figure 2.2.1.

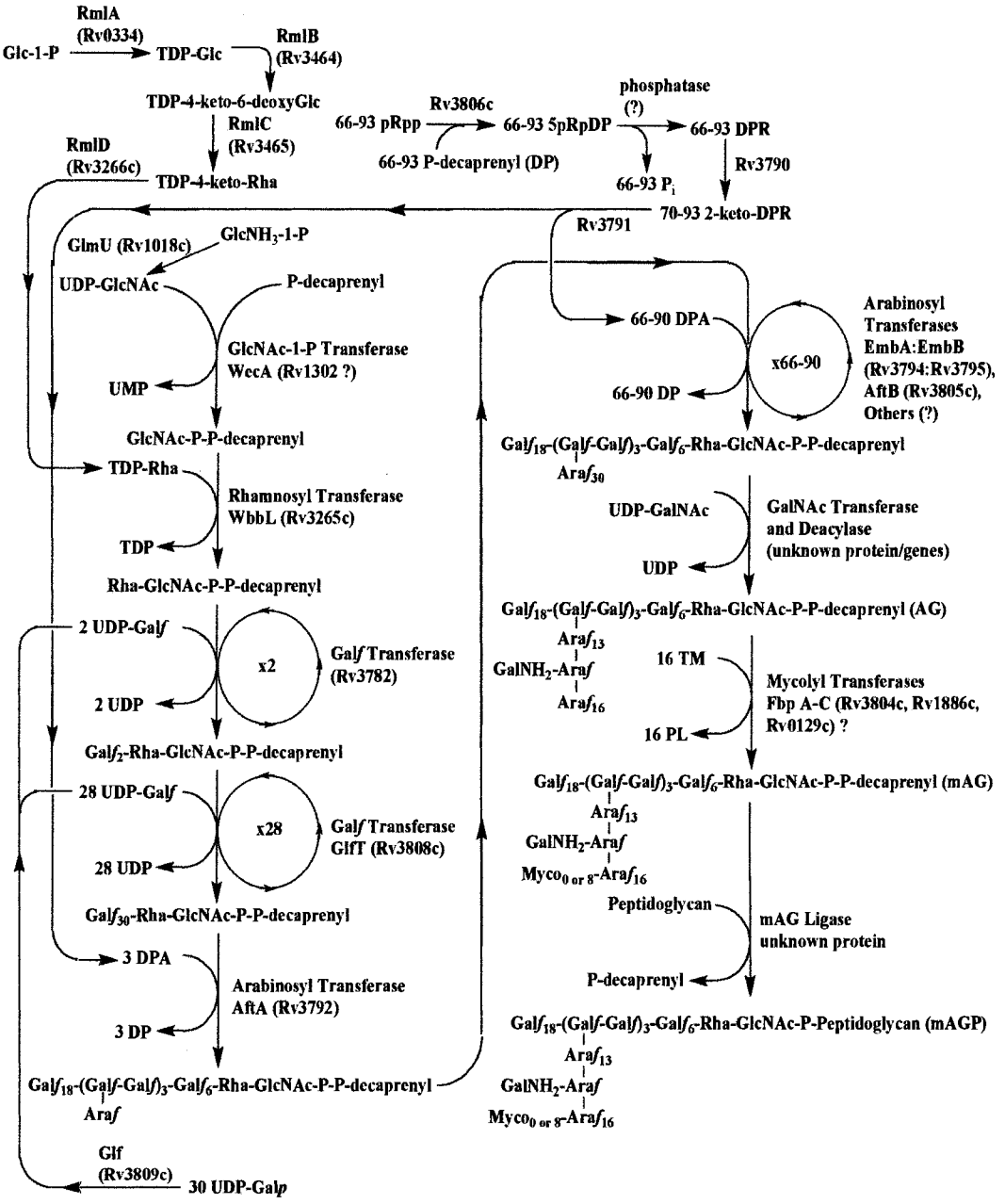

Figure 2.2.1. Key enzymes involved in the synthesis of the mAGP cell wall core. The predominant core of the mycobacterial cell wall consists of sections of arabinogalactan, peptidoglycan, and the mycolic acids all connected to one another covalently. Glycosyltransferases and polyprenyl phosphates play central roles in the synthesis and elongation reactions of these macromolecules.

Peptidoglycan (PG), or murein, is a structural component essential for bacterial cell life, providing the bacterial cell mechanical integrity (especially against osmotic stress) and cell shape (van Heijenoort, 2001). It provides the covalent attachment point for the complex outer cell wall of the mycobacteria and is involved in the cellular division process, as well as providing a rather impermeable barrier to larger molecules. Disruption of PG invariably leads to cellular lysis and death. In the 1970's, the structure of the mycobacterial peptidoglycan was found to be somewhat different from that of other Gram-positive bacteria. Though its structure is classified as the common A1γ type, it is different from that of other species (Schleifer and Kandler, 1972). The general structure of peptidoglycan is that of a heteropolymer, in which the linear glycan chains have repeating disaccharide peptide units in which the peptide cross-bridges are directly established between the peptide of two monomer units (van Heijenoort, 2001).

The mycobacterial peptidoglycan consists of linear glycan chains of an alternating polymer of GlcNAc and *N*-acetylmuramic acid (MurNAc), although the typical *N*-acetyl functional group on the muramic acid is oxidized to *N*-glycolyl functionalities. *N*-glycolylmuramic acid in peptidoglycan is also found in a few other bacteria, for example, *Norcardia* and *Corynebacterium* species. This implies a similar synthetic mechanism to other bacteria. In *M. tuberculosis, M. leprae,* and *M. smegmatis*, the peptidoglycan contains modified free carboxylic acid functions of amidated diaminopimelic acid (DAP) and amidated D-glutamic acid as well as direct DAP-DAP crosslinks (Wietzerbin *et al.*, 1974; Mahapatra *et al.,* 2008). The muramic acid residues have a tetrapeptide side chain moiety consisting of L-alanyl-D-isoglutaminyl-*meso*-diaminopimelyl-D-alanine. The muramic acid residues of *M. tuberculosis* and *M.*

smegmatis PGs are comprised of a mixture of *N*-glycolylmuramic acid (MurNGlyc) and MurNAc (Mahapatra *et al.,* 2005). The peptidoglycan of *M. leprae* lacks MurNGlyc and utilizes only MurNAc, as the orthologue of *namH* (ML0085c, which synthesizes MurNGlyc) was found to be a pseudogene in its degraded genome. The *namH* orthologue in *M. tuberculosis* is Rv3818 (Cole *et al.,* 2001; Mahapatra *et al.,* 2008). It seems as though the absence of MurNGlyc is not necessary for survival in the host but may confer increased resistance to lysozyme (Raymond *et al.,* 2005).

2.3. THE POLYPRENYL PHOSPHATES

The cytoplasmic membrane plays a crucial role in the biosynthesis of mycobacterial cell wall components and many of the involved proteins are either embedded in the lipid bilayer as integral membrane proteins, or associated with the membrane surface by hydrophobic and/or electrostatic interactions. Events such as protein translocation, membrane protein folding, and protein binding and function depend on the lipid composition of biomembranes (Karlsson *et al.*, 1994; Simons and Ikonen, 1997; van Klompenburg *et al.*, 1997; Bogdanov and Dowhan, 1999). The plasma membrane is also central in the synthesis and function for many of the lipoglycans present in mycobacteria. A key isoprenoid phosphate glycosyl accepter, Pol-P, accepts sugar residues from sugar nucleotide donors and serves as anchor points on the plasma membrane for various macromolecule assemblies. Therefore, Pol-P is required for the synthesis of mAGP, AG, LAM, galactan, LM, and PG (Brennan and Crick, 2007). The amount of Pol-P appears to be rate limiting in such macromolecule synthesis as was demonstrated for cell wall components in *Staphyloccus aureus* and *Bacillus* species

(Higashi *et al.,* 1970 and Anderson *et al.,* 1972). Peptidoglycan synthesis in *Escherichia coli* is likewise limited by Pol-P levels in the synthesis of Lipid I, and therefore the synthesis of the cell wall can be regulated in such a manner (Baddiley, 1972; van Heijenoort, 1996). Moreover, Crick *et al.* has shown that the slow growing *M. tuberculosis* synthesizes Pol-P at much lower rates than the faster growing *M. smegmatis* (Crick *et al.,* 2000). Therefore it seems evident that the cell wall core of mycobacteria and cell walls of bacteria in general, begin with the synthesis of Pol-P.

Polyprenyl phosphates structures fall into four groups, the 1) all-*E*-prenol, 2) di-*E*, poly-*Z*-prenol, 3) tri-*E*, poly-*Z*-prenol, and 4) all-*Z*-prenol (Crick *et al.*, 2001). Three Pol-P commonly encountered in bacteria are the C_{35}-P, C_{50}-P, and C_{55}-P varieties; heptaprenyl phosphate (di-*E,* mono-*Z* configuration), decaprenyl phosphate (DP; mono-*E*, poly-*Z* configuration), and undecaprenyl phosphate (bactoprenyl phosphate; di-*E*, poly-*Z* configuration), respectively (Besra *et al.,* 1994; Wolucka *et al.,* 1994; Crick *et al.,* 2001). Undecaprenyl phosphate is the carrier of activated sugar species (lipid I and lipid II) in peptidoglycan synthesis in most bacteria, while DP is used exclusively in many key glycosylation reactions and synthesis of the cell wall core in *M. tuberculosis*. In *M. smegmatis*, both heptaprenyl phosphate and DP are found (Takayama *et al.*, 1973). In any case, these Pol-P's operate within the membrane as key participants in extracellular glycosylation of macromolecules and glycoproteins. Figure 2.3.1 illustrates the structure and proposed synthesis of DP in detail.

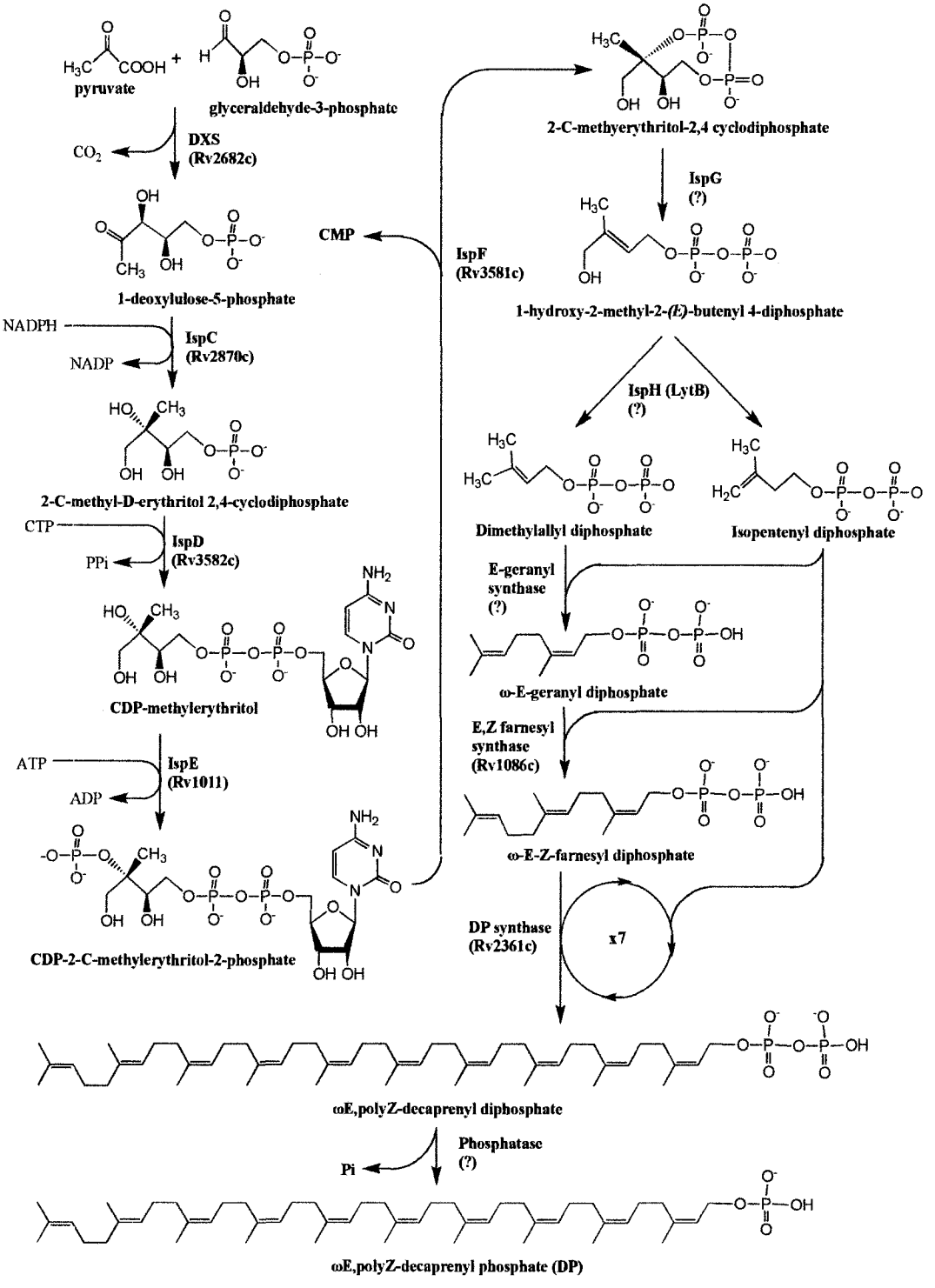

Figure 2.3.1. Proposed synthesis of decaprenyl phosphate in mycobacteria. (Adapted from Crick *et al.*, 2000; Crick *et al.*, 2001; Brennan and Crick, 2007).

Subgroups of isoprenoids include sterols, carotenoids, cholorophylls, quinones, as well as dolichol. Their synthesis proceeds through varying and differing pathways in animals and bacteria, but are likely to proceed through the methylerythritol phosphate pathway in mycobacteria (Crick *et al.*, 2001). The exact details of polyprenyl phosphate isoprenoid synthesis in bacteria are unclear. Experiments relating to the biosynthesis of Pol-P in mycobacteria have shown that *M. tuberculosis* synthesizes decaprenyl diphosphate, but not heptaprenyl diphosphate (Crick *et al.*, 2000). This suggests a possible pathway through geranyl diphosphate catalyzed by distinct prenyl diphosphate synthetases in a series of cyclization reactions, rearrangements, and further oxidations or reductions of the carbon skeleton, thereby responsible for the enormous diversity of structures (Connolly and Hill, 1989; Connolly *et al.,* 1994; Crick *et al.*, 2001). All Pol-P isoprenoids derive from the biological equivalents of isoprene, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), which are ubiquitous precursors in all living organisms (Gabrielsen *et al.,* 2004). In mycobacteria, 1-deoxy-D-xylulose-5-phosphate-synthase (DXS) catalyzes the first step of the mevalonate independent pathway by the condensation of pyruvate and glyceraldehyde-3-phosphate. Actions by the subsequent enzymes ending with IspH give IPP and DMAPP (Crick *et al.,* 2001; Brennan and Crick, 2007). ω,*E*-geranyl diphosphate is then converted to ω-*E,Z*-farnesyl diphosphate by Rv1086 (in mycobacteria) which is then further elongated by prenyl diphosphate synthases to give the varying chain lengths of the isoprenoids. DP is made in this fashion in *M. tuberculosis* by Rv2361c (DP synthase) from the ω-*E,Z*-farnesyl diphosphate and IPP substrates (Schulbach *et al.,* 2000).

2.4. LAM, AND RELATED GLYCOLIPIDS AND LIPOGLYCANS

Lipoarabinomannan (LAM), and its phosphatidylinositol containing synthetic precursors, lipomannan (LM) and the phosphatidyl *myo*-inositol mannosides (PIMs), are important glycolipids employed by *M. tuberculosis* and other mycobacteria which play key roles in the immunology and pathogenesis of TB. The structure of LAM is reminiscent of the lipoteichoic acids of Gram-positive bacteria; however many of its functions mimic those of Gram-negative lipopolysaccharides. LM is thought to influence mycobacterial virulence via its strong proinflammatory response and apoptosis-inducing activity (Briken *et al.*, 2004). These lipoglycans are also likely to be involved in the survival and pathogenicity of mycobacteria (Chatterjee and Khoo, 1998; Patterson *et al.*, 2003).

LAM is a high molecular weight amphipathic lipoglycan, which makes up one of the major components of the cell wall of mycobacteria and exhibits a wide spectrum of immunomodulatory effects. Its structure is complex and heterogeneous; comprised of three distinct structural domains, including a phosphatidylinositol anchor (PI anchor), a branched mannan region, and a branched arabinan. LAM is a key ligand in the interaction between the *M. tuberculosis* bacterium, macrophages, and dendritic cells and is therefore implicated in the inhibition of apoptosis, phagosome maturation, and IFN-γ signaling in macrophages and IL-12 cytokine secretion of dendritic cells (Briken *et al.*, 2004). Alveolar macrophages are the targets of *M. tuberculosis*, and bacteria survive the threat posed by macrophages by blocking the fusion of the phagosome with lysosome as was discussed in chapter 1 (Nigou *et al.*, 2003). Two types of LAM present in mycobacteria

are utilized in host invasion, ManLAM and PILAM (Madea *et al.,* 2003, McGreal *et al.,* 2005). More recently, a third class of LAM was observed that is not capped, called AraLAM, and was described in the fast grower *M. chelonae* (Guérardel *et al.*, 2002; Wieland *et al.,* 2004).

ManLAM, has been described in slow growing mycobacteria such as *M. tuberculosis*, *M. leprae*, *M. avium*, and *M. bovis* (as well as the *M. bovis* BCG strain), and is involved in the induction of phagocytosis, phagosomal alteration and fusion inhibition, and induction of innate, humoral, and acquired T-cell mediate immunity (Nigou *et al.,* 2003; McGreal *et al.*, 2005). ManLAM also inhibits activation of macrophages, production of Th1 proinflammatory cytokines IL-12 and TNF-α, and *M. tuberculosis* induced apoptosis, thereby allowing bacteria to persist in the human host.

PILAM has been described in fast growing mycobacteria such as *M. smegmatis* and *M. fortuitum*, and is able to induce the release of a variety of proinflammatory cytokines through the activation of Toll-like 2 receptors (TLR-2), as well as inhibiting pro-inflammatory cytokine production by lipopolysaccharide (LPS)-activated macrophages through a TLR2-independent pathway, thus favoring the killing of fast growing mycobacteria by activating macrophages (Means *et al.*, 1999; Doz *et al.,* 2007). ManLAM and PILAM interact with the phagocytic cells differently. As discussed in Chapter 1, ManLAM binds to the C-type lectins cell surface receptors, mannose receptor, and dendritic cell-specific ICAM-3 binding non-integrin (DG-SIGN), while PILAM binds to the LPS co-receptor CD14 (Tailleux *et al.,* 2003; Quesniaux *et al.*, 2004) . Both types of LAM can insert into the host cell plasma membrane without a receptor (Nigou *et al.,* 2003).

AraLAM (but not ManLAM) is a potent inducer of TNF expression in human and murine macrophages (Chatterjee *et al.*, 1992; Underbill *et al.*, 1999). AraLAM induces a profound inflammatory response in the lungs, which is characterized by a rapid induction of proinflammatory cytokines and chemokines, then followed by a large influx of neutrophils into the infected area (Wieland *et al.,* 2002; Wieland *et al.,* 2004).

Until now, the specific steps in the formation of LAM remain unclear. Therefore, the identification and characterization of the unknown glycosyltransferases is needed for an understanding of the fundamentals of how these complex macromolecules are synthesized and assembled. Moreover, the definitive structure of LAM is not fully understood, however a great deal of its structure has been elucidated. Figure 2.4.1 shows a current representation of the structure of LAM and the related precursor lipoglycans, LM and PIMs.

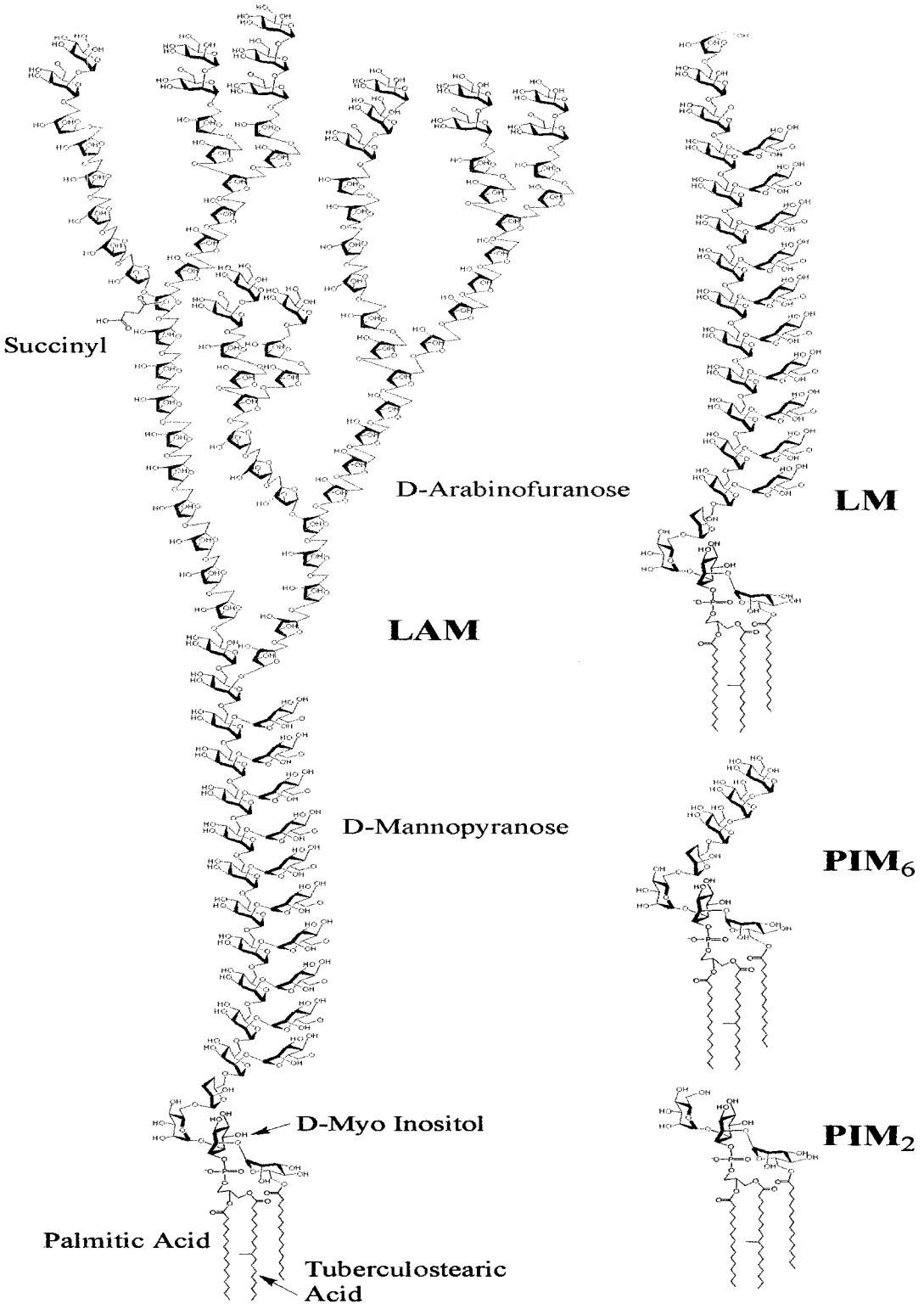

Figure 2.4.1 Proposed structures based on current data of ManLAM, LM, and the PIMs. There is some question as to the exact attachment points of the arabinan portions to the LM core in LAM, and the number of Ara*f* residues and their arrangement can vary.

LAM is thought to be non-covalently anchored in the plasma membrane as well as out in the mycolic acid layer. The reducing end of LAM is similar to the PIMs structurally, with the *myo*-inositol residue being mannosylated at the 2 and 6 positions. The mannan portion of LAM is simply an extension of the PIMs and is composed of a linear α(1→6)linked mannan chain thought to be approximately 14 Man*p* residues long. There are perhaps ten single α(1→2)linked Man residues that branch off the linear mannan chain, as illustrated in Figure 2.4.1 (Berg *et al.*, 2007). This LM core of LAM is thought to contain about 20-25 Man*p* residues in total (Chatterjee *et al.*, 1991; Khoo *et al.*, 1996). The arabinan portion of LAM is far more variable than that of arabinogalactan, though somewhat similar with the presence of an Ara_{18} motif. The arabinan portion contains between 50-80 Ara*f* residues, and its exact attachment to the LM core is unknown. The terminal extensions on the non-reducing ends of this motif vary in length in the arabinan chain (Shi *et al.*, 2006).

The predominant species of PIMs found in mycobacteria are the mono- and diacylated forms of phosphatidyl-*myo*-inositol dimannosides ($AcPIM_2$ and Ac_2PIM_2) and phosphatidyl-*myo*-inositol hexamannosides ($AcPIM_6$ and Ac_2PIM_6). Other PIM species (PIM_1, PIM_3-PIM_5) are typically metabolic intermediates present only in very small amounts in mycobacterial cells. PIMs are multiacylated at levels of up to four acylated chains which are shown in Figure 2.4.2.

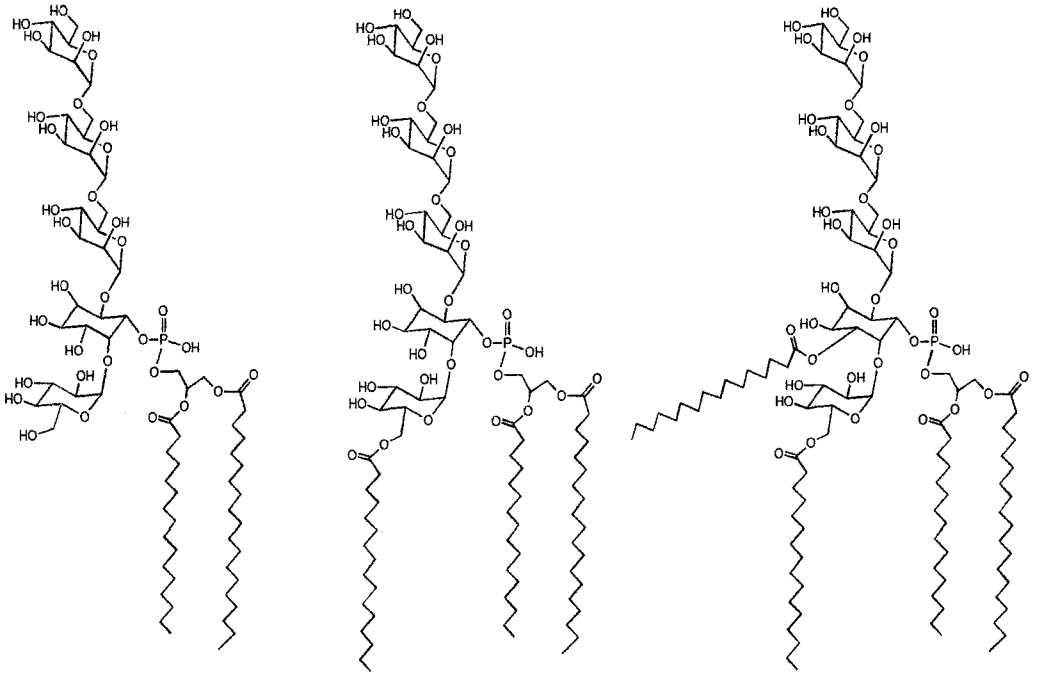

Figure 2.4.2 Examples of the differing levels of acylation in PIMs.

2.5 LAM-RELATED GLYCOLIPID AND LIPOGLYCAN SYNTHESIS

The phospholipids in the plasma membrane of mycobacteria are all derivatives of phosphatidic acid, such as phosphatidylinositol (PI), phosphatidylglycerol, cardiolipin, phosphatidylethanolamine, and the phosphatidylinositol mannosides (PIMs) (Brennan and Nikaido, 1995; Haites *et al.*, 2005). The related PIMs, LAMs, and LM, share a common phosphatidylinositol (PI) anchor with mannosylation extension at the C-6 position of the *myo*-inositol, suggesting a similar biosynthetic relationship (Khoo *et al.*, 1995; Kaur *et al.*, 2007). The final formation of LM and the various flavors of LAM of *M. tuberculosis* is believed to occur extracytoplasmically in the periplasmic space (between the cell membrane and peptidoglycan) or in the cell wall via the lipid-linked

sugar donor, decaprenyl phosphomannose (C_{50}-P or DPM) (Besra *et al.*, 1997). PI is the precursor molecule in PIM, LM, and LAM biosynthesis. It has been shown that *pgs*A (*Rv2611c*) is responsible for the synthesis of PI, which allows the exchange of the CMP-moiety of CDP-diacylglycerol (DAG) for inositol (Salman *et al.*, 1999; Jackson *et al.*, 2000). The mannosyl donor for the initial steps of PIM biosynthesis (synthesis of PIM_1 and PIM_2) has been shown to be GDP-mannose (Schaeffer *et al.*, 1999; Kordulavova *et al.*, 2002).

PimA and PimB are responsible for the addition of the first and second mannose respectively, to position 2 of the *myo*-inositol of PI, forming PIM_1 and PIM_2, which occurs on the cytoplasmic face of the cell membrane (Schaeffer *et al.*, 1999; Kordulakova *et al.*, 2002). The first mannose has been shown to be acetylated by the *Rv2611c* gene product (Kordulakova *et al.*, 2003). The transfer of the third mannose to $AcPIM_2$ is catalyzed by PimC, forming $AcPIM_3$ (Kremer *et al.*, 2002). However, *M. tuberculosis* $H_{37}Rv$, many clinical strains of *M. tuberculosis* and *M. smegmatis*, the saprophytic mycobacteria, lack PimC, indicating that there might be another mannosyltransferase responsible for this step (Kremer *et al.*, 2002). It is thought that PIM_3 (and/or $AcPIM_3$) is then translocated (flipped) through the plasma membrane to the extra-cytoplasmic side via a processes suggested to be mediated by flippases (Bugg and Brandish, 1994; Rush and Waechter, 2005).

The Man*p* at position 6 of the inositol of PIM_3 is then further elongated with additional Man*p* residues to give the higher form of PIMs (PIM_{4-6}, also called polar PIMs) by PimE and other unidentified mannosyltransferases (ManT). PimE transfers a

Man*p* residue from GDP-Man to the 2-position of mannose of PIM_4, forming PIM_5 (Morita *et al.*, 2006). At PIM_4, there is a probable branch point in the synthetic pathway, with one direction leading to LM/LAM via α(1→6) linked mannan backbone and the other direction leading to PIM_6, via the addition of two consecutive α(1→2) linked Man*p* residues (Morita *et al.*, 2004). It is apparent that PIM_6 is the dead-end product of PIM synthesis, and not involved in LM and subsequent LAM synthesis, since it contains two α(1→2) linked mannoses, a structure which has not been found in LM and LAM (Khoo *et al.*, 1995; Morita *et al.*, 2004; Kaur *et al.*, 2006). Therefore, the only possible intermediate precursor for LM/LAM synthesis should be PIM_4 (Morita *et al.*, 2004). The mannosyl elongation of PIM_4 is the probable route for the formation of LM and mature branched LM. PimE (Rv1159), a probable C_{50}-P-Man-dependent mannosyltransferase, was found to be responsible for the formation of PIM_5 from PIM_4 (Morita *et al.*, 2006). Whether PimE also transfers the sixth mannose to form PIM_6 remains to be determined. Finally, EmbC utilizes DPA and is partially responsible for the arabinosyl decoration of LM to give rise to LAM (Zhang *et al.*, 2003). ManLAM is then formed by the capping enzyme encoded by Rv1635c (Dinadayala *et al.*, 2006).

Early studies propose that the mannosylation of the more polar (higher levels of mannosylation) PIMs and LM involve both GDP-Man and C_{50}-P-Man (Yokoyama and Ballou, 1989). However, based on data from inhibition studies, amphomycin was shown to inhibit the synthesis of PIM_4, PIM_5, and PIM_6. This suggests that these enzymatic steps actually utilize C_{50}-P-Man as donor substrate (Morita *et al.*, 2004). A knockout *M. smegmatis* mutant △*MSMEG4250* (an ortholog to *Rv2181* of *M. tuberculosis*), resulted

in the LM lacking α(1→2) linked Man*p* on the LM backbone, strongly suggesting this protein to be an α(1→2) ManT in the synthesis of mature LM (Kaur *et al.*, 2006).

Mannosyl residues are required for the mannosylation of the later forms of PIM as well as LM and LAM. This is carried out by the activity of various glycosyltransferases. GDP-Man serves as the mannosyl donor for the formation of the early PIMs, occurring on the cytosolic side of the plasma membrane. However, due to the lack of a transporter, GDP-Man is unable to cross the plasma membrane. In eukaryotes, dolicholphospho-mannose (Dol-P-Man) translocates mannosyl residues across the membrane of the endoplasmic reticulum in to the lumen (Helenius *et al.,* 2002). Dol-P-Man synthase (EC 2.4.1.83) catalyzes the transfer of D-mannose (D-Man*p*) from GDP-Man to dolichol monohosphate. In mycobacteria, the phospholipid carrier is typically Pol-P, and the transfer of a D-Man*p* from GDP-Man to Pol-P gives polyprenyl phosphomannose (PPM). In *M. tuberculosis*, decaprenyl phosphomannose (C_{50}-P-Man or DPM) is the PPM which is the mannosyl donor in polar PIMs, LM, and LAM synthesis. Synthesis of PPM in mycobacteria has been found to be catalyzed by at least one enzyme, Ppm1 (Rv2051c), a GDP-Man dependent mannosyltransferase (Gurcha *et al.,* 2002). Figure 2.5.1 illustrates a proposed synthetic scheme for PIM, LM, and LAM.

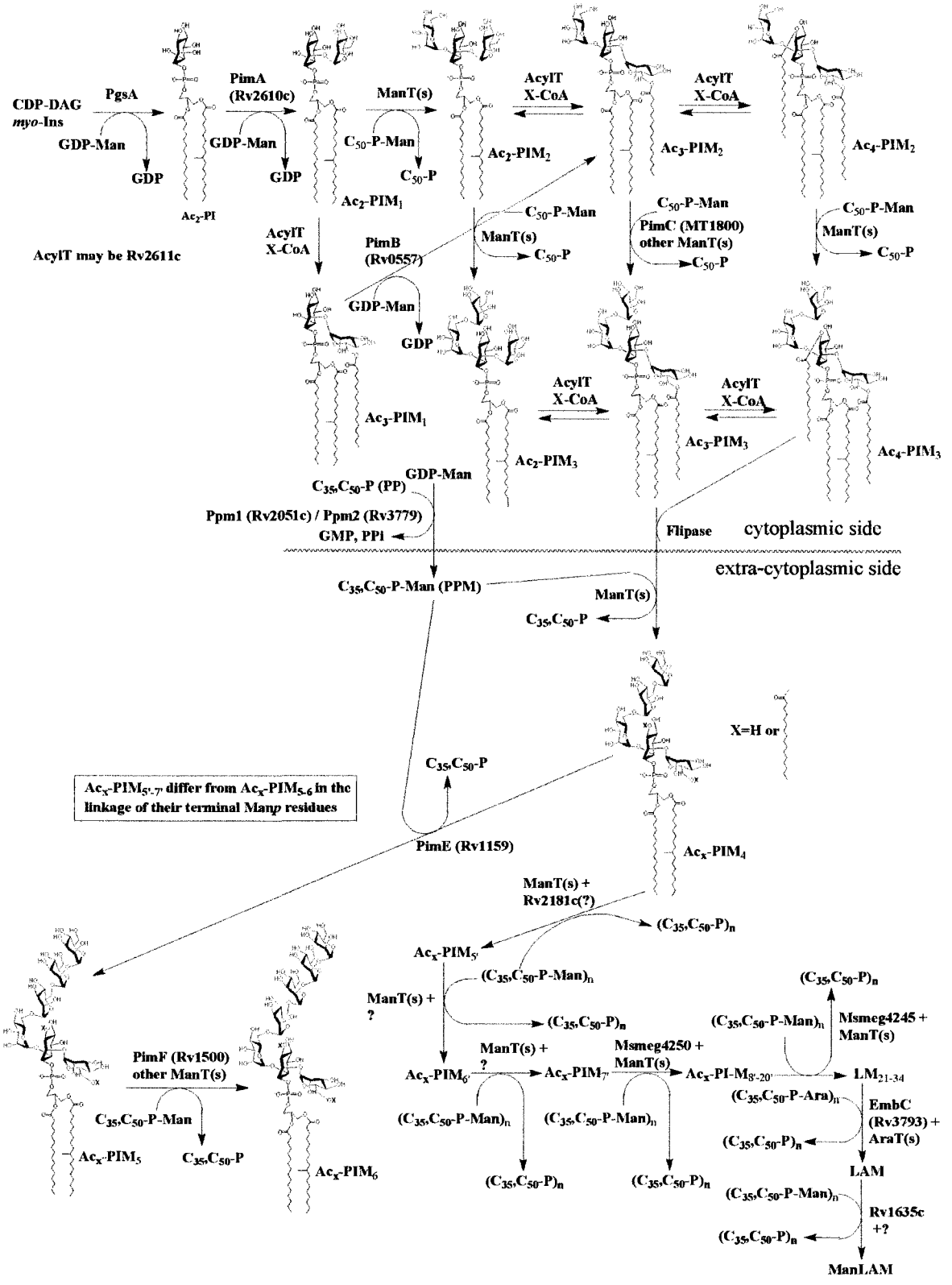

Figure 2.5.1. Proposed synthetic pathways for PIM, LM and LAM in mycobacteria. This scheme combines most of the data available on LAM biosynthesis pathways.

Polyprenyl phosphate is thereby involved in the biosynthesis of bacterial cell walls as a glycosyl acceptor (Hemming, 1974). Pol-P can be mannosylated to give PPM, or arabinosylated to give polyprenyl phosphoarabinose such as C_{50}-P-Ara (decaprenylphosphoarabinose or DPA). Likewise, C_{35}-P (heptaprenyl phosphate), also found in mycobacteria such as *M. smegmatis*, plays a similar role. The relative amount of C_{35}-PPM was found to be comparable to the amount of C_{50}-P-pentoses, and over an order of magnitude higher than that of C_{50}-P-Man (Wolucka *et al.*, 1998). As was discussed earlier, the availability of Pol-P was shown to be the rate limiter for cell wall synthesis in mycobacteria and also in cell-free preparations from *Staphylococcus aureus* and *Bacillus* species (Higashi *et al.*, 1970; Anderson *et al.*, 1972; Takayama *et al.*, 1973). Interestingly, it was shown that the slow growing *M. tuberculosis* actually synthesizes Pol-P at a much lower rate than the fast growing *M. smegmatis* (Crick *et al.*, 2000). Findings such as these clearly indicate that the level of the Pol-P pool is the main limiting factor in transmembrane steps of cell wall synthesis.

2.6. THE GLYCOSYLTRANSFERASES

Glycosyltransferases (GTs) catalyze arguably one of the most important transfer reactions on earth, considering the tremendous biomass involved in turnover of such polysaccharides as starch, glycogen, chitin, cellulose, and those of microbial cell wall components (Breton *et al.*, 2006). Moreover, glycosylation of proteins mediates crucial regulatory events in the biochemical interactions of prokaryotes and eukaryotes alike. Therefore, GTs are likewise among the most important enzymes in mycobacterial cell biology and are central in the synthetic roles of the PIMs, LM, and LAM, in particular.

GTs are now grouped into 90 families based on sequence homology and by differentiating between inverting and retaining enzymes by the Carbohydrate-Active enZymes database (CAZy: http://afmb.cnrs-mrs.fr/CAZY/ as November 2007). The CAZy database of enzymes involved in carbohydrate metabolism is maintained by the Glycobiology unit at Architecture et Fonction des Macromolécules Biologiques Centre National De La Recherche Scientifique (AFMB-CNRS) in Marseille, France, and is the authoritative source of information about glycosyltransferases. The glycosyltransferase section of CAZy contains over 7000 sequences, organized into 90 families on the basis of high-sequence similarity to one or more founding members with experimentally demonstrated GT activity. In addition, proteins with similar sequences, but different catalytic mechanisms, tend to be placed in separate families (Liu and Mushegian, 2003). Of the roughly 3,900 ORFs found in *M. tuberculosis,* approximately 41 encode putative GTs, a majority of which require NDP-sugar donors. Analysis of the resolved X-ray structures of the various GTs has suggested there are far fewer families, all with a possible common evolutionary origin (Murzin *et al.,* 1995). Moreover, classification of GTs can likewise be described in terms of functional parameters, as the catalytic mechanism used by enzymes within a GT family is consistent, and similar structural elements are employed in families having the same fold, irrespective of the stereochemistry of the catalyzed reaction (Persson *et al.*, 2001). Three superfamilies of GTs have been thus identified; GT-A, GT-B, and GT-C (Unligil and Rini, 2000; Liu and Mushegian, 2003).

The GT-A and GT-B superfamilies contain mainly soluble and peripheral membrane associated proteins which use NDP-sugars as glycosyl donor substrates. The GT-C superfamily contains integral membrane proteins with dependency for polyprenyl-linked glycosyl donors, and this superfamily was discovered by iterative BLAST searches and by structural comparisons. No X-ray crystal structure has been solved for any of its members (Oriol *et al.*, 2002; Liu and Mushegian, 2003). However, families within a superfamily can use different mechanisms. The catalytic mechanisms by which the glycosyl hydrolysis and transfer reaction occurs leads to either inversion or retention of the anomeric sugar binding, and the enzymatic formation of an α- or β-glycosidic bond is therefore determined by the mechanism used by the enzyme and the anomeric configuration of the donor substrate (Sinnott, 1991).

In the inverting mechanism, the acceptor is thought to perform a nucleophilic attack at carbon 1 of the NDP-sugar donor, and the anomeric configuration of the added sugar is changed. In the retaining mechanism, the process is a two-step double displacement reaction, which first involves the formation of a glycosyl-enzyme intermediate, followed by the release of the nucleoside diphosphate and the subsequent attack of the glycosyl enzyme by the acceptor. The anomeric configuration of the transferred sugar is retained (Withers *et al.*, 2002). Figure 2.6.1 illustrates the proposed mechanisms of inverting and retaining glycosyltranferases.

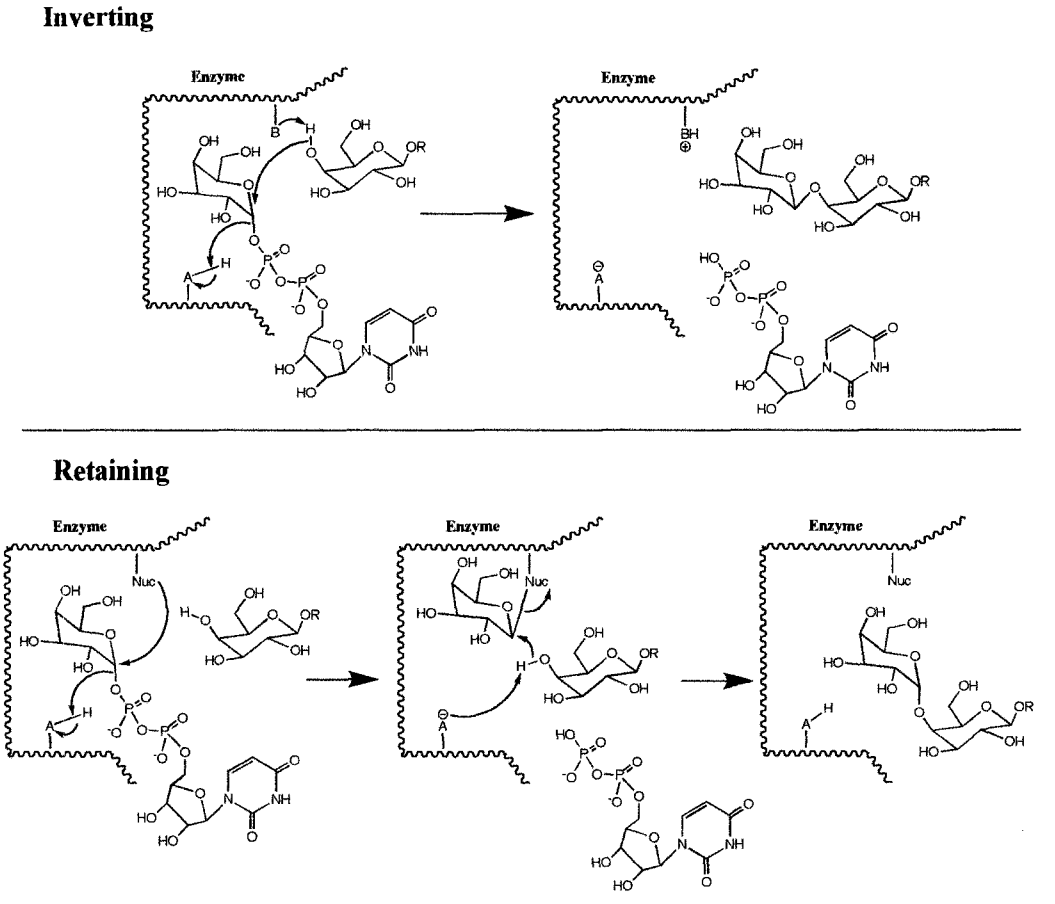

Figure 2.6.1. Proposed catalytic mechanisms of inverting and retaining α-glycosyltransferases. LgtC catalyzes the transfer of D-galactopyranose from UDP-Gal to the lipopolysaccharide core oligosaccharide of *Neisseria* as a retaining α-galactosyltransferase. This figure however, illustrates how both the inverting and retaining mechanisms might operate for such an α-galactosyltransferase with these substrates. A nucleophile is required to attack the anomeric center of the donor sugar to form the glycosidic bond to the enzyme. Either an acid catalyst (A) or a metal ion may be required to provide general acid assistance to the cleavage of the exocyclic carbon 1, oxygen 1 bond, as well as a general base (B) to deprotonate the reactive hydroxyl of the acceptor. Figure adapted from Persson *et al.*, 2001.

The involvement of a general acidic amino acid such as a glutamate or an aspartate residue is typically utilized to initiate the catalytic glycosyl transfer (Unligil and Rini, 2000; Davies, 2001; Ramakrishnan *et al.*, 2002). In the inversion and retention

mechanisms of glycosyl binding and hydrolysis, the residues with acidic or polar side chains, typically aspartate residue, function as the general base and nucleophile (Sinnott 1991; McCarter and Withers 1994). The so-called DxD motif, which is found in many groups of both inverting and retaining GTs is thought to be involved in the binding of a divalent cation, most commonly Mn^{2+} or Mg^{2+}, and in catalysis (Breton *et al.* 1998; Wiggins and Munro, 1998; Breton and Imberty, 1999; Unligil and Rini, 2000). For example, when Mn^{2+} and UDP-galactose are complexed with the retaining galactosyltransferase LgtC, a single Mn^{2+} is coordinated by the two phosphate oxygens of the uridine diphosphate as well as the side chain atoms of His244, Asp103, and Asp105 (the two Asp residues that are in the DXD motif). The Asp103 provides 1 side chain oxygen, and Asp105 provides both side chain oxygen atoms in a bidentate interaction (Persson *et al.,* 2001). This is similar to the mechanism involving the inverting N-acetylglucosaminyltranferase I (GnT-1) family 13 glycosyltransferase belonging to superfamily GT-A (Kozmon and Tvaroska, 2006). GnT-1 adds the GlcNAc residue to carbon 2 of a terminal mannose in the $Man_5GlcNAc_2$-Asn-X oligosaccharide acceptor in the beginning stages of the biosynthesis of hybrid and complex *N*-linked glycans in the Golgi apparatus (Figure 2.6.2).

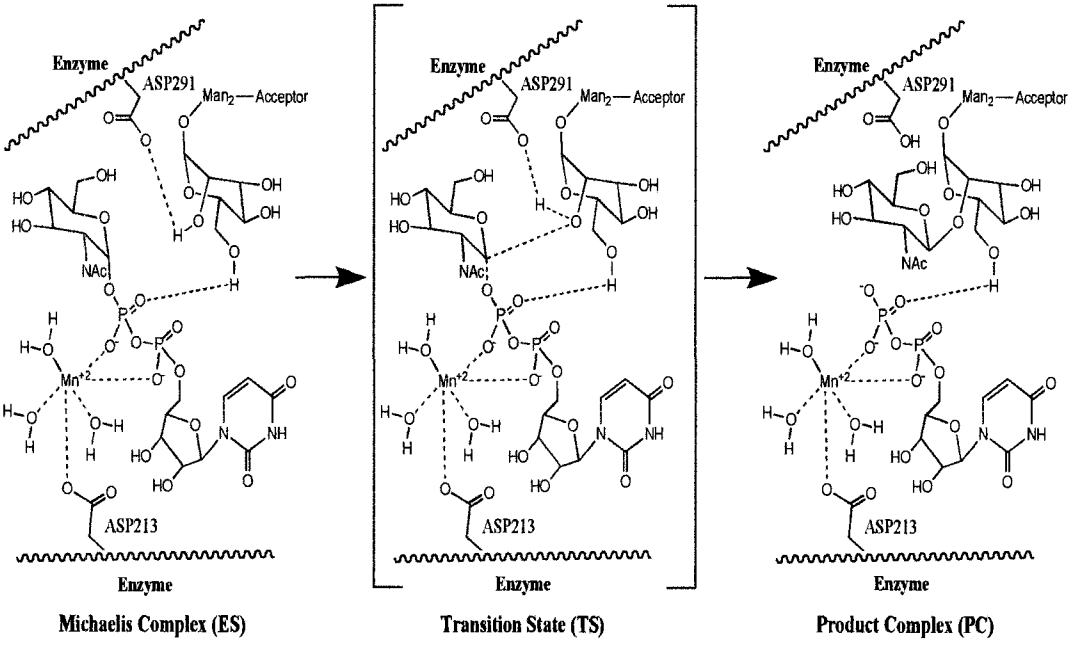

Figure 2.6.2. Schematic of the proposed inverting mechanism by GnT-1. *N*-acetyl-glucosamine (GlcNAc) is transferred from UDP-GlcNAc to carbon 2 of a mannose in the $Man_5GlcNAc_2$-Asn-X oligosaccharide acceptor via the inverting mechanism by GnT-1. Mn^{2+} is required to provide general acid assistance to the cleavage of the exocyclic carbon 1, oxygen 1 bond. This is a nucleophilic displacement of UDP at the anomeric carbon 1 of the GlcNAc residue of UDP-GlcNAc by the hydroxyl group of at carbon 2 of the oligosaccharide acceptor. Figure adapted from Kozmon and Tvaroska, 2006.

It is possible that the bound divalent cation acts in the catalysis reaction by polarizing a water molecule, which may then attack carbon 1 of the bound sugar, however, the exact identity of either nucleophile or general base has not been directly elucidated in these cases (Liu and Mushegian, 2003). However, in the GTs in the GT-B superfamily, there is no evidence of a bound metal ion associated with catalysis, but there are several partially conserved acidic residues that are involved in interactions with the substrate, and in some enzymes, the catalytic role has been proposed for two glutamic

acid residues in the carboxy-terminal E-X_7-E motif (Cid *et al.*, 2000; Bourne and Henrissat, 2001). The GT-B family includes other enzymes involved in sugar metabolism, such as sugar epimerases (Wrabl and Grishin, 2001), adding to the growing list of examples in which the catalytic activity is thought to have changed during the evolution of sequence family (Mushegian and Koonin, 1994; Copley and Bork, 2000; Smit and Mushegian, 2000; Nagano *et al.*, 2002). There is no evidence of the common evolutionary origin of the putative DxD loop in GT-C and the DxD motif in GT-A. The DxD tripeptide in the GT-C superfamily is located at the carboxy-terminal end of the first transmembrane helix, and is often followed by a small patch of hydrophobic amino acids, which are predicted to be part of the same extracellular loop.

Functionally important short amino acid motifs such as the DxD motif, may be clues to the common ancestry of the enzymes that share them, or could have evolved convergently in different lineages of evolutionarily unrelated GTs. Analysis of the similarity of the three-dimensional structures of GTs is also used in prediction of evolutionary common origins of proteins sharing a short sequence motif. GTs with the DxD motif typically share common structural elements, in particular the Rossmann-like αβα three-layer with seven-stranded β-sheets of the 3214657 topology, in which strand 6 is antiparallel to the rest (Liu and Mushegian, 2003; http://scop.mrc-lmb.cam.ac.uk/scop/data/scop.b.d.hj.A.html). However, there are some nucleotidyltransferases which also possess the same fold and variations of the functionally important DxD tripeptide (Blankenfeldt *et al.*, 2000; Mosimann *et al.*, 2001; Olsen and Roderick, 2001). However there is no way to statistically differentiate between convergent and divergent three-dimensional structures by direct comparison of the atomic coordinates. In contrast,

statistical analysis of random versus non-random (evolutionarily relevant) sequence matches is well understood (Karlin and Altschul, 1990). Therefore, the strongest support for the common origin and divergent evolution of the three-dimensional protein structures comes from matching their sequences in the context of large sequence databases (Aravind and Koonin, 1999; Copley and Bork, 2000; Nagano *et al.*, 2002).

While glycosyltransferase members of the GT-A and GT-B superfamily are found throughout bacterial species, the GTs that belong to the GT-C superfamily are unique to the order of *Actinomycetales* and mostly form their own GT families. It is assumed that GTs utilizing the soluble NDP-sugar donors are catalytically active on the cytosolic side of the plasma membrane while GTs dependent on lipid-linked donors (*i.e.* polyprenyl-dependent GTs) are active mainly on the extracytoplasmic side. Resolved X-ray structures of members of the GT-A superfamily are based on the Rossmann-like fold, one of the most common arrangements of protein spatial structure, observed in dozens of diverse families of enzymes (Lesk, 1995). They consist of a mixed α/β fold, organized into two tightly associated domains, an N-terminal NDP-sugar binding domain, and a C-terminal acceptor binding domain (Tarbouriech *et al.*, 2001). In the most basic arrangement, GT-A superfamily members have extended β-stranded and α-helical regions that alternate along the length of the protein, with all strands forming a central relatively planar β-sheet, and with helices filling two layers, one on each side of the plane. As with many other Rossmann-like folds, the amino-terminal β-strand of the GT-A proteins is located in the middle of the sheet (Liu and Mushegian, 2003). Another typical feature of Rossmanoid enzymes is that the functionally important, conserved residues are

often located in the carboxy-termini of the α-strands or in the adjoining loops (Lesk, 1995).

The typical structure of GT-B proteins is comparable and comprises two distinct domains of Rossmann type folds (α-β-α sandwiches) connected by a flexible hinge, thereby giving rise to a cleft between the domains (Ha *et al.*, 2000; Mulichak *et al.*, 2001). The binding domains for acceptor and donor substrates are reversed in GT-B enzymes, consequently leading to a NDP-sugar binding site in the C-terminal domain (Coutinho *et al.*, 2003). Generally the most conserved domain of GTs in those two superfamilies is that for NDP-glycosyl binding. Distant evolutionary relationships between GTs and a monophyletic origin of 15 families in the CAZy database have been demonstrated recently using PSI-BLAST searches (Wrabl and Grishin, 2001). The newly established superfamily is an extension of the GT-B superfamily and includes over 2700 proteins, which represent all three domains of living organisms and almost every completely sequenced genome so far. The CAZy database uses a classification scheme that entails capturing both evolutionary divergence, in the form of groups on the basis of high-sequence similarity, and functional variation, in the form of separating retaining and inverting enzymes, even if their sequences are similar. Such classification is based only on sequence relationships, and in many cases, the distance between the related sequences is too high for extrapolation of the exact mechanism of glycosyl transfer (Liu and Mushegian, 2003). Therefore, as more information comes from structural and mechanistic studies of various glycosyltransferases, sequence-based computational prediction of inverting versus retaining mechanisms in these enzymes should become more reliable.

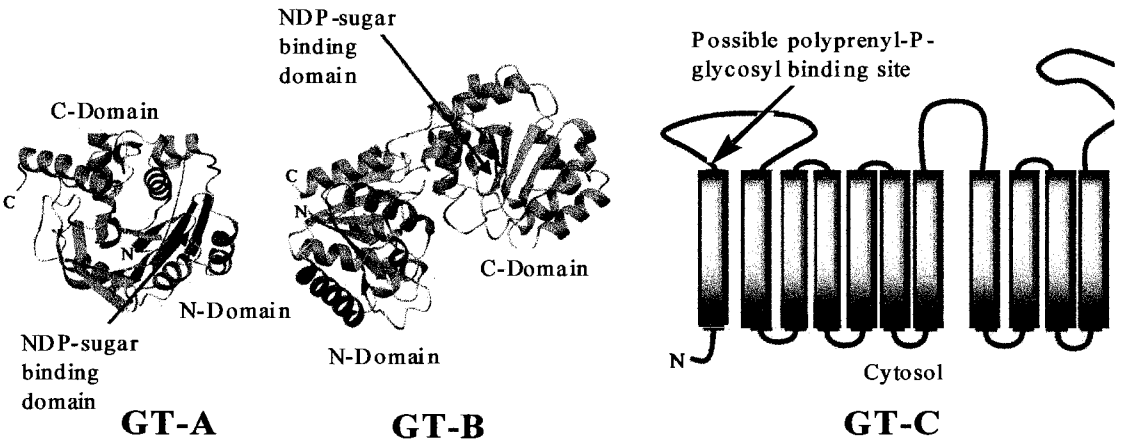

Figure 2.6.3. Schematic representations of glycosyltransferases from the GT superfamilies. X-ray resolved structures of SpsA (PDB 1QGS) from *Bacillus subtilis* and MurG (PDB 1F0K) from *Escherichia coli* represent GT-A and GT-B respectively in this illustration. The generalized topology model for the GT-C in *M. tuberculosis* is that of an integral membrane protein. Figure adapted from Ha *et al.*, 2000; Tarbouriech *et al.*, 2001; Mulichak *et al.*, 2001 and Berg *et al.*, 2007.

In *M. tuberculosis* $H_{37}Rv$, the characterized GT genes are more or less evenly distributed on the $H_{37}Rv$ chromosome. A survey of the *M. tuberculosis* genome by searching for genes with predicted polysaccharide-associated functions and linkage to known AG biosynthetic genes led to the identification two prominent GT containing gene clusters, each holding nine proposed GT genes. One cluster is located in a region bound by *Rv1500* to *Rv1526c* and contains at least eight putative GTs, all classified as inverting enzymes of the GT-1 and the GT-2 families utilizing NDP-sugars, and the other in a region bound by *Rv3779* to *Rv3809c,* described as "the cell wall biosynthetic cluster" (Cole *et al.*, 1998; Belanger and Inamine, 2000). This cluster includes 31 genes implicated in AG, LAM and mycolic acid biosynthesis (Cole *et al.,* 1998; Mikušová *et al.*, 2000).

Some prominent members of this cluster include the arabinosyltransferases involved in AG and LAM synthesis; the Emb proteins. They are a family of large transmembrane GTs of over 1100 amino acid residues in size with a cytoplasmic N-terminal domain, 13–15 transmembrane regions, and a large extracytoplasmic C-terminal domain (Berg *et al.,* 2003; Seidel *et al.,* 2007; Amin *et al.,* 2008). As many as five ORFs in this cluster are possible Pol-P-sugar dependent GTs (Berg *et al.,* 2007), and at least eight GTs overall are thought to be present here. Many of the GTs involved in cell wall biosynthesis are membrane-associated, particularly those with their substrates located in the cytoplasmic membrane.

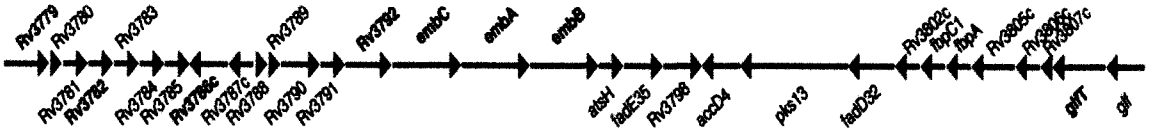

Figure 2.6.4. The cell wall biosynthetic cluster of *M. tuberculosis.* Figure is from Berg *et al.*. 2007.

Berg *et al.* investigated 18 amino acid sequences of all of the ORFs of $H_{37}Rv$ thought to belong to any of the three GT superfamilies. Since most of the classified GTs of *M. tuberculosis* are thought to be NDP-sugar dependent, they are classified within the families GT-1, GT-2, GT-4, GT-20, GT-28, and GT-35. *M. tuberculosis* has five ORFs classified into GT-1, a family of GTs using the inverting mechanism, since most of them use α-linked glycosyl donors, leading to glycosyl transfers in the β-configuration. Conversely, this family also includes many GTs with specificity for β-linked glycosyl

donors, such as TDP-vancosamine and dTDP-Rha, and thereby produce α-linked products. Furthermore, the five GT-1 proteins from *M. tuberculosis* seem to use β-linked glycosyl donors. Rv1524, Rv1526c and Rv2739 may utilize β-linked glycosyl donors since they share 25-60% amino acid identity with the rhamnosyltransferase RtfA of *M. avium* (Berg *et al.*, 2007). Rv2958c and Rv2962c use dTDP-β-Rha as a donor substrate and are involved in the biosynthesis of PGLs and *p*-HBADs (Perez *et al.*, 2004). Only members of the GT-2 family are ones with a GT-A fold; as many as 16 ORFs of *M. tuberculosis* H_{37}Rv are members, including LosA, Ppm1, WbbL, Rv3782, and GlfT. The remaining five belong to superfamily GT-B. Family GT-2 enzymes carry the hallmark DxD motif that binds a divalent cation to lock the NDP-sugar donor in an inverting mechanism, thereby forming a glycosidic bond in the β-configuration (Charnock and Davies, 1999).

The GT-4 family is the largest family of retaining GTs that bind glycosyl residues in the anomeric α-configurations. This family contains many bacterial GTs involved in synthesis of cell envelope structures, such as lipopolysaccharides and capsular polysaccharides. It is probable that GT-4 family members Rv3032, Rv0225, Rv1212c, and Rv2188c are involved in biosynthesis of LAM, glycogen, α-glucan and polymethylated polysaccharides (PMPS) of mycobacteria, since their products contain glycosidic bonds in α-configurations (Stadthagen *et al.*, 2007; Berg *et al.*, 2007). Mycobacteria produce 6-*O*-methylglucosyl-containing lipopolysaccharide (MGLP) and 3-*O*-methylamnnose polysaccharide, which serve to regulate fatty acid biosynthesis.

Experimentally, overexpression of the glycosyltransferase Rv3032 in *M. smegmatis* stimulates MGLP production, while its disruption in *M. tuberculosis* can lead to a reduction of MGLP. The glycogen content of the cell was therefore reduced, suggesting Rv3032 is a glucosyltransferase responsible for elongating MGLP (Stadthagen, *et al.*, 2007). As such, seven members of this class from *M. tuberculosis*, including the two mannosyltransferases PimA (Rv2610c) and PimB (Rv0557), are involved in PIM synthesis (Kordulakova *et al.*, 2002; Schaeffer *et al.*, 1999). Interestingly, *M. tuberculosis* has no GT belonging to either GT-3 or GT-5 families.

Ppm1, a nonessential polyprenylphosphomannnose synthase, is one of the most conserved GTs in the GT-2 family (Gurcha *et al.*, 2002), and forms C_{50}-P-Man (DPM) from GDP-Man in higher PIM, LM, and LAM synthesis. This protein probably has dual functionality as its N-terminal domain contains seven predicted transmembrane segments showing high sequence and topology similarities to an apolipoprotein *N*-acyltransferase that was characterized in *E. coli* (Robichon *et al.*, 2005), while its soluble C-terminal domain possesses ManT activity (Baulard *et al.*, 2003). Interestingly, these two domains are expressed as two separate proteins in other mycobacteria (Gurcha *et al.*, 2002).

The polyprenyl-dependent glycosyltransferases are a diverse lot that spans eleven families across eukaryotes and prokaryotes. The dolichyl-dependent glucosyltransferases (ALG6, ALG8, and ALG10) and ManTs (ALG3, ALG9, ALG12, PIG-B, PIG-M, PIG-V, and SMP3) form the basis for the CAZy grouping of GTs and has assigned them to six families, GT-22, GT-50, GT-57, GT-58, GT-59, and GT-76, which

so far only contain members of the eukaryotic kingdom (Burda and Aebi, 1999; Kang *et al.*, 2005a). All of these enzymes have been proposed to have a common evolutionary path (Oriol *et al.*, 2002). Polyprenyl-dependent GTs associated with protein *O*-mannosylation function are organized into the GT-39 family which has both eukaryotic and prokaryotic proteins. Besides these seven GT families, CAZy has classified other proposed polyprenyl-dependent GTs into another four families (GT-53, GT-85, GT-86, and GT-87) which consist of proteins restricted to mycobacteria and related species of the order *Actinomycetales.* The GT-87 family contains no members from species beyond *Actinomycetales.* These have recently been characterized as AraTs and ManTs involved in AG and LAM biosynthesis. All eleven families of polyprenyl-dependent GTs consist of integral membrane proteins having 8-13 predicted transmembrane domains. The sequence homology between them is in general very low, but conserved amino acid motifs have been found (Oriol *et al.*, 2002). In common is a modified DxD motif (e.g. DxE, ExD, DDx, DEx, or EEx), typically located in the first or the second predicted extracytoplasmic loop. The position of this motif and a similar topology pattern among these polyprenyl-dependent GTs have suggested that they are structurally related, and therefore they have been organized into the GT-C superfamily (Liu and Mushegian, 2003). A topology study of the putative mycobacterial GT-C proteins was performed (TMHMM 2.0) and discovered that the N-terminus of these proteins is most often predicted to be in the cytosol, and the C-terminus is often a larger soluble domain. Generally, three sections of clustered TM domains were observed, and these clusters are connected with two longer loops of which the GT-C motif is part of the first of these loops (Berg *et al.,* 2007). This topology is similar to what was predicted for GT-C

proteins of eukaryotic origin (Oriol *et al.*, 2002), indicating that they may have a common ancestor of which only the GT-C motif has remained fairly conserved.

Site directed mutagenesis has shown the importance of the modified DxD motif, as substitution of an aspartic acid in this motif in the human PIG-M (a ManT in PIG biosynthesis) (Maeda *et al.*, 2001), in PimE of *M. smegmatis* (ManT in PIM_5 biosynthesis) (Morita *et al.*, 2006), and in EmbC of *M. smegmatis* (AraT in LAM biosynthesis) (Berg *et al.*, 2005), resulted in all cases, in loss or reduction of the enzyme activity. Although the exact function of this motif has not been elucidated, a comparison can be made to NDP-sugar dependent GTs, many of which carry also a DxD motif involved in binding of the donor substrate via a divalent cation (Unligil and Rini, 2000). The acidic motifs of PIG-M, PimE, and EmbC and the corresponding motifs of other GT-C proteins appear to be involved in the binding site for Pol-P-sugar donors. Moreover, there is an aromatic residue commonly clustered together with one or more prolines next to an additional partially conserved acidic residue about 20-40 amino acids downstream of the modified DxD motif (Liu and Mushegian, 2003). These conserved residues of the GT-C motif are part of the same predicted loop in the GT-C proteins and may constitute elements important for binding of a lipid-linked sugar donor and/or for catalytic activity (Berg *et al.,* 2007). Sequencing of the complete genome of *M. tuberculosis* by Cole *et al.* elucidated only one ORF, *Rv1002c* (GT-39), with similarity to known polyprenyl-dependent GTs. Rv1002c is characterized as a ManT involved in a Sec-dependent pathway for protein *O*-mannosylation (Cole *et al.*, 1998; VanderVen *et al.*, 2005). Further bioinformatic investigation identified 10 additional ORFs from *M. tuberculosis* $H_{37}Rv$ as possible members of the GT-C superfamily and four of these candidates were therefore

classified as such, *Rv3792*, *Rv1635c*, *Rv1159*, and *Rv2181* (Liu and Mushegian, 2003; Morita *et al.*, 2006; Kaur *et al.*, 2006).

Polyprenyl dependent GTs are involved in LAM synthesis. The recently identified glycosyltransferase Rv1635c in *M. tuberculosis* is involved in mannose capping of ManLAM by adding of the first mannose residue in an α(1→5) linkage to the terminal β-Ara*f* moieties at the non-reducing ends of the LAM arabinan, and utilizes C_{50}-P-Man as a donor (Chatterjee *et al.*, 1993; Dinadayala *et al.*, 2006). BLAST searches in sequenced genomes of *Actinomycetales* species have identified orthologs to *Rv1635c* only in species known to have ManLAM. Furthermore, the α(1→2)mannosyltransferases, Rv1159 (PimE) and Rv2181 share 25% identity to each other in amino acid sequence, also utilize C_{50}-P-Man as their donor substrate, and are therefore responsible for biosynthesis of PIM_5 and branching of the mannan backbone of LM/LAM, respectively (Morita *et al.*, 2006; Kaur *et al.*, 2006). As such, these two proteins also share sequence similarities with the uncharacterized protein, Rv2673, suggesting that this is also a putative GT-C protein with an analogous function as a C_{50}-P-Man dependent ManT involved in PIM_6 biosynthesis. Therefore, CAZy has grouped these three proteins of *M. tuberculosis* into the GT-87 family.

Another seven ORFs which utilize lipid-linked sugar donors have been identified with characteristics of the GT-C superfamily including *Rv3779*, however the functions for these proteins are most probably involved in the pathways of AG and LAM biosynthesis and in protein mannosylation. *M. tuberculosis* possesses various

glycoproteins, such as the 45 KDa glycoprotein, that are glycosylated with linear α(1→2)- and α(1→3)-oligomannosides that are likely mannosylated by Rv1002c on the extracytoplasmic side of the plasma membrane (Dobos *et al.*, 1996; Michell *et al.*, 2003; VanderVen *et al.*, 2005). Subsequent glycosylations are catalyzed by other GT-C proteins, possibly Rv0541c, judging by its conservation of the amino acid patterns DEx and HPP of its GT-C motif with Rv1002c and other GT-39 proteins. Furthermore, *Rv0541c* is clustered on the chromosome of *M. tuberculosis* with *Rv0539*, a proposed PPM synthase gene homologous to *ppm1* (Berg *et al.*, 2007). Thus, this suggests that Rv0541c may utilize the C_{50}-P-Man substrate generated by Rv0539 for subsequent protein mannosylation.

2.7 CONCLUSION

The mycobacteria possess unique cell walls composed primarily of a mycolyl-arabinogalactan core layer superimposed upon peptidoglycan. Interspersed in this later are various glycolipids such as the PIMs, and lipoglycans such as LM and LAM which confer pathogenesis to many of the mycobacteria. The polyprenyl phosphates are phospholipid anchors central to the synthesis and build-up of many of these cell wall macromolecules. The PIMs, LM, and variants of LAM, are unique molecules that serve to modulate the host immune response. Detailed knowledge of their biosynthesis is therefore important. Many enzymes play a role in their synthesis, and glycosyltransferases in particular, are important to the extracytoplasmic polymerization of these macromolecules. The GTs are a diverse and metabolically critical group of enzymes that are worthy of study, not only from the standpoint of functional elucidation,

but for secondary implications in disease and pathogenesis. A continuing investigation of these enzymes, in particular the Ppm1 analogous Pol-P dependent mannosyltransferase that polymerizes the later forms of PIM, LM, and LAM is warranted, and a topic of Chapter 3. Moreover, separate but relevant studies into the biosynthesis of AG are likewise a topic of reflection in the upcoming Chapter 4.

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CHAPTER 3

CHARACTERIZATION OF *RV3779* IN *MYCOBACTERIUM TUBERCULOSIS*

3.1 INTRODUCTION

The mycobacterial cell envelope consists primarily of a complex multilayered structure of covalently linked peptidoglycan, arabinogalactan (AG), and mycolic acids (thereby forming the mAGP complex) and various non-covalently bound glycosylated lipids (Brennan and Nikaido, 1995; Daffe and Draper, 1998; Dmitriev *et al.*, 2000; Brennan, 2003). Among the most prominent glycolipid components of the cell wall are the phosphatidyl-*myo*-inositol mannosides (PIMs) which can be further glycosylated to form the lipoglycans, lipomannan (LM), and variants of lipoarabinomannan (LAM) (Hunter and Brennan, 1990; Chatterjee *et al.*, 1992; Khoo *et al.*, 1995). These glycolipids and lipoglycans exhibit a broad range of immunomodulatory activities which are implicated in the pathogenesis of tuberculosis and leprosy (Chatterjee and Khoo, 1998; Nigou *et al.*, 2003).

The proposed biosynthesis pathway, PI → PIM → LM → LAM, has been postulated from the extensive evidence obtained from biochemical and genetic approaches (Khoo *et al.*, 1995; Besra and Brennan, 1997; Schaeffer *et al.*, 1999;

Kordulakova *et al.*, 2002; Kremer *et al.*, 2002). The predominant species of PIMs found in mycobacteria are the monoacylted and diacylated forms of phosphatidyl-*myo*-inositol dimannosides ($AcPIM_2$ and Ac_2PIM_2) and phosphatidyl-*myo*-inositol hexamannosides ($AcPIM_6$ and Ac_2PIM_6). Other PIM species (PIM_1, PIM_3-PIM_5) are typically metabolic intermediates present only in very small amounts in mycobacterial cells. The biosynthesis of the higher forms of PIM (polar PIMs; PIM_4-PIM_6), is still poorly understood. Recently, a novel mannosyltransferase (ManT) encoded by the *pimE* (*Rv1159*) gene involved in the synthesis of polar PIMs has been discovered. PimE catalyzes the transfer of the fifth mannose to $AcPIM_4$ leading to the formation of $AcPIM_5$ (Bifani *et al.*, 1999; Kordulakova *et al.*, 2003; Morita *et al.*, 2006). Mannosyltransferases involved in polar PIM synthesis were shown to require polyprenyl monophosphomannose (PPM), specifically C_{35}-P-Man or C_{50}-P-Man as a sugar donor, unlike the early steps in PIM biosynthesis which directly utilize GDP-Man as a mannosyl donor. In prokaryotes, the utilization of lipid-linked monosaccharides (polyprenol-P sugar) is enzymatically associated with reactions that occur on the cytosolic side of the cell membrane. Similarly in eukaryotes, dolichol-P-sugar is utilized in the endoplasmic reticulum lumen. However, the larger lipid-linked polysaccharides polar PIMs and LAM are likely synthesized extracytoplasmically (Bifani *et al.*, 1999; Morita *et al.*, 2006).

PPM is a key intermediate in transferring mannosyl residues through the plasma membrane and is therefore centrally involved in PIM, LM, and LAM synthesis. Besra and co-workers demonstrated that PPM was utilized in the synthesis of a novel α (1→6)-linked linear form of LM by mycobacterial membranes (Besra *et al.*, 1997).

Moreover, inhibition studies with the lipopeptide antibiotic amphomycin were shown to inhibit the synthesis of PIM_4, PIM_5, and PIM_6 (from acyl-PIM_2), suggesting these enzymatic steps utilize C_{50}-P-Man as the mannosyl donor, rather than GDP-Man (Morita *et al.*, 2004). Amphomycin forms a complex with the Pol-P precursor of PPM (Besra *et al.* 1997). Therefore, identification of the enzyme(s) responsible for synthesizing PPM from GDP-Man are important in the understanding of PIM, LM, and LAM biosynthesis. However, only one polyprenyl monophosphomannose synthase has yet been discovered thus far in *M. tuberculosis*. It is encoded by *ppm1* (*Rv2051c*), is not essential, and catalyzes the synthesis of PPM from GDP-Man and Pol-P (Gurcha *et al.*, 2002). The disruption of the *ppm1* ortholog in *Corynebacterium glutamicum* (Cg-*ppm1*) produced lipoglycan-less mutants (Gibson *et al.*, 2003). However, PPM synthase mutants of mycobacteria have not yet been reported.

In this report, we have identified an uncharacterized mannosyltransferase encoded by the *M. tuberculosis Rv3779* gene. We have found it to be involved in PIM/LM/LAM synthesis. A survey of the *M. tuberculosis* genome by searching for genes with predicted polysaccharide-associated functions and linkage to known AG biosynthetic genes led to the identification of a cluster of 31 genes thought to be involved in AG biosynthesis (Cole *et al.*, 1998; Belanger and Inamine, 2000). This cluster is anchored by *embCA*, and *B*, and *glf* (*Rv3809c*). Genes involved in the biosynthesis of surface and cell envelope polysaccharides in other bacteria are likewise clustered (Schnaitman and Klena, 1993; Heinrichs *et al.*, 1998; Whitfield and Roberts, 1999). Throughout this cluster are genes encoding enzymes with similarity to other polysaccharide biosynthetic enzymes, including AG synthetic proteins, members of the

ABC transporter system, mycolyl transferases, and Rv3779. Interestingly, there is little intragenic space within the cluster, as these genes are tightly packed, suggesting the presence of several operons within this region (Belanger and Inamine, 2000). Moreover, five open reading frames (ORF) in this cluster are possibly PP-sugar dependent GTs (Berg *et al.*, 2007).

Rv3779 was preliminarily identified by Berg *et al.* as a putative glycosyltransferase (GT) that likely belongs to the GT-C superfamily, due to a conserved DXD motif found in the membrane bound GTs of this family (Berg *et al.*, 2007). GTs are now grouped into 90 families (as of November 2007) based on sequence homology and differentiating inverting and retaining enzymes by the Carbohydrate-Active enZymes database (CAZy: http://afmb.cnrs-mrs.fr/CAZY/) (Campbell *et al.*, 1997; Campbell *et al.*, 1998). Of the roughly 3,900 ORFs found in *M. tuberculosis,* approximately 41 encode putative GTs, a majority of which require NDP-sugar donors. Resolved X-ray structures of the various GTs has suggested there are far fewer families, all with a possible common evolutionary origin (Murzin *et al.*, 1995). Currently three superfamilies of GTs are identified, GT-A, GT-B, and GT-C. The GT-A and GT-B families contain mostly soluble and peripheral membrane proteins using NDP-sugars as donor substrates. The GT-C superfamily comprises integral membrane proteins that utilize PP-linked sugar donors. The catalytic mechanism used by members of a superfamily are consistent; however families within a superfamily can utilize different mechanisms. Most of the GT genes found in *M. tuberculosis* are more or less evenly distributed across the genome; however, there are two GT-containing gene clusters. One is framed by *Rv1500* to *Rv1526c*, and the other framed by *Rv3779* to *Rv3809c* (Berg *et*

al., 2007). Therefore, after further investigation and biochemical characterization of the role of Rv3779 presented herein, we have concluded that Rv3779 possesses PPM synthase activity homologous to Ppm1, and thus plays a central role in the synthesis of the PIMs, LM, and LAM.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial strains and other materials

Mycobacterium tuberculosis $H_{37}Rv$ strain TM102 was propagated in Middlebrook 7H9 broth (BD) supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC). *Escherichia coli* DH5α and *M. smegmatis* mc^2155 strains were grown in Luria-Bertani broth. All liquid cultures were grown at 37°C with constant agitation. Cell growth density was monitored by optical density (OD) at 600 nm using a spectrophotometer (Bausch & Lomb). When needed, Middlebrook 7H11 agar supplemented with 10% OADC was used as the solid selection medium for *M. tuberculosis* and *M. smegmatis*, whereas *Escherichia coli* was grown on LB agar. Antibiotics were used at the following concentrations: 100 μg/ml of ampicilin, 25 μg/ml of kanamycin, and 50 μg/ml hygromycin. Sucrose at a final concentration of 2% was added to the solid medium to select for the double crossover mutants of *M. tuberculosis* $H_{37}Rv$.

M. tuberculosis $H_{37}Rv$ genomic DNA and monoclonal antibody CS-35 were received from NIH/NIAID Contract N01-AI-75320, "Tuberculosis Research Material and Vaccine Testing" at Colorado State University, Fort Collins, CO. Restriction enzymes for cloning were obtained from New England BioLabs (Ibswich, MA). Most

chemicals were at least analytical grade and purchased from Sigma Aldrich (St. Louis, MO). The radiolabeled GDP-[^{14}C]Mannose was obtained from PerkinElmer Life Sciences (Waltham, MA).

3.2.2 Sequence analysis of *tbRv3779*

DNA and amino acid sequence information was obtained from the National Center for Biotechnology Information (NCBI) and the homologous sequences were aligned at EBI server using EBI tools – ClustalW (www.ebi.ac.uk/clustalW). Advanced BLAST searches were performed online at the NCBI. Transmembrane Hidden Markov Model (TMHMM 2.0) and SOSUI were used to predict the membranous nature of the protein, secondary structure, hydrophobicity, and transmembrane topology (Krogh *et al.*, 2001; Kahsay *et al.*, 2005; Hirokawa *et al.*, 1998). Protein family and domain architecture search were performed using Pfam 22.0, a Pfam HMM searcher at http://pfam.janelia.org, from Janelia farm, Howard Hughes Medical Institute (Finn *et al.*, 2006).

3.2.3 Construction of *tbRv3779* mutant (△*Rv3779*)

Disruption of the *M. tuberculosis Rv3779* gene was performed by use of a two-step homologous recombination procedure as previously described (Pelicic *et al.*, 1997). Standard PCR was used to amplify an approximately 3-kb fragment of the *tbRv3779* gene and its flanking region by using the forward primer 5′-CCGGTGGCATACGGATTG GTCGAAA-3' and the reverse primer containing *Xba*I restriction site (bold and underlined) 5′-TAAAA**TCTAGA**GTGTCCGACCA GACCGAC-3′. PCR amplifications were carried out with a PerkinElmer Gene Amp

2400 PCR system using Expand High Fidelity Polymerase (Roche Applied Science, Indianapolis, IN). The PCR fragment was directly cloned into pGEM-T Easy vector (Promega, Madison, WI) yielding pGEM*Rv3779,* which was later digested with *Age*I and blunt-ended with T4 DNA polymerase (Fermentas, Glen Burnie, MD). A 1.2-kb kanamycin-resistance cassette (*km*) was excised from pUC4k (GE Healthcare) using *Hinc*II and ligated to the vector prepared above, resulting in pGEM*Rv3779km*. The plasmid containing the disrupted copy of *Rv3779* was digested with *Not*I and *Xba*I, yielding the 3.1-kb *Rv3779km* fragment, which was then ligated to the *Not*I and *Xba*I site of the pPR27xylE vector giving the final construct pPR27xylE*Rv3779Km* (Jackson *et al.*, 2001). Plasmid pPR27xylE*Rv3779Km* was introduced in *M. tuberculosis* by electroporation, and transformants were selected at 32°C on 7H11-kanamycin. Several colonies were grown in liquid culture and then plated at 39°C on 7H11-kanamycin with 2% sucrose plates.

3.2.4 Electrotransformation of mycobacterial cells

Electrocompetent cells were prepared as described with few modifications. *M. tuberculosis* H_{37}Rv and *M. smegmatis* mc^2155 were grown in 200 ml 7H9-0.05% Tween 80 with 10% OADC to an optical density of 0.6-0.8 at 600 nm (Pelicic *et al.*, 1996). Cells were washed once in 0.05% Tween 80 (in double distilled water), twice in 10% glycerol, and resuspended in 1 ml 10% glycerol. Aliquots (100 µl) of freshly prepared competent cells were mixed with 1 µg of vector DNA in 0.2-cm cuvettes and electroporated with a single pulse (2.5 kV; 25 µF; 1000 ohms). Then 5 ml of fresh media

was added and the culture was incubated for 24 h before plating. Transformants were selected after 4-5 days incubation for *M. smegmatis* or 7-8 weeks for *M. tuberculosis*.

3.2.5 Southern blot analysis

Chromosomal DNA from the *M. tuberculosis* $H_{37}Rv$ wild type and the Δ*Rv3779* strains were extracted as previously described (Belisle and Sonnenberg, 1998). A 6 μg DNA aliquot was digested overnight with *Kpn*I and *Hind*III, resolved by electrophoresis on 0.9% (w/v) agarose gel in 1X Tris-acetate-EDTA (TAE) buffer at 30 V for 12 h, and blotted onto a Hybond N+ nylon membrane (GE Healthcare). Probe hybridization and signal detection were performed using Gene Images AlkPhos Direct Labeling and Detection System (GE Healthcare) following the manufacturer's instructions.

3.2.6 Overexpression of *Rv3779* and complementation

The open reading frame of the *Rv3779* gene was amplified by PCR from *M. tuberculois*s $H_{37}Rv$ genomic DNA using the primer pairs containing the *Nde*I and *Hind*III restriction sites (bold and underlined); Rv3779-forward: TATAA**CATATG**GGCCTGTGGTTCGGTACG and Rv3779-reverse: TATAA**AAGCTT**GGAGTGTGTTGTTGCGGC. Expand High Fidelity polymerase was used and PCR amplification consisted of; 1 denaturation step at 95°C for 4 minutes, followed by 29 additional cycles of denaturation (95°C, 1 minute), annealing (60 °C, 30 seconds), primer extension (72°C, 3 minutes), and a final extension step at 72°C for 10 minutes. The *Rv3779* PCR product was then directly cloned into the pGEM-T Easy

vector, which gave pGEM-T-*Rv3779*. This was confirmed by sequencing for integrity at Macromolecular Resources (Colorado State University).

For protein expression of Rv3779, pGEM-*Rv3779* was digested with *Nde*I and *Hind*III and ligated into the *E. coli* expression vector, pET28b (Novagen, Madison, WI), as well as the mycobacterial expression vector pVV16. pVV16 is a derivative of pMV261 and contains a hygromycin-resistance cassette (*hyg*), a kanamycin-resistance cassette, a C-terminal six-histidine tag, and the hsp60 promoter, and allows genes to be constitutively expressed (Stover *et al.*, 1991). pET28b-*Rv3779* was transformed into *E.coli* BL21(DE3) pLysS with selection by kanamycin and chloramphenicol resistance, while pVV16-*Rv3779* was transformed into the *M. tuberculosis* $\triangle$*Rv3779* mutant and *M. smegmatis* mc^2155 wild-type strains with selection by kanamycin and hygromycin resistance.

3.2.7 Lipid extraction and analysis

Whole cell lipids were extracted in 10 volumes of chloroform/methanol (2:1, v/v) for two consecutive overnight extractions at room temperature, combined, Folch-washed, and dried under a nitrogen stream (Folch *et al.*, 1957). The polar glycolipids were extracted in 1 volume of chloroform/methanol/water (10:10:3, v/v/v) overnight, dried under a stream of nitrogen and resuspended in the same solvent. These lipids were applied to aluminum-backed thin layer chromatography silica Gel 60 (EMD Chemicals, Gibbstown, NJ) and resolved in chloroform/methanol/water (58:25:4, v/v/v). Lipids were also analyzed by two-dimensional TLC were developed with chloroform/methanol/water (60:30:6, v/v/v) in the first dimension and chloroform/acetic

acid/methanol/water (40:25:3:6, v/v/v/v) in the second dimension (Minnikin *et al.*, 1984). Lipids were visualized by charring with α-naphthol/H_2SO_4.

The remaining pellet was further extracted with 1 volume of chloroform/methanol/water (10:10:3, v/v/v) for extraction of polar-lipid-linked polymers which were applied to aluminum-backed thin layer chromatography silica gel 60 plate (EMD Chemicals) and developed in chloroform/methanol/water (60:35:8, v/v/v) (Rush *et al.*, 1993). Visualization of lipid-linked polymers was done by spraying plates with α-naphthol/H_2SO_4.

3.2.8 Extraction and analysis of cell wall bound mycolic acids from *M. tuberculosis* strains

Cells were grown as previously described, harvested, washed and dried. Then, 50 mg dry weight of cells were extracted by two consecutive extractions of 2 ml of chloroform/methanol/water (10:10:3, v/v/v) for 3 hours at 50°C. The bound lipids from the delipidated extracts were released by the addition of 2 ml of 15% aqueous tetrabutylammonium hydroxide at 100 °C overnight, followed by the addition of 1 ml of dichloromethane, 250 µl of iodomethane, and 2 ml of water. The entire reaction mixture was then mixed for 30 minutes. The upper, aqueous phase was discarded, and the lower, organic phase was washed with 3 ml of 1 M hydrochloric acid, followed by 3 ml of water and evaporated to dryness. The crude mycolic acid methyl esters (MAMEs) were dissolved in a mixture of 0.2 ml toluene and 0.1 ml acetonitrile, followed by the addition of another 0.2 ml of acetonitrile. This was incubated at 4°C for 1 hour. The precipitated MAMEs were removed from the supernatant by centrifugation and resuspended in

dichloromethane prior to TLC analysis. TLC of MAMEs was performed on Silica Gel-60 plates (EMD chemicals) and developed a total of six times using petroleum ether/diethyl ether (95:5, v/v). Individual MAMEs were visualized by charring at 100°C using 5% molybdophosphoric acid in ethanol (Besra, 1998).

3.2.9 MALDI-TOF mass spectrometry analysis

Preparative TLC was performed to isolate lipids of interest for analysis by mass spectrometry. Briefly, polar lipids were separated on a set of TLC plates that were run concurrently. One plate was charred with the α-naphthol/H_2SO_4 spray to determine the locations of lipids. The corresponding areas were scraped from the uncharred plates, dissolved in chloroform/methanol (2:1, v/v), and subjected to MALDI-TOF MS analysis at Macromolecular Resources, Colorado State University, Fort Collins, CO.

MALDI-TOF MS was performed with a matrix of 2,5-dihydroxybenzoic acid at a concentration of 10 mg/ml in a solution consisting of water/acetonitrile (1:1, v/v) containing 0.1% trifluroacetic acid and 10 μg of chloroform/methanol (2:1, v/v) extract mixed with 1.0 μl of the matrix solution. A Uniflex MALDI-TOF/TOF (Bruker, Bremen, Germany) was utilized for the analysis using reflector mode detection. The mass spectra were recorded in negative mode for the underivatized sample with a 30 ns time delay, a grid voltage of 94%, and a full accelerating voltage of 25 kV. The mass spectra were then mass aligned through external calibration.

3.2.10 Lipoglycan extraction and analysis

The remaining pellet from 3.2.8 containing LM and LAM was extracted by the hot phenol-water method, as described previously with slight modification (Sutcliffe, 2000). Briefly, the pellet was resuspended in a mixture of 200 µl PBS-saturated phenol and 200 µl water and incubated at 80°C for 2 h. Centrifugation at 12,000 rpm for 5 min was performed after adding 200 µl chloroform to form the biphase. The upper aqueous phase was removed and dialyzed to remove traces of phenol. The crude aqueous extract containing lipoglycan was then lyophilized and analyzed by SDS-PAGE, followed by periodic acid Schiff staining on 10–20% gradient Tricine SDS-polyacrylamide gels (Invitrogen, Carlsbad, CA) (Prinzis *et al.*, 1993). Western blotting was performed on nitrocellulose membranes at 45 V for 1 h. Immunodetection was performed with monoclonal antibody CS-35, specific for the arabinan component of LAM, and concanavalin A (ConA) which binds specifically to terminal Man*p* residues (Kaur *et al.*, 2002). The nitrocellulose membranes probed with ConA-peroxidase were subsequently developed with the 4-chloro-1-naphthol/3,3′-diaminobenzidine, tetrahydro chloride substrate kit (Pierce, Rockford, IL). In the case of ^{14}C-radiolabeled products, membranes were exposed to Kodak Biomax MR film at -70 °C for 20 days.

3.2.11 Monosaccharide composition

Alditol acetates were prepared as described (McNeil *et al.*, 1989). Gas chromatography (GC) of alditol acetates was performed on an HP Gas Chromatography model 5890 fitted with an SP 2380 column with 30 m x 0.25 mm internal diameter (Supelco, Bellefonte, PA) at an initial temperature of 50 °C, held for 1 min, rising to 170 °C at 30 °C/min before increasing to 260 °C at 5 °C/min (Berg *et al.*, 2005). The results

were compared with reference to known sugars; *scyllo*-inositol was used as an internal standard. The areas of ions from each component were compared with the corresponding areas generated from a standard with known molar ratios (Sweet and Albersheim, 1975).

3.2.12 Preparation of Mycobacterial membrane enriched enzyme extracts

Preparation of enzymatically active membranes was carried out by methods described by Mikusova *et al.* with slight modification (Mikuova *et al.*, 2006). Briefly, *M. smegmatis* pVV16 and *M. smegmatis* pVV16-*Rv3779* were harvested at the mid-logarithmic growth phase and washed once with buffer A (50 mM MOPS buffer, 10 mM $MgCl_2$, 5 mM mercaptoethanol, pH 8.0, pH adjusted with KOH). Then, 10 g (wet weight) cell pellet was resuspended in the same buffer and disrupted by sonication using a Sanyo model Soniprep 150. The cells were sonicated at 4°C for 10 cycles of 60-s pulses with 90-s cooling period between each cycle. The lysate was then centrifuged at 27,000 x g for 30 min. The resulting supernatant was ultracentrifuged in a Beckman model L7-80 ultracentrifuge at 100,000 x g for 2 h. The cell membrane enriched pellet was resuspended in buffer A and final protein concentrations were measured using the bicinchoninic acid (BCA) protein assay (Smith *et al.*, 1985).

3.2.13 Preparation of *E. coli* cell lysate and membrane enriched extracts

E. coli BL21 (DE3) pLysS with pET28b for control and pET28b-*Rv3779* were grown as previously described. Cell pellets were resuspended in buffer A and disrupted by sonication at 4°C for 7 cycles of 20-s pulses with 90-s cooling period between each cycle. Then, 10% of this cell lystate was aliquoted and stored at -70°C for the enzymatic assay, and the rest was centrifuged at 27,000 x g for 30 min. The resulting supernatant

was ultracentrifuged in a Beckman model L7-80 ultracentrifuge at 100,000 x g for 2 h. The cell membrane enriched pellet was resuspended in buffer A. Protein concentrations of the cell lysate and membrane enriched fraction were measured by BCA protein assay.

3.2.14 *In vitro* mannosyltransferase assays

Membrane fractions from *M. smegmatis*/pVV16 were assayed and compared with *M. smegmatis*/pVV16-*Rv3779* for *in vitro* mannosyltransferase activity. The reaction mixtures contained 0.25 μCi of GDP-[^{14}C]Man (specific activity 305 mCi/mmol), 250 μg of membranes, 62.5 μM ATP, and buffer A in a final volume of 50 μl. Exogenous lipid monophosphate substrates (C_{35}, heptaprenyl monophosphate or C_{50}, decaprenyl monophosphate) were added to the reaction mixtures at a final concentration of 0.5 mM in 0.3% Triton X-100. After incubation at 37 °C for a period of 30 and 60 min, 3 ml of chloroform/methanol (2:1, v/v) was added to stop the reaction. This was further incubated at room temperature for 15 minutes before centrifugation at 3,500 x *g* for 10 min. The resulting pellet was washed 3 times to remove residues of GDP-[^{14}C]Man in the following order ofwashes; 50% methanol in 0.9% NaCl (v/v), 50% methanol (v/v), and 100% methanol respectively (Mikusova *et al.*, 2000). Then, 1 ml of chloroform/methanol/water (10:10:3, v/v/v) was added to the final pellet to extract the more polar lipid-linked product (Rush *et al.*, 1993). To the chloroform/methanol (2:1, v/v) fraction, 340 μl of water was added and then centrifuged briefly to define a biphase. The upper aqueous phase, which contains residual GDP-[^{14}C]Man, was removed and discarded, and the bottom phase was backwashed with 1 ml of chloroform/methanol/water (3:47:48, v/v/v) (Folch *et al.*, 1957). The bottom phase was

dried under a stream of nitrogen and reconstituted in 100 µl of chloroform/methanol (2:1, v/v). The incorporation of [^{14}C]Man from GDP-[^{14}C]Man into the lipid-linked intermediates was quantified by scintillation counting on a Beckman LS6500 Scintillation counter (Beckman, Fullerton, CA) using 7 ml of Ultima Gold^{TM} liquid scintillation cocktail (PerkinElmer) and analyzed by TLC, which was developed in chloroform/methanol/water/ammonium hydroxide (65:25:4:0.5, v/v/v/v). The TLC plate was subjected to autoradiography using a Kodak Biomax MR film.

3.2.15 Analytical procedures

Mild alkaline hydrolysis of the glycolipid extracts was performed in 0.2 N NaOH in methanol at 37°C for 30 min, neutralized with acetic acid, and partitioned into a mixture of chloroform/methanol/water (8:4:3, v/v/v) (Wolucka *et al.*, 1994). Mild acid hydrolysis was likewise conducted on the glycolipid extracts by resuspending each in 50 µl of 1-propanol with 0.02 N HCl and briefly sonicating in a bath sonicatior, heating at 60°C for 30 minutes, and then neutralizing with 10 µl of 0.2 N NaOH. The final product was finally partitioned into a mixture of chloroform/methanol/water (8:4:3, v/v/v) (Lucas *et al.*, 1975).

3.3 RESULTS

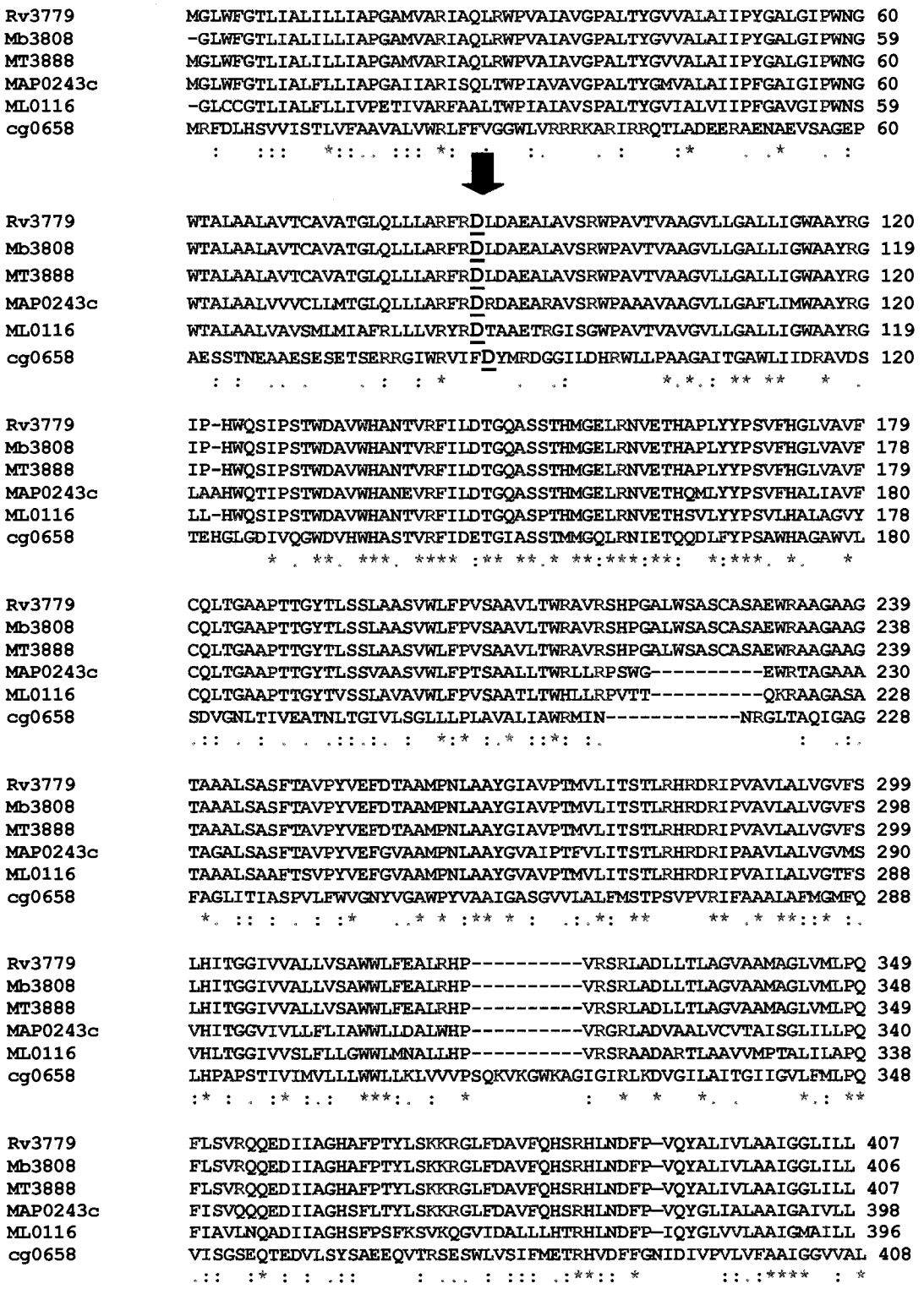
3.3.1 Topology prediction and sequence analysis of the Rv3779 protein

M. tuberculosis Rv3779 encodes a probable conserved transmembrane protein that is alanine and leucine rich with an unknown function, according to the Tuberculist web site. Amino acid sequences of Rv3779 from *M. tuberculosis* H_{37}Rv and CDC1551

(MT3888), *Mycobacterium bovis* (MB3808), *Mycobacterium paratuberculosis* (MAP0243c), and *Mycobacterium leprae* (ML0116) as well as *Corynebacterium glutamicum* ATCC 13032 (cg0658) were analyzed in detail. The overall CLUSTALW alignment scores of Rv3779 orthologs from other mycobacteria and paralogs from corynebacteria are summarized in Table 3.3.1 and the amino acid sequence alignment is shown in Figure 3.3.1. No homolog of Rv3779 in *M. smegmatis* and other fast-growing mycobacteria was found. *Rv3779* encodes a 666 amino acid protein that has been found to be non-essential based on results from the Tuberculist web site. Its gene lies within a cluster of genes involved in cell wall biosynthesis (Figure 2.6.4 and Figure 3.3.2).

Table 3.3.1. CLUSTALW Alignment scores of Rv3779 orthologs from other mycobacteria species and paralogs from corynebacteria. Strains are: *M. tuberculosis* $H_{37}Rv$ (Rv3779), *M. tuberculosis* CDC1551 (MT3888), *M. bovis* (MB3808), *M. avium subsp. paratuberculosis* (MAP0243c), *M. leprae* (ML0116) and *C. glutamicum* (cg0658). Rv3779 is a protein of 666 amino acid in length with no homologs in *M. smegmatis* and other fast-growing mycobacteria.

Name	Len(aa)	Name	Len(aa)	Score
Rv3779	666	MT3888	666	99
Rv3779	666	Mb3808	665	100
Rv3779	666	ML0116	653	67
Rv3779	666	MAP0243c	657	77
Rv3779	666	cg0658	743	20
MT3888	666	Mb3808	665	99
MT3888	666	ML0116	653	67
MT3888	666	MAP0243c	657	77
MT3888	666	cg0658	743	20
Mb3808	665	ML0116	653	67
Mb3808	665	MAP0243c	657	77
Mb3808	665	cg0658	743	20
ML0116	653	MAP0243c	657	68
ML0116	653	cg0658	743	25
MAP0243c	657	cg0658	743	24


Rv3779 MGLWFGTLIALILLIAPGAMVARIAQLRWPVAIAVGPALTYGVVALAIIPYGALGIPWNG 60
Mb3808 -GLWFGTLIALILLIAPGAMVARIAQLRWPVAIAVGPALTYGVVALAIIPYGALGIPWNG 59
MT3888 MGLWFGTLIALILLIAPGAMVARIAQLRWPVAIAVGPALTYGVVALAIIPYGALGIPWNG 60
MAP0243c MGLWFGTLIALFLLIAPGAIIARISQLTWPIAVAVGPALTYGMVALAIIPFGAIGIPWNG 60
ML0116 -GLCCGTLIALFLLIVPETIVARFAALTWPIAIAVSPALTYGVIALVIIPFGAVGIPWNS 59
cg0658 MRFDLHSVVISTLVFAAVALVWRLFFVGGWLVRRRKARIRRQTLADEERAENAEVSAGEP 60
Rv3779 WTALAALAVTCAVATGLQLLLARFRDLDAEALAVSRWPAVTVAAGVLLGALLIGWAAYRG 120
Mb3808 WTALAALAVTCAVATGLQLLLARFRDLDAEALAVSRWPAVTVAAGVLLGALLIGWAAYRG 119
MT3888 WTALAALAVTCAVATGLQLLLARFRDLDAEALAVSRWPAVTVAAGVLLGALLIGWAAYRG 120
MAP0243c WTALAALVVVCLLMTGLQLLLARFRDRDAEARAVSRWPAAAVAAGVLLGAFLIMWAAYRG 120
ML0116 WTALAALVAVSMLMIAFRLLLVRYRDTAAETRGISGWPAVTVAVGVLLGALLIGWAAYRG 119
cg0658 AESSTNEAAESESETSERRGIWRVIFDYMRDGGILDHRWLLPAAGAITGAWLIIDRAVDS 120
Rv3779 IP-HWQSIPSTWDAVWHANTVRFILDTGQASSTHMGELRNVETHAPLYYPSVFHGLVAVF 179
Mb3808 IP-HWQSIPSTWDAVWHANTVRFILDTGQASSTHMGELRNVETHAPLYYPSVFHGLVAVF 178
MT3888 IP-HWQSIPSTWDAVWHANTVRFILDTGQASSTHMGELRNVETHAPLYYPSVFHGLVAVF 179
MAP0243c LAAHWQTIPSTWDAVWHANEVRFILDTGQASSTHMGELRNVETHQMLYYPSVFHALIAVF 180
ML0116 LL-HWQSIPSTWDAVWHANTVRFILDTGQASPTHMGELRNVETHSVLYYPSVLHALAGVY 178
cg0658 TEHGLGDIVQGWDVHWHASTVRFIDETGIASSTMMGQLRNIETQQDLFYPSAWHAGAWVL 180
Rv3779 CQLTGAAPTTGYTLSSLAASVWLFPVSAAVLTWRAVRSHPGALWSASCASAEWRAAGAAG 239
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MT3888 CQLTGAAPTTGYTLSSLAASVWLFPVSAAVLTWRAVRSHPGALWSASCASAEWRAAGAAG 239
MAP0243c CQLTGAAPTTGYTLSSVAASVWLFPTSAALLTWRLLRPSWG----------EWRTAGAAA 230
ML0116 CQLTGAAPTTGYTVSSLAVAVWLFPVSAATLTWHLLRPVTT----------QKRAAGASA 228
cg0658 SDVGNLTIVEATNLTGIVLSGLLLPLAVALIAWRMIN----------NRGLTAQIGAG 228
Rv3779 TAAALSASFTAVPYVEFDTAAMPNLAAYGIAVPTMVLITSTLRHRDRIPVAVLALVGVFS 299
Mb3808 TAAALSASFTAVPYVEFDTAAMPNLAAYGIAVPTMVLITSTLRHRDRIPVAVLALVGVFS 298
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MAP0243c TAGALSASFTAVPYVEFGVAAMPNLAAYGVAIPTFVLITSTLRHRDRIPAAVLALVGVMS 290
ML0116 TAAALSAAFTSVPYVEFGVAAMPNLAAYGVAVPTMVLITSTLRHRDRIPVAILALVGTFS 288
cg0658 FAGLITIASPVLFWVGNYVGAWPYVAAIGASGVVLALFMSTPSVPVRIFAAALAFMGMFQ 288
Rv3779 LHITGGIVVALLVSAWWLFEALRHP----------VRSRLADLLTLAGVAAMAGLVMLPQ 349
Mb3808 LHITGGIVVALLVSAWWLFEALRHP----------VRSRLADLLTLAGVAAMAGLVMLPQ 348
MT3888 LHITGGIVVALLVSAWWLFEALRHP----------VRSRLADLLTLAGVAAMAGLVMLPQ 349
MAP0243c VHITGGVIVLLFLIAWWLLDALWHP----------VRGRLADVAALVCVTAISGLILLPQ 340
ML0116 VHLTGGIVVSLFLLGWWLMNALLHP----------VRSRAADARTLAAVVMPTALILAPQ 338
cg0658 LHPAPSTIVIMVLLLWWLLKLVVVPSQKVKGWKAGIGIRLKDVGILAITGIIGVLFMLPQ 348
Rv3779 FLSVRQQEDIIAGHAFPTYLSKKRGLFDAVFQHSRHLNDFP—VQYALIVLAAIGGLILL 407
Mb3808 FLSVRQQEDIIAGHAFPTYLSKKRGLFDAVFQHSRHLNDFP—VQYALIVLAAIGGLILL 406
MT3888 FLSVRQQEDIIAGHAFPTYLSKKRGLFDAVFQHSRHLNDFP—VQYALIVLAAIGGLILL 407
MAP0243c FISVQQQEDIIAGHSFLTYLSKKRGLFDAVFQHSRHLNDFP—VQYGLIALAAIGAIVLL 398
ML0116 FIAVLNQADIIAGHSFPSFKSVKQGVIDALLLHTRHLNDFP—IQYGLVVLAAIGMAILL 396
cg0658 VISGSEQTEDVLSYSAEEQVTRSESWLVSIFMETRHVDFFGNIDIVPVLVFAAIGGVVAL 408

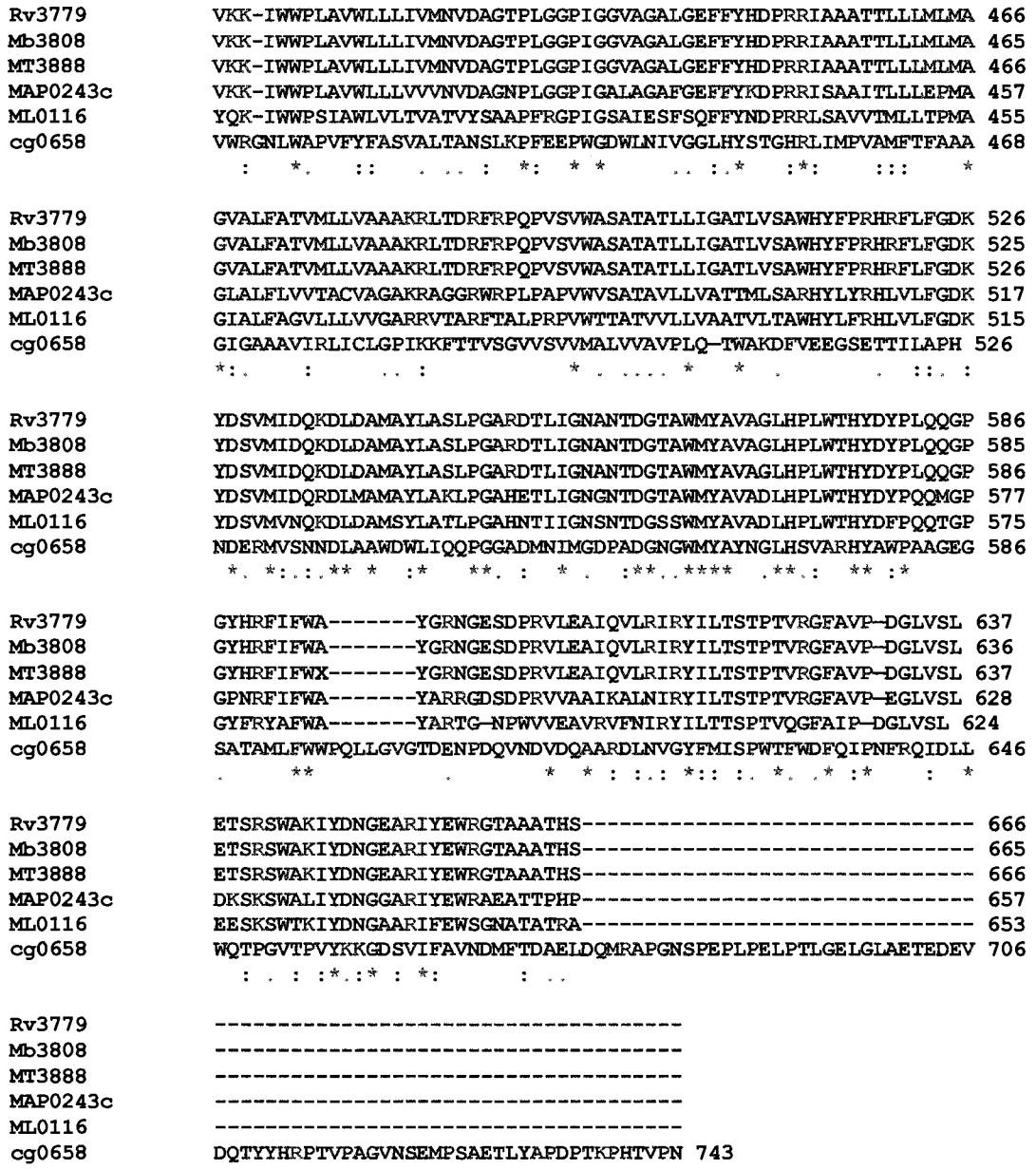


Figure 3.3.1. Multiple sequence alignment of the *Rv3779* locus within *Mycobacterium* and *Corynebacterium* species. CLUSTAL W (1.83) multiple sequence alignment: the arrow indicates the DxD motif and which aspartyl residues (underlined) are conserved among slow-growing mycobacteria, including *M. tuberculosis* $H_{37}Rv$ (Rv3779), *M. tuberculosis* CDC1551 (MT3888), *M. bovis* (MB3808), *M. avium subsp. paratuberculosis* (MAP0243c), *M. leprae* (ML0116) and *C. glutamicum* (cg0658).

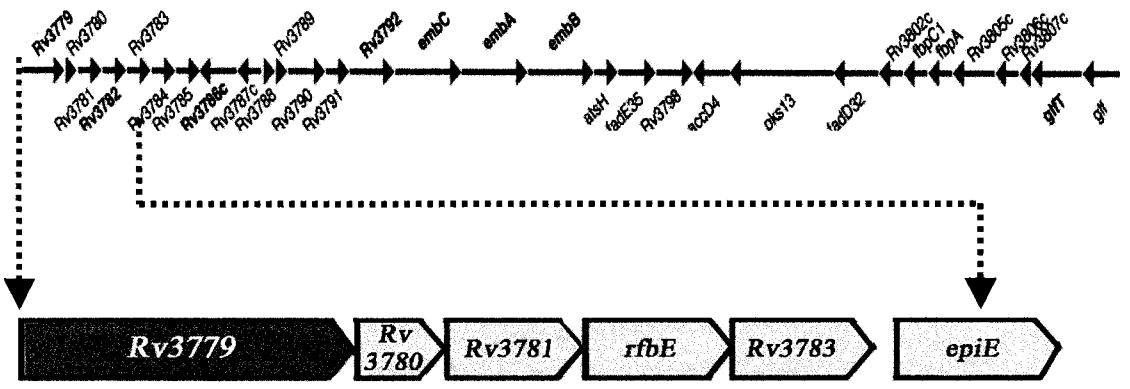


Figure 3.3.2. Schematic representation the cell wall biosynthetic gene cluster (*Rv3779-Rv3809c*) of *M. tuberculosis*. This gene cluster contains many genes directly or indirectly involved in the synthesis of AG, mycolic acid, and LAM in *M. tuberculosis*. Notable members of this cluster include Rv3779, a probable mannosyltransferase involved in LAM biosynthesis, as well as Rv3781 and Rv3783, which are probable ABC-2 type transport system ATP-binding proteins responsible for the translocation of lipid-linked galactan chains across the plasma membrane (Braibant *et al.*, 2000; Berg *et al.*, 2007). Other prominent members of this cluster include Rv3782, a galactosyltransferase that transfers the first Gal*f* residue to the linker rhamnose of the araninogalactan peptidoglycan complex (Mikusova *et al.* 2006), and the embA, B, and C, arabinosyltransferases, which are involved in arabinan synthesis and chain extension of LAM (Berg *et al.*, 2007; Shi *et al.*, 2006). Moreover, Rv3806c forms 5-P-DPR, a precursor of the C_{50}-P-Ara*f* donor of all Ara*f* residues in the cell wall of mycobcteria. Glf is a well characterized UDP-Gal mutase, and GlfT is a bifunctional galactosyltransferase that forms the linear galactan chain (Berg *et al.*, 2007; Mikušová *et al.*, 2000; Kremer *et al.*, 2001; Rose *et al.*, 2006).

From our preliminary studies, the region containing Rv3779 was recognized as a large motif preserved among a group of membrane-bound GTs, which have been classified as members of the GT-C superfamily (Berg *et al.*, 2007). TMHMM and SOSUI prediction has shown that Rv3779 consists of 12 transmembrane domains by SOSUI (Figure 3.3.3) and 14 transmembrane domains by TMHMM (Figure 3.3.4), and has the same conserved DxD motif as other members of the GT-C superfamily. This conserved motif has been observed to play an important role in the enzymatic function of GTs utilizing the lipid-linked sugar donors (Lucas *et al.*, 1975; Berg *et al.*, 2005). Rv3779 contains a DLD motif at amino acid position 82, located on the second cytoplasmic loop (Figure 3.3.3) (Berg *et al.*, 2007). Interestingly, the DLD motif of Rv3779 lies in the cytoplasm on the second cytoplasmic loop, where it can interact with a substrate. The signature DxD motif is typically on the first extracytoplasmic loop in the GT-C superfamily, however. This strongly implies that the gene product of Rv3779 utilizes a substrate in the cytoplasm. However Rv3779 was shown to contain a lower score of homology (scores = 37 bits and E value = 1.8) to the *M. tuberculosis* EmbA protein. From this evidence, we hypothesized that *Rv3779* may encode a putative GT utilizing a cytoplasmic NDP-sugar donor as its substrate.

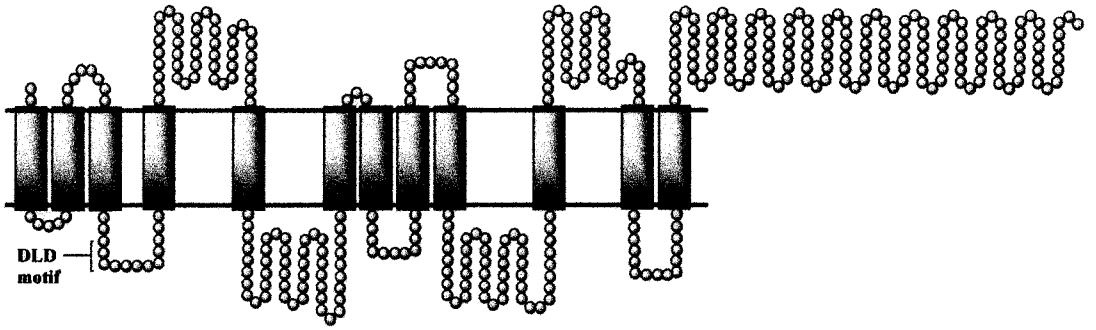


Figure 3.3.3. Membrane topology of *M. tuberculosis* Rv3779 protein (predicted by SOSUI). Twelve transmembrane domains are shown, in addition to A DLD motif which is located at amino acid position 82 on the second cytoplasmic loop, suggesting a cytosolic substrate is utilized.

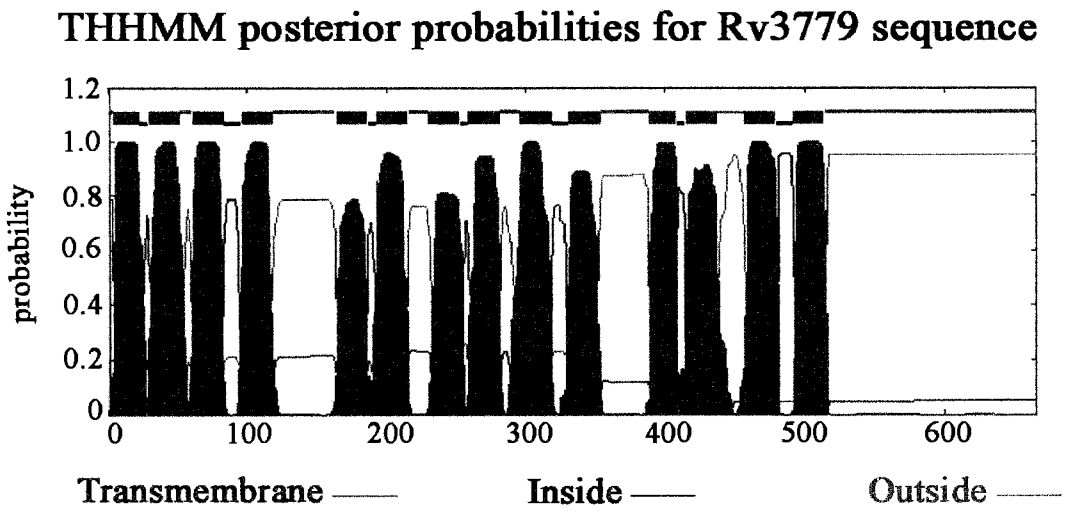


Figure 3.3.4. Membrane topology of *M. tuberculosis* Rv3779 protein (predicted by TMHMM 2.0). Here 14 transmembrane domains are predicted. Inside is cytosolic, outside is extracytosolic.

A specific domain search of *M. tuberculosis* Rv3779 sequence using the Pfam HMM database (http://pfam.janelia.org) revealed the presence of 12 protein families (Pfam-A) as shown in table 3.3.2. One of the distinct families at the C-terminal domain (residues 454-513) was assigned by Pfam as a part of the UbiA prenyltransferase family (PF01040), a family predicted to be involved in the transfer of a prenyl group from one compound (donor) to another (acceptor). The well-described example in *M. tuberculosis* Rv3806c, contains the UbiA domain (Pfam) and encodes decaprenylphosphoryl-5-phosphoribose (DPPR) synthase (UbiA). UbiA catalyzes the transfer of 5-phospho-ribofuranose-pyrophosphate (pRpp) to decaprenol phosphate to form DPPR, the precursor for AG biosynthesis in mycobacteria. According to the membrane topology prediction by HMTMM model (Figure 3.3.4), the UbiA domain of Rv3779 (amino acids 454-514) covers 2 transmembrane domains (13 and 14), which link together with the last cytoplasmic loop on the C-terminal of the protein. Rv3806c (302 amino acids in length) contains a distinct UbiA domain covering the entire protein sequence from amino acid 29-299 with 7 transmembrane domains. This implies that Rv3779 contains only part of the entire UbiA domain (~20%).

Table 3.3.2. Major functional domains of Rv3779 of *M. tuberculosis* $H_{37}Rv$. The functional domains of Rv3779 have similarities to that of other major polyprenyltransferases. The Pfam database compares the functional domain in question to generate related bits score and e-values. These parameters estimate the extent of the relationship of matched sequences. The E-value is the number of hits that would be expected to have a score equal or better than this by chance alone. Therefore a good E-value is much less than 1, and around 1 is what is expected just by chance.

Pfam-A Protein Family	**Description**	**Sequence (residues)**	**E-value**
H_PPase	Inorganic H+ pyrophosphatase	8-473	0.33
Na_H_Exchanger	Sodium/hydrogen exchanger family	64-519	0.96
CbtB	Probable cobalt transporter subunit (CbtB)	86-123	0.072
Cuticle_2	Cuticle protein 7 isoform family	132-180	0.022
Voltage_CLC	Voltage gated chloride channel	193-507	0.059
Competence	Competence protein	222-413	0.31
Trep_Strep	Hypothetical bacterial integral membrane protein (Trep_Strep)	240-410	0.58
NrfD	Polysulphide reductase, NrfD	265-309	0.98
Peptidase_A24	Type IV leader peptidase family	269-408	0.61
CTP_transf_1	Cytidylyltransferase family	284-509	0.72
C4dic_mal_tran	C4-dicarboxylate transporter/malic acid transport protein	287-524	0.089
UbiA	**UbiA prenyltransferase family**	**454-513**	**0.65**

3.3.2. Construction and growth of the *M. tuberculosis* Δ*Rv3779* mutants

The putative glycosyltransferase gene *Rv3779* was disrupted using the strategy previously described by Jackson *et al.*, (2001). Briefly, a two-step homologous recombination was performed which resulted in an allelic exchange at the *Rv3779* locus. *M. tuberculosis* $H_{37}Rv$ was transformed with the temperature-sensitive plasmid pPR27*xylE* containing a kanamycin-disrupted copy of *Rv3779* from *M. tuberculosis* as an insert. The selection for a single homologous recombination event at the *Rv3779* locus was done on 7H11-OADC with 25 μg/ml kanamycin at 32°C. Thus, 70% of the arising clones were found to be positive for the XylE marker (resulting in yellow colonies after spraying with catechol). These XylE-positive clones were further grown in 7H9-OADC and plated onto 7H11-OADC agar with kanamycin (at the same concentration) and 2% sucrose at 39°C to select for the clones that had undergone a second recombination event leading to the allelic exchange at the *Rv3779* locus on the *M. tuberculosis* chromosome (Figure 3.3.5). After spraying with catechol, 50% of colonies observed on the plates remained white in color (*xylE*-negative, Figure 3.3.6). Ten colonies were obtained and subjected to Southern blot analysis, and all were found to contain the kanamycin-disrupted *Rv3779* gene (Figure 3.3.7).

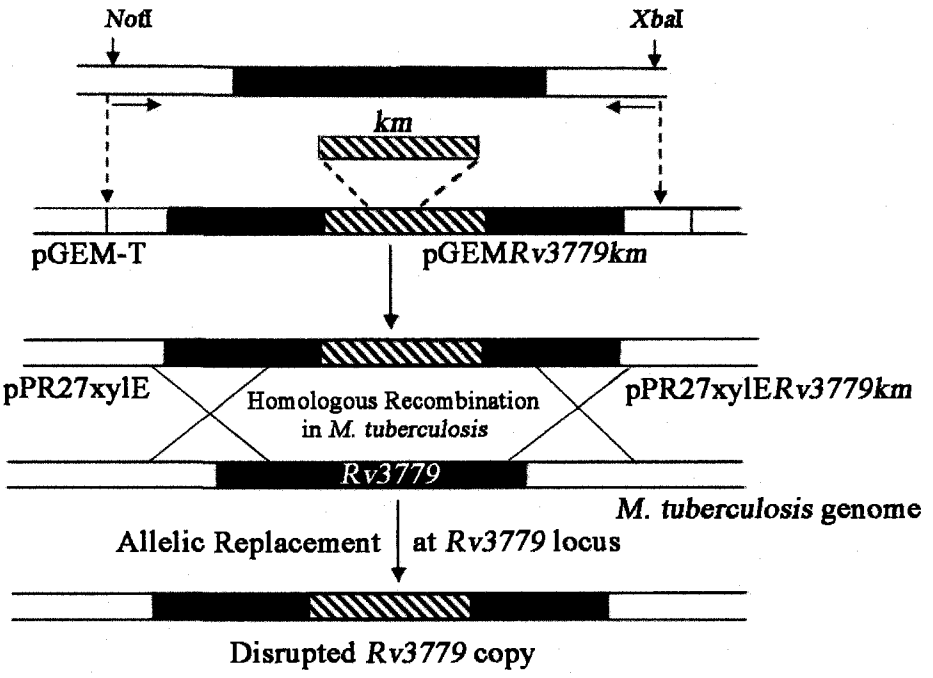
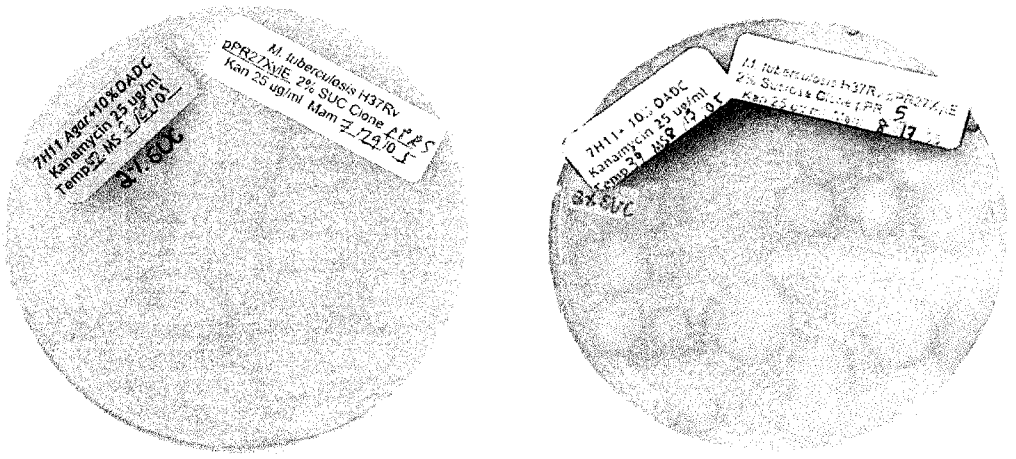


Figure 3.3.5. Schematic representation of the generation of *M. tuberculosis* Δ*Rv3779* by allelic replacement. The locations of primers for PCR are indicated by small arrows. Black regions represent the coding sequence of *Rv3779*. Hatched boxes indicate the intragenic DNA fragment replaced with a kanamycin (*km*) cassette from pUC4K. Homologous recombination and selection for kanamycin and sucrose resistance and *xylE* resulted in colonies with a disrupted *Rv3779*, which remained white upon spraying with catechol ($XylE^-$).



A) Positive selection **B) Negative selection**

Figure 3.3.6. Positive (A) and negative selection (B) of *M. tuberculosis* $H_{37}Rv$ colonies which contained the disrupted copy of *Rv3779*. Transformation of *M. tuberculosis* with the vector carrying a kanamycin disrupted copy of *Rv3779* and selection at 39°C for kanamycin resistance (Km^r) and sucrose resistance (Suc^r) led to the identification of white colonies when sprayed with catechol, suggesting that allelic replacement had occurred (Km^r-Suc^r-$XylE^-$, positive selection (A)). The negative selection (B) represented the colonies with the phenotype of Km^r-Suc^r-$XylE^+$.

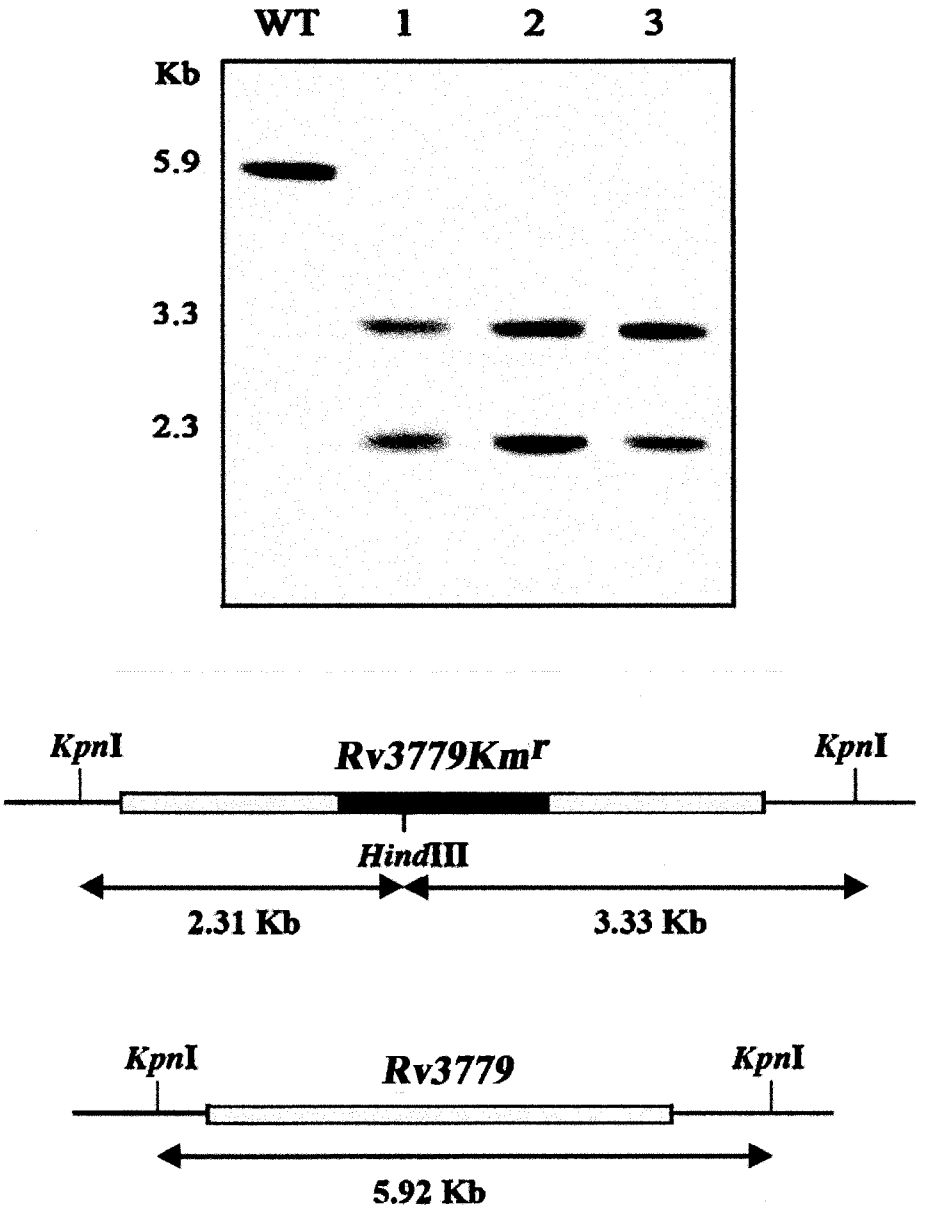


Figure 3.3.7. Southern blot analysis of *M. tuberculosis* △*Rv3779* mutants and expected schematic pattern of a double crossover mutant. Chromosomal DNA of *M. tuberculosis* H_{37}Rv WT (WT) and △*Rv3779* mutant (clones 1-3) were digested with *Kpn*I and *Hind*III and probed for hybridization with the PCR fragment generated for cloning in the initial step.

The △*Rv3779* mutant grew at a much slower rate than the wild-type *M. tuberculosis* at 37°C on 7H9-OADC broth with 0.05% Tween 80 and kanamycin (Figure 4.3.8). The growth of △*Rv3779* has slowed dramatically after a few days of incubation with agitation at 30°C; however growth of the wild type continued at a normal rate. The colony morphology of both strains was examined by scanning electron microscopy (SEM) and the mutant cells were found to be significantly shorter than the wild type strain. The cell length of the △*Rv3779* mutant cells was on average 1.2 μm (+/-), whereas the average length of the wild type cells was 2.5 μm (+/-) (Figure 3.3.9 and 3.3.10). These results suggested that the mutant phenotypes arose from the disruption of *Rv3779*. We speculated that Rv3779 might be responsible for the biosynthesis of cell wall components, since the disruption of *Rv3779* affects cell shape or integrity.

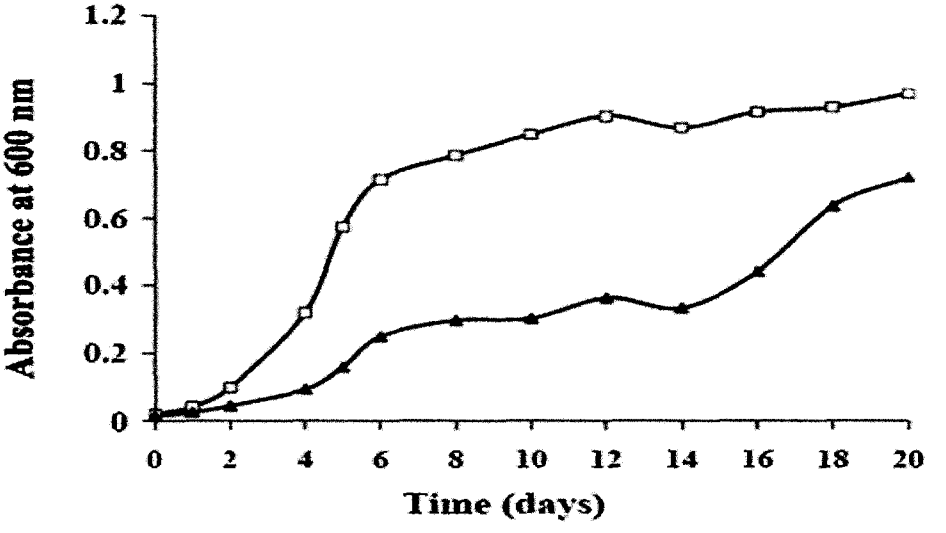


Figure 3.3.8. Growth curve of *M. tuberculosis* △*Rv3779* mutant compared with the wild type strain of *M. tuberculosis* H_{37}Rv. The growth rate was monitored in a single assay at an optical density of 600 nm over a period of 20-days - *M. tuberculosis* WT (—□—) and △*Rv3779* mutant (—▲—).

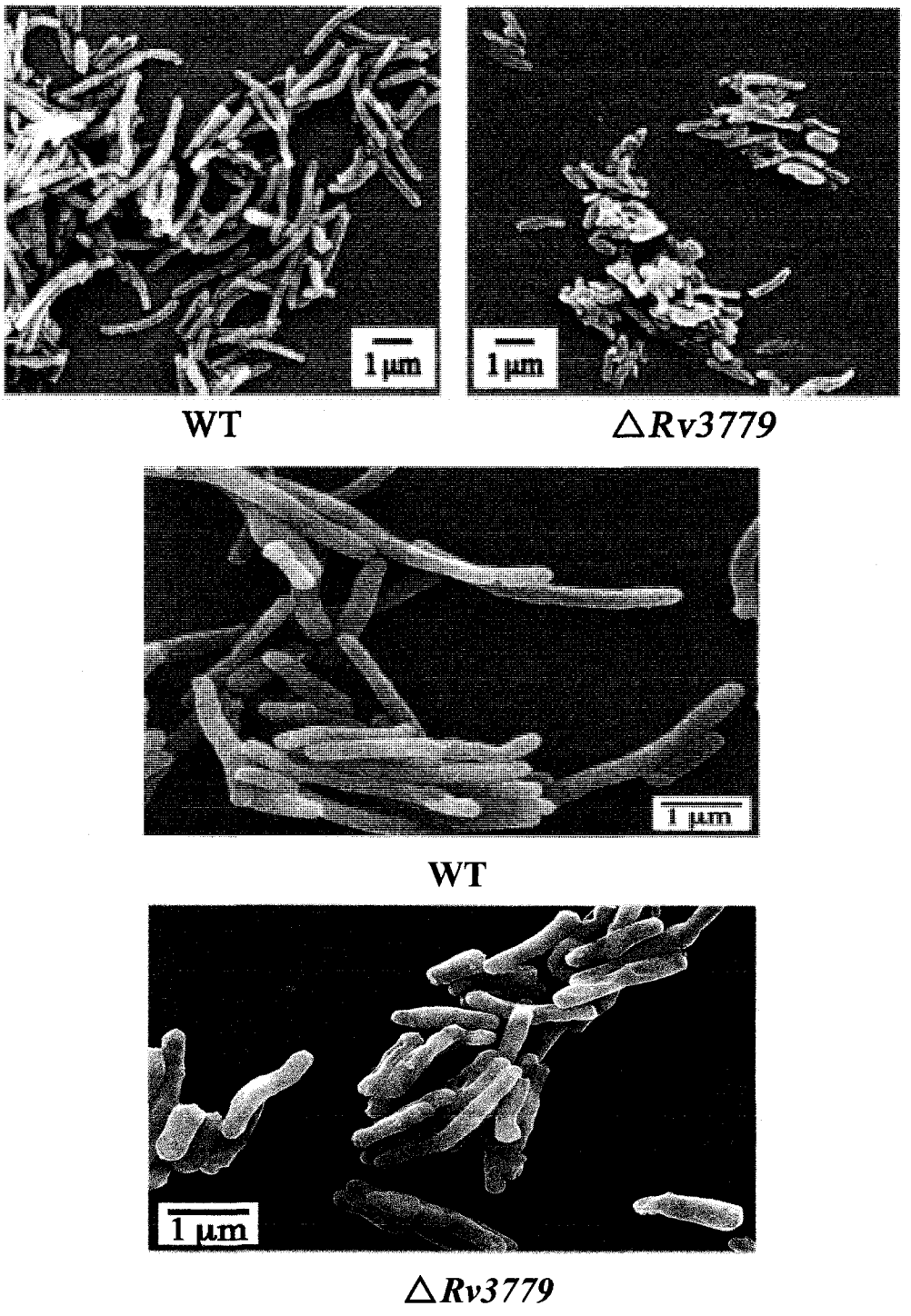


Figure 3.3.9. Scanning electron micrograph demonstrating the effects of inactivation of *Rv3779* on the cell length of *M. tuberculosis* (*ΔRv3779* mutant) as compared of the wild type strain of *M. tuberculosis* $H_{37}Rv$ (WT). A dramatic shortening of the cells is visually evident in the absence of Rv3779.

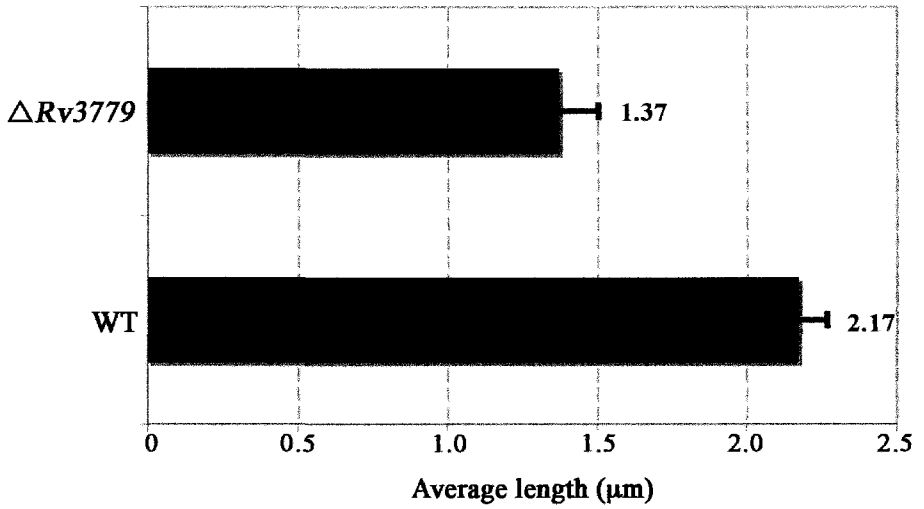


Figure 3.3.10. Average cell length of wild type *M. tuberculosis* $H_{37}Rv$ and *ΔRv3779*. Cell length was measured using the length-scale on the scanning electron micrographs of 25 individual cells of each strain of *M. tuberculosis*. The average length in micrometers (μm) was calculated and is shown above. A dramatic shortening of the average cell length by 37% is evident in the absence of Rv3779.

3.3.3 Biochemical phenotype of *M. tuberculosis ΔRv3779* mutant

3.3.3.1 Mycolic methyl ester profiles

TLC analysis of purified mycolic acid methyl esters (MAME) isolated from *M. tuberculosis* $H_{37}Rv$ wild type and the *ΔRv3779* mutant showed three spots corresponding to α-, methoxy- and ketomycolic acid methyl esters respectively, corresponding to results in a previous study by Besra *et al.* (Besra, 1998). *M. tuberculosis* $H_{37}Rv$ wild type and the *ΔRv3779* mutant showed a similar pattern, although the relative amount of each mycolic acid subclass in the mutant was less than the wild type based on dry weight of cells (Figure 3.3.11).

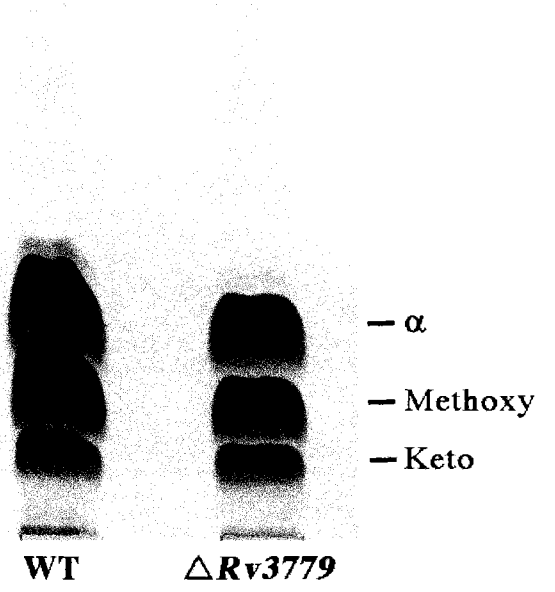


Figure 3.3.11. TLC profile of mycolic acid methyl esters of *M. tuberculosis* H_{37}Rv WT and the *ΔRv3779* mutant. The MAME profile is largely unaffected by the loss of *Rv3779*.

3.3.3.2 Lipid profiles

The lipid profiles from the whole cell extract with chloroform/methanol (2:1, v/v) and chloroform/methanol/water (10:10:3, v/v/v) were obtained by TLC. The chloroform/methanol (2:1, v/v) extract of the *M. tuberculosis* *ΔRv3779* mutant differed from that of the wild type strain in that a profound decrease in the amounts of $AcPIM_6$ and Ac_2PIM_6 was observed (Figure 3.3.12 and 3.3.13). The two lipid extracts from the wild type and *ΔRv3779* mutant were subjected to MALDI-TOF MS analysis, and molecular ions with the expected m/z corresponding to $AcPIM_2$ and Ac_2PIM_2 were observed in both strains. However, molecular ions corresponding to $AcPIM_6$ and Ac_2PIM_6 were less abundant in the *ΔRv3779* mutant. The ratio of the $AcPIM_2$ and $AcPIM_6$ molecular ion intensity of the mutant was 4.4 times higher than the wild type, because of the sharp decrease in the amount of $AcPIM_6$, as shown in Figures 3.3.14A

and 3.3.14B. For further confirmation of these results, the bands of the glycolipids were scraped from the TLC plates, extracted with chloroform/methanol (2:1, v/v), and subjected to MALDI-TOF MS analysis. Molecular ions with the expected m/z ratios were detected in the purified $AcPIM_2$, Ac_2PIM_2, $AcPIM_6$, and Ac_2PIM_6, confirming their identities (Figure 3.3.15, 3.3.16, 3.3.17 and 3.3.18). TLC of the chloroform/methanol/water (10:10:3, v/v/v) extract of the wild type and the mutant strain showed no differences in the polar glycolipids profiles (data not shown). From this evidence, we conclude that Rv3779 is involved in PIM biosynthesis in *M. tuberculosis*.

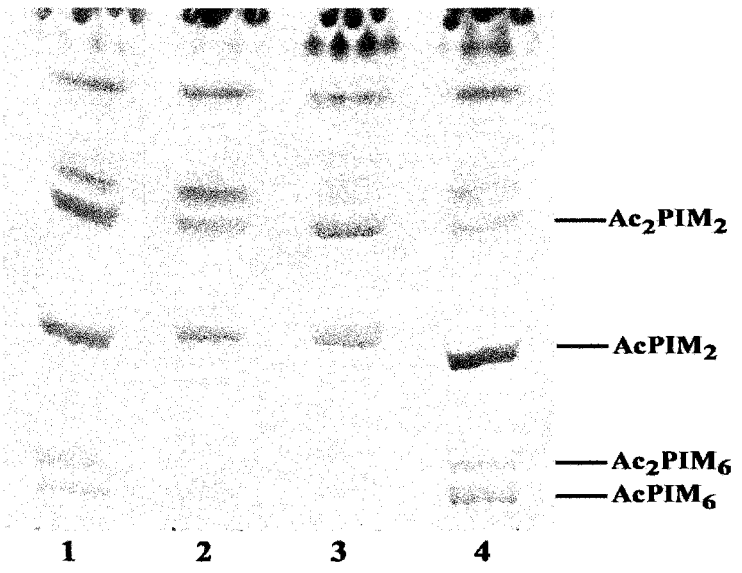


Figure 3.3.12. Effect of *Rv3779* deletion on the composition of PIMs of *M. tuberculosis*. TLC analysis of PIMs from *M. tuberculosis* $H_{37}Rv$ wild type (lanel), The profound loss of the higher PIMs (PIM_6) is evident in the $\triangle$*Rv3779* mutant (lane 2) and the $\triangle$*Rv3779*/pVV16 (lane 3). The formation of the higher PIMs is restored in the $\triangle$*Rv3779*/pVV16-*Rv3779* (lane 4). PIMs were detected after charring the TLC plate at 100°C with α-naphthol/H_2SO_4.

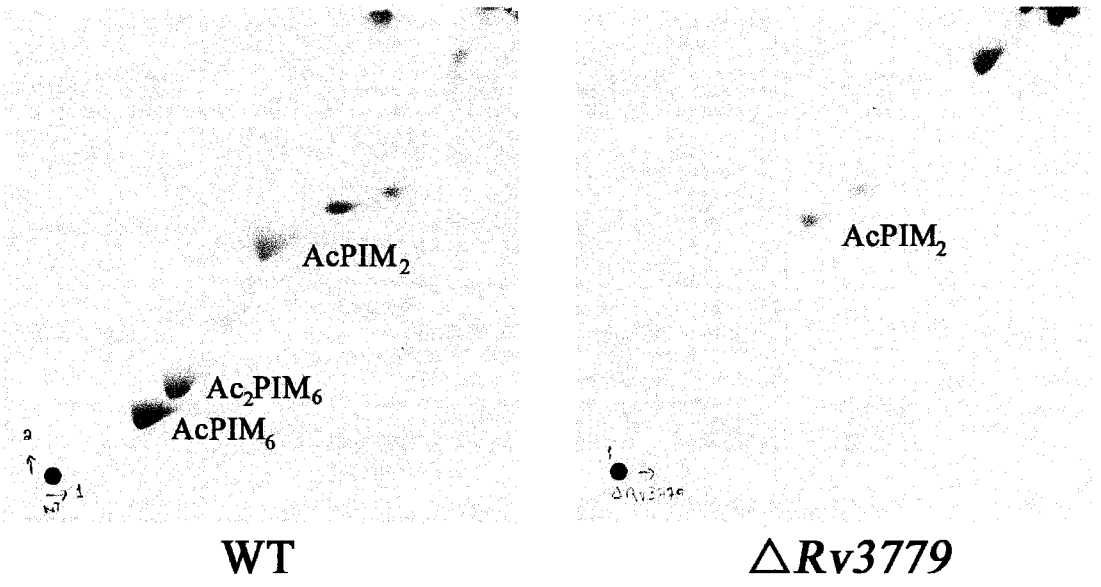


Figure 3.3.13. Two-dimensional TLC analysis demonstrating the effects of *tbRv3779* deletion on the composition of PIMs of *M. tuberculosis*. The chloroform/methanol (2:1, v/v) soluble lipids from the WT strain *M. tuberculosis* $H_{37}Rv$ and the mutant strain *M. tuberculosis* r*Rv3779* were separated with chloroform/methanol/water (60:30:6) in the first dimension and chloroform/acetic acid/methanol/water (40:25:3:6) in the second dimension. Lipids were detected by charring with α-naphthol/H_2SO_4.

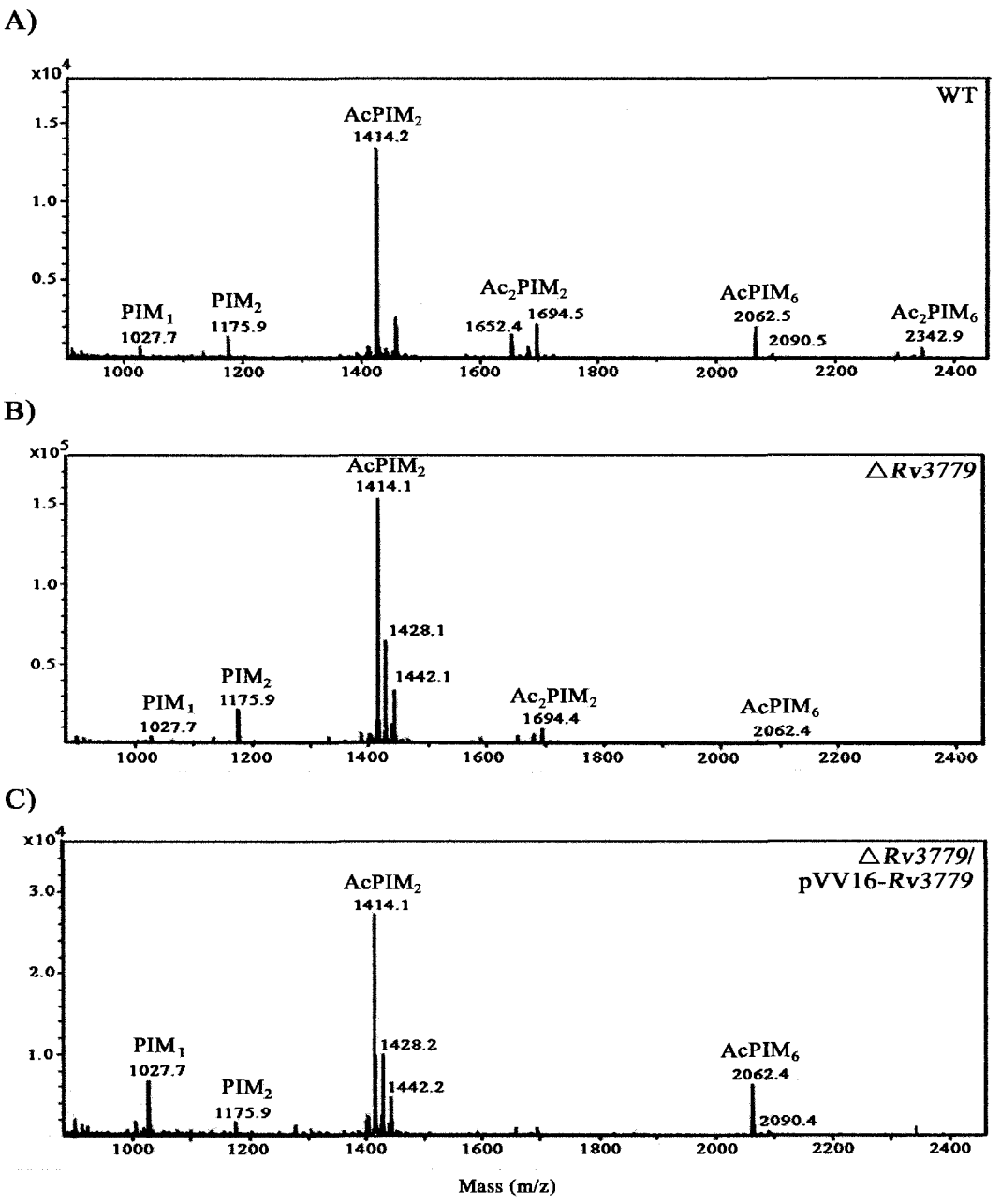


Figure 3.3.14. MALDI-MS analysis in the total lipids from A) *M. tuberculosis* H_{37}Rv WT, B) Δ*Rv3779*, and C) Δ*Rv3779*/pVV16-*Rv3779*. Lipids were extracted with chloroform/methanol (2:1, v/v) and subject to MALDI-TOF MS analysis in the negative ion mode as described in "3.2 Materials and Methods ". Peaks identified are m/z 1027.7, PIM_1 with C_{18} + C_{18}; m/z 1175.9, PIM_2 with C_{16} + C_{19}; m/z 1414.1 or 1414.2 $AcPIM_2$ with $2C_{16}$ + C_{19}; m/z 1428.2, $AcPIM_2$ with C_{16} + $2C_{18}$; m/z 1442.2, $AcPIM_2$ with C_{16} + C_{18} + C_{19}; m/z 1652.4, Ac_2PIM_2 with $3C_{16}$ + C_{19}; m/z 1694.4 or 1694.5, Ac_2PIM_2 with $2C_{16}$ + $2C_{19}$; m/z 2062.4 or 2062.5, $AcPIM_6$ with $2C_{16}$ + C_{19}; m/z 2090.4 or 2090.5, $AcPIM_6$ with C_{16} + C_{18} +C_{19}; m/z 2342.9, Ac_2PIM_6 with

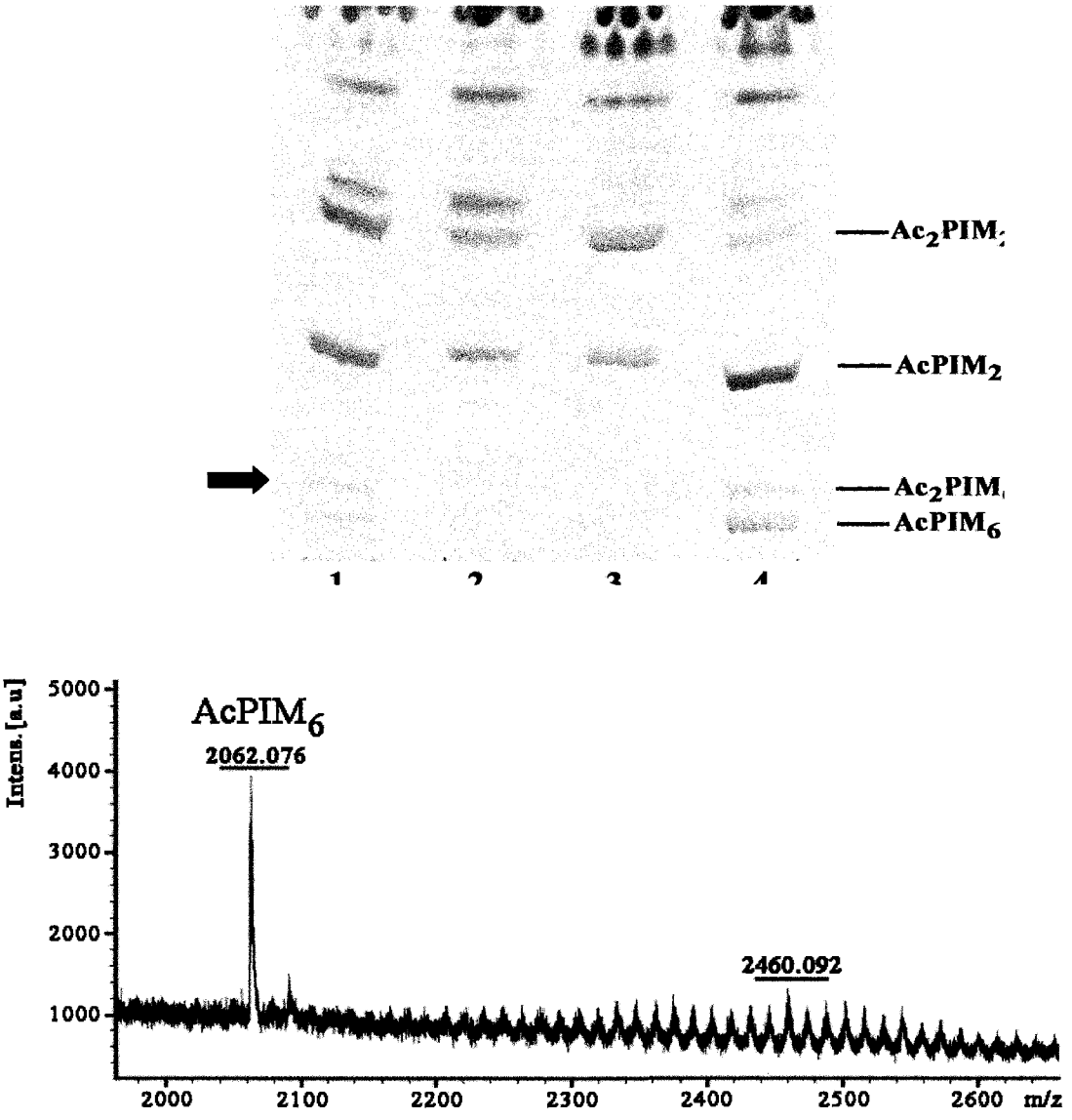


Figure 3.3.15. MALDI-TOF mass spectrum of putative $AcPIM_6$ species derived from *M. tuberculosis* $H_{37}Rv$ WT cells. Glycolipid band (black arrow) was scraped from TLC sheets, extracted with chloroform/methanol (2:1, v/v) and further purified by Folch wash, prior to subjecting to MALDI-TOF MS analysis in negative mode. Peaks identified are m/z 2062.076, $AcPIM_6$ with $2C_{16} + C_{19}$.

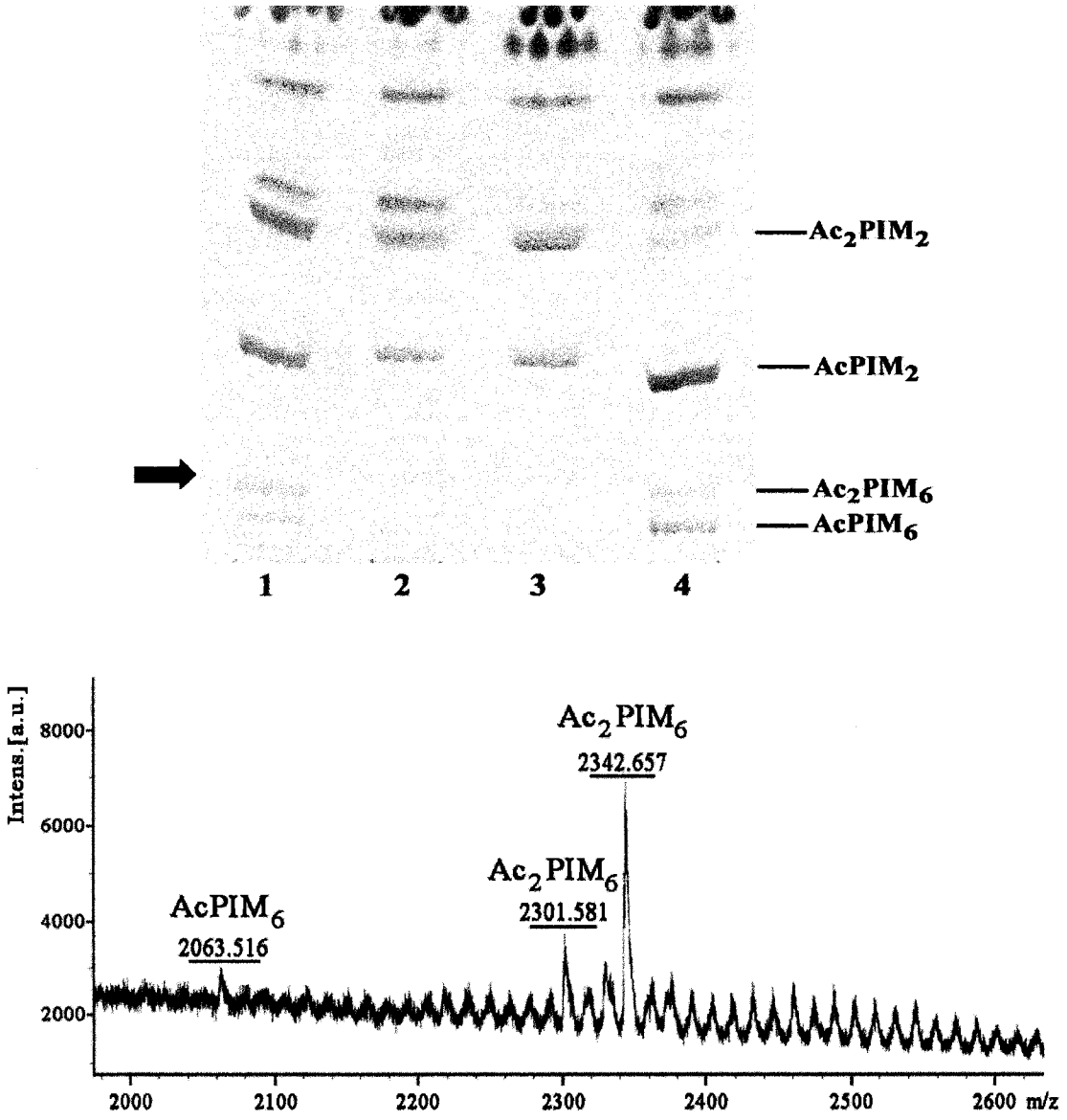


Figure 3.3.16. MALDI-TOF mass spectrum of putative Ac_2PIM_6 species derived from *M. tuberculosis* $H_{37}Rv$ WT cells. Glycolipid band (black arrow) was scraped from TLC sheets, extracted with chloroform/methanol (2:1, v/v) and further purified by Folch wash, prior to subjecting to MALDI-TOF MS analysis in the negative mode. Peaks identified are m/z 2301.581, Ac_2PIM_6 with $3C_{16} + C_{19}$; m/z 2342.657, Ac_2PIM_6 with $2C_{16} + 2C_{19}$.

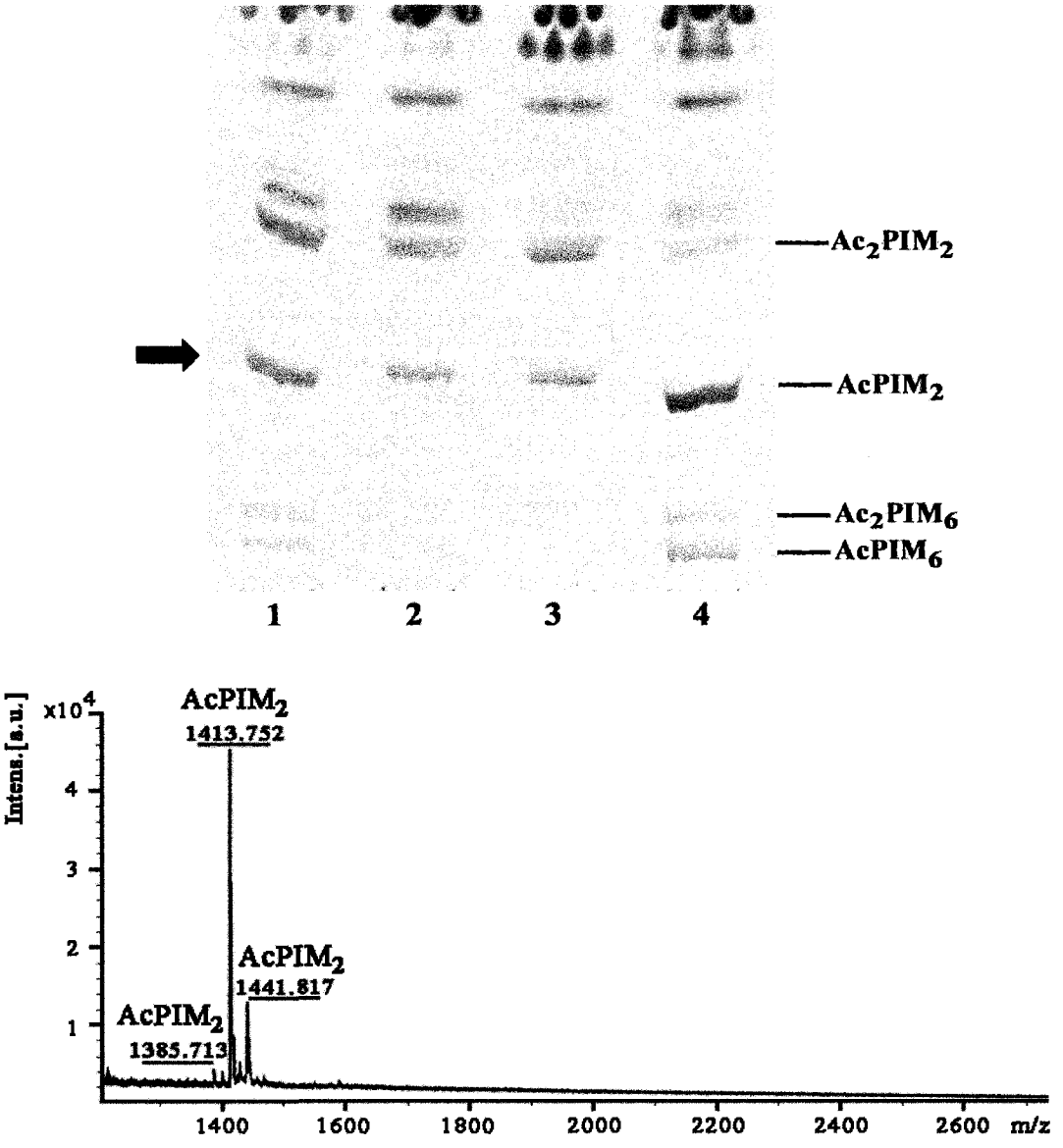
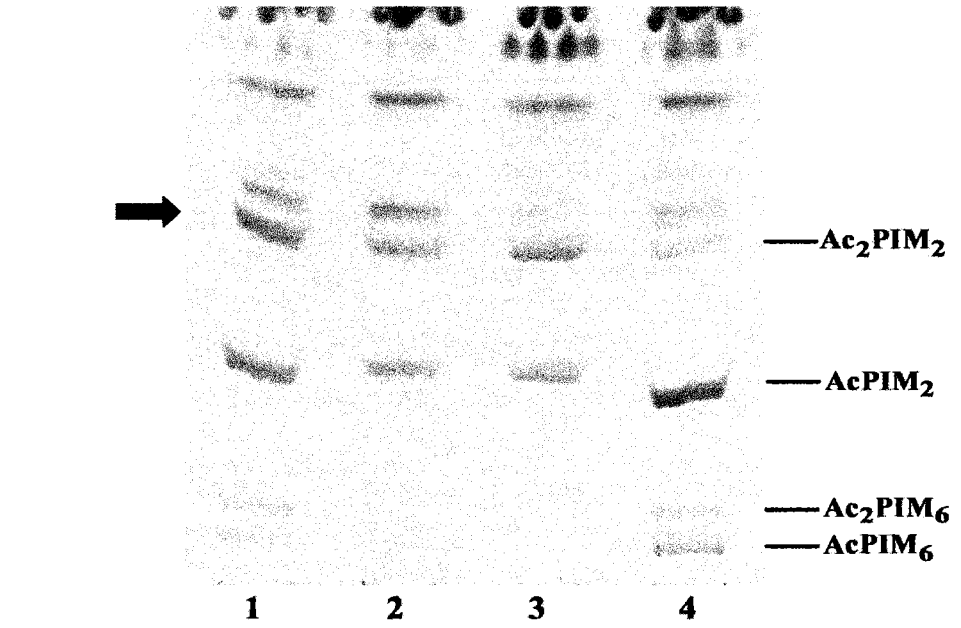


Figure 3.3.17. MALDI-TOF mass spectrum of putative $AcPIM_2$ species derived from *M. tuberculosis* $H_{37}Rv$ WT cells. The glycolipid band (black arrow) was scraped from the TLC sheets, extracted with chloroform/methanol (2:1, v/v) and further purified by Folch wash, prior to subjecting to MALDI-TOF MS analysis in the negative mode. Peaks identified are m/z 1413.752, $AcPIM_2$ with $2C_{16} + C_{19}$; m/z 1441.817, $AcPIM_2$ with $C_{16} + C_{18} + C_{19}$.



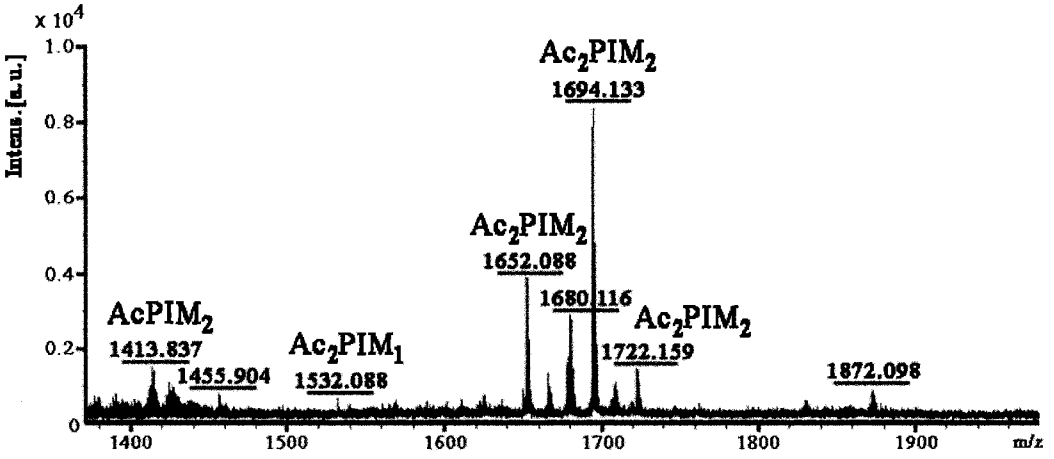


Figure 3.3.18. MALDI-TOF mass spectrum of putative Ac_2PIM_2 species derived from *M. tuberculosis* $H_{37}Rv$ WT cells. The glycolipid band (black arrow) was scraped from TLC sheets, extracted with chloroform/methanol (2:1, v/v) and further purified by Folch wash, prior to subjecting to MALDI-TOF MS analysis in the negative mode. Peaks identified are m/z 1413.837, $AcPIM_2$ with $2C_{16} + C_{19}$; m/z 1455.904, $AcPIM_2$ with $C_{16} + 2C_{19}$; m/z 1532.088, Ac_2PIM_1 with $C_{16} + 3C_{18}$; m/z 1652.088, Ac_2PIM_2 with $3C_{16} + C_{19}$; m/z 1680.116, Ac_2PIM_2 with $2C_{16} + C_{18} + C_{19}$; m/z 1694.133, Ac_2PIM_2 with $2C_{16} + 2C_{19}$; m/z 1722.159, Ac_2PIM_2 with $C_{16} + C_{18} + 2C_{19}$.

To confirm this conclusion, we constructed the complemented strain of the △*Rv3779* mutant by transforming it with pVV16-*Rv3779,* which is expressed constitutively under the hsp60 promoter. The expression of Rv3779 in the mutant restores the biosynthesis of PIM_6 while the mutant cells transformed with the empty vector, pVV16, remains the same (Figure 3.3.12 and 3.3.14). Next we examined the effect of *Rv3779* deletion on LM/LAM synthesis. Analysis of the crude phenol-extracted LM/LAM fraction from *M. tuberculosis* WT and *M. tuberculosis* △*Rv3779* mutant dried cells (which were weighed equally) were visualized on SDS-PAGE and revealed lesser amounts of both LM an LAM in the mutant compared to the wild type (Figure 3.3.19A). The binding specificity to monoclonal antibody CS-35 (known to react with the arabinan chain of LAM) and concanavalin A (ConA, specific to t-Man*p* residues) was also strongly reduced in the LAM and LM of the mutant, respectively (Figure 3.3.19B and 3.3.19C). As expected, the complementation of the mutant with *Rv3779* restored the amount and binding specificity to CS-35 and ConA of LM/LAM to the same level as the wild type. Considering these observations together, Rv3779 gene deletion resulted in a decrease in the synthesis of PIM_6 species and concomitant decrease in amount of LM and LAM. This alteration in PIM, LM, and LAM profiles had significant effects on cell growth, shape and viability.

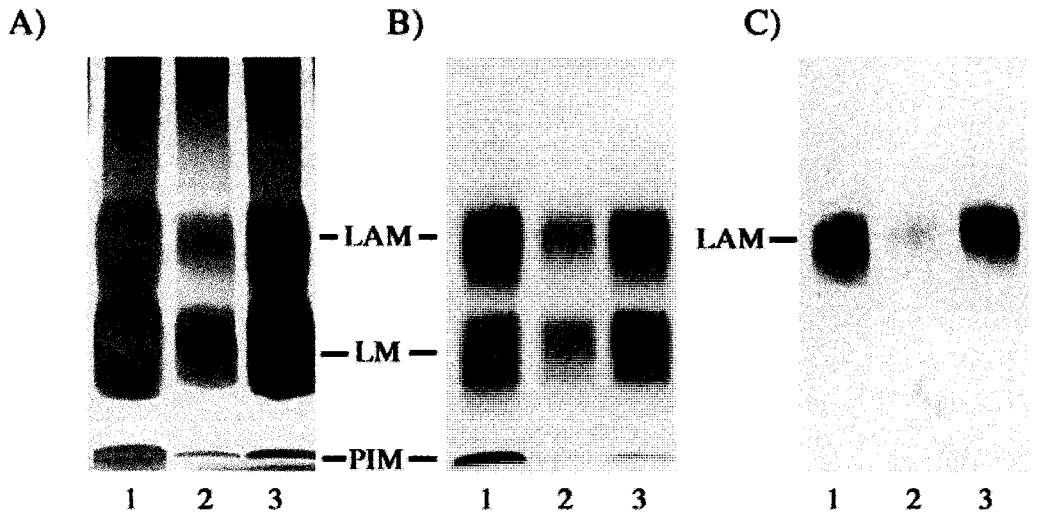


Figure 3.3.19. Analysis of LM/LAM from *M. tuberculosis* H_{37}Rv, *M. tuberculosis* △*Rv3779* and *M. tuberculosis* △*Rv3779*/pVV16-*Rv3779*. LM/LAM was extracted with phenol from equal weight cells of the wild type H_{37}Rv strain (lane 1), the *M. tuberculosis* △*Rv3779* mutant (lane 2) and the complemented mutant *M. tuberculosis* △*Rv3779*/pVV16-*Rv3779* (lane 3), separated on 10-20% Tricine gel, and revealed by periodic-Schiff staining (A). The Western blot analyses were performed on the same samples using the lectin ConA (B) and the CS-35 monoclonal antibody (C).

3.3.3.3 Sugar composition of cell wall lipids

The whole cell chloroform/methanol (2:1, v/v) extracts from *M. tuberculosis* H_{37}Rv WT, *M. tuberculosis* △*Rv3779*, and complemented mutant *M. tuberculosis* △*Rv3779*/pVV16-Rv3779 were analyzed for sugar composition by alditol acetate derivatization and GC analysis. The amount of each sugar was calculated in relation to the internal standard (*scyllo*-inositol) (Table 3.3.3). The ratio of mannose to *myo*-inositol between each strain of *M. tuberculosis* was compared as shown in Table 3.3.4. The chloroform/methanol (2:1, v/v) extract of the △*Rv3779* mutant strain of *M. tuberculosis*

had a lower ratio of mannose to *myo*-inositol than the wild type strain, and the complemented mutant strain (△*Rv3779*/pVV16-*Rv3779*). This result parallels the TLC and MALDI-TOF MS analysis in which the knock-out mutant contained less polar PIMs (PIM_6) than the wild type $H_{37}Rv$ and the complemented mutant.

Table 3.3.3. Sugar composition of the chloroform/methanol (2:1, v/v) extract of *M. tuberculosis* $H_{37}Rv$ WT, *M. tuberculosis* △*Rv3779*, and *M. tuberculosis* △*Rv3779*/pVV16-*Rv3779*.

	Ratio of area of each sugar peak to area of *scyllo*-inositol peak		
	WT	**△*Rv3779***	**△*Rv3779*/pVV16-*Rv3779***
Arabinose	0.06	0.038	0
Galactose	0	0.108	0
Glucose	0.181	0.142	0.095
Mannose	1.161	0.62	0.847
myo-Inositol	0.594	0.46	0.38

Table 3.3.4. Ratio of mannose to *myo*-inositol in the chloroform/methanol (2:1, v/v) extract of *M. tuberculosis* $H_{37}Rv$ WT, *M. tuberculosis* △*Rv3779*, and *M. tuberculosis* △*Rv3779*/pVV16-*Rv3779*.

Ratio	***M. tuberculosis* strains**		
	WT	**△*Rv3779***	**△*Rv3779*/pVV16-*Rv3779***
Mannose/*myo*-Inositol	1.955	1.348	2.229

3.3.4. Effects of overexpressing *Rv3779* in *M. smegmatis*

To further investigate the role of Rv3779 in lipoglycan biosynthesis, we analyzed the effects of overexpressing Rv3779 on the production of these lipoglycans in *M. smegmatis*. Since *Rv3779* is not naturally present in fast-growing mycobacteria, including *M. smegmatis*, aspects of the phenotype of the overexpressed strain (which differs from the wild type), probably results from the function of Rv3779 itself. *M. smegmatis* mc^2155 transformed either with pVV16 or pVV16-*Rv3779* (the same plasmid used to complement the *M. tuberculosis* $\triangle$*Rv3779* mutant) was utilized to examine the *in vivo* and *in vitro* synthesized PIMs and lipoglycans. The chloroform/methanol (2:1, v/v) extracted lipids from *M. smegmatis* pVV16 and *M. smegmatis* pVV16-*Rv3779* known to contain the lipids were analyzed by TLC. Both strains produced similar PIM profiles, except for a slight increase in $AcPIM_6$ synthesis and lack of $AcPIM_4$ in the pVV16-*Rv3779* construct (Figure 3.3.20). Likewise, the TLC analysis of the chloroform/methanol/water (10:10:3, v/v/v) extract (Figure 3.3.21) and SDS/PAGE analysis of crude phenol LM/LAM extract did not demonstrate any differences in the polar glycolipids (Figure 3.3.22).

However, when we incubated the cell lysate extract from *M. smegmatis* pVV16 and *M. smegmatis* pVV16-*Rv3779* with GDP-[^{14}C]Man for 4 hours and 24 hours, the Rv3779 overexpressed cell lysate was able to synthesize [^{14}C]LM 4 hours after incubation and more so at 24 hours of incubation. The vector control however, gave no production of [^{14}C]LM at 4 hours and some lower molecular weight [^{14}C]LM at 24 hours (Figure 3.3.23). Moreover, there is more higher molecular weight [^{14}C]LM present in the overexpressed lysate at 24 hours as compared to the plasmid control, indicative of

increased Rv3779 activity.

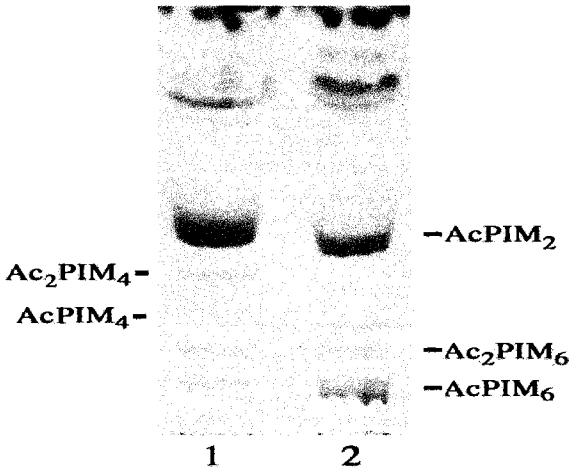


Figure 3.3.20. Effect of overexpression of Rv3779 on PIM biosynthesis in *M. smegmatis*. Chloroform/methanol (2:1, v/v) extracts from *M. smegmatis* pVV16 (lane 1) and *M. smegmatis* pVV16-*Rv3779* (lane 2) were separated on the TLC plate with chloroform/methanol/water (58:25:4). Glycolipids were visualized by charring the TLC plate at 100°C with α-naphthol/H_2SO_4 spray.

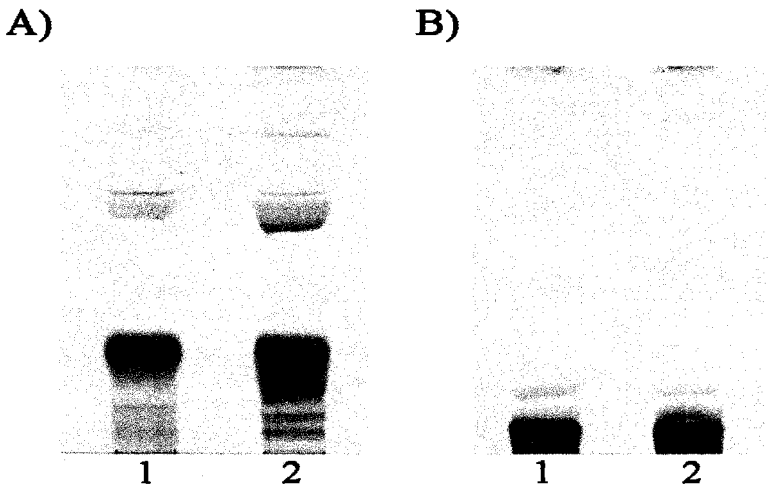


Figure 3.3.21. Effect of overexpression of Rv3779 on polar glycolipid biosynthesis in *M. smegmatis*. TLC analysis of chloroform/methanol/water (10:10:3, v/v/v) extracts from *M. smegmatis* pVV16 (lane 1) and *M. smegmatis* pVV16-*Rv3779* (lane 2) were developed in either solvent system A) chloroform/methanol/water (60:35:8, v/v/v) or B) chloroform/methanol/water (58:25:4, v/v/v). Glycolipids were revealed by charring the TLC plate at 100°C with α-naphthol/H_2SO_4 spray.

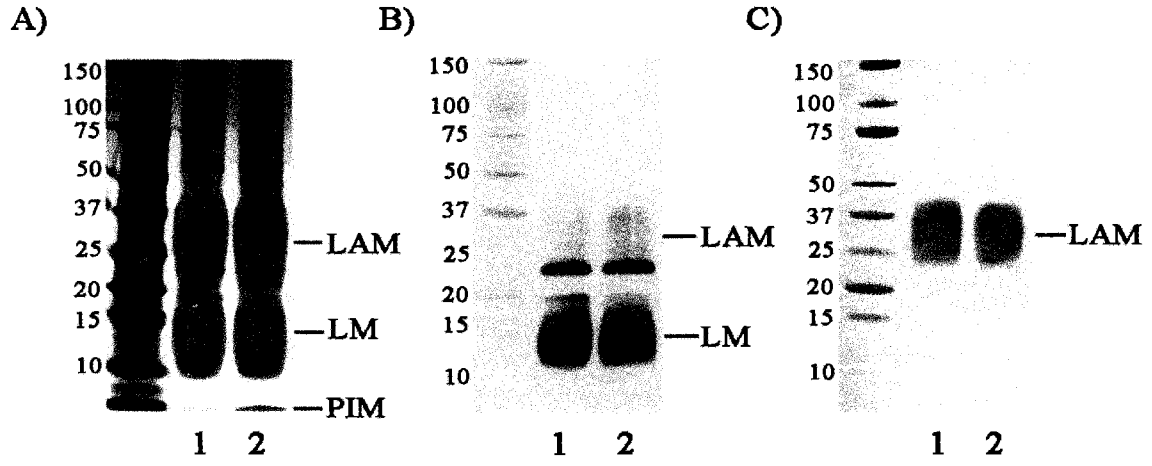


Figure 3.3.22. Analysis of LM/LAM from *M. smegmatis* pVV16 and *M. smegmatis* pVV16-*Rv3779*. LM/LAM was extracted with phenol from cells (equal dry weight) of *M. smegmatis* transformed with the vector control, pVV16 (lane 1) and the Rv3779-overexpressed strain of *M. smegmatis* (lane 2), separated on 10-20% Tricine gel, visualized by periodic-schiff staining (A). The Western blot analyses were performed on the same samples using the concavalin A (B) and the CS-35 monoclonal antibody (C).

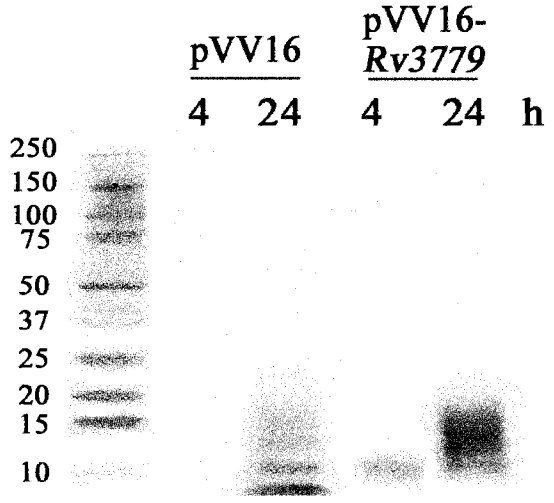
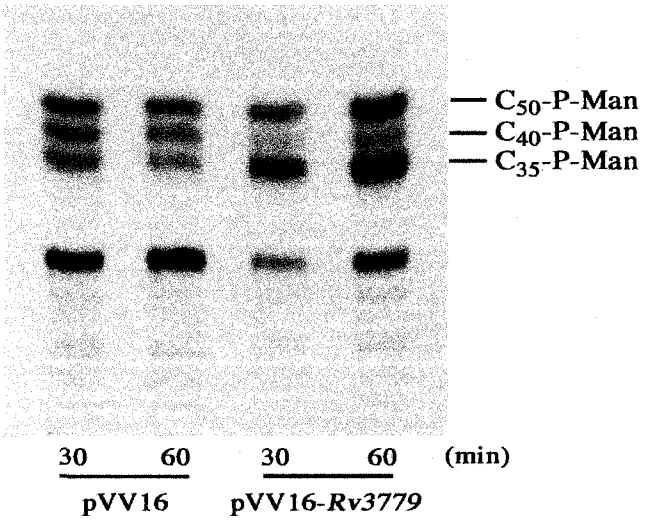


Figure 3.3.23. *In vitro* LM biosynthesis in Rv3779-overexpressed strain of *M. smegmatis*. Crude cell extract (4 mg protein) of *M. smegmatis* pVV16 (lane 1) or *M. smegmatis* pVV16-Rv3779 (lane 2) was incubated with 1.0 μci of GDP-[^{14}C]Man for 4 and 24 h. LM synthesis is enhanced with the pVV16-Rv3779. The reaction mixture was stopped by adding chloroform/methanol (2:1, v/v) and the cell pellet was extracted with hot phenol for LM/LAM. LM/LAM was separated on 10-20% Tricine gel and subsequent blotting to nitrocellulose membrane, which later was exposed to Kodak MR film at -70 °C for 3 weeks.

We also performed an *in vitro* mannosyltransferase activity assay to further illuminate which step Rv3779 is involved in PIM and LM/LAM biosynthesis. The overall incorporation of [^{14}C]Man from GDP-[^{14}C]Man into both membrane and cell lysate prepared from cells transformed with pVV16-*Rv3779* was relatively similar as compared to membrane prepared from cells transformed with pVV16. However TLC autoradiography demonstrated a clear linear increase in a time-dependent manner in the synthesis of the C_{50} and C_{35}-linked mannose in the overexpressed construct as compared with the empty plasmid control, *M. smegmatis* pVV16 (Figure 3.3.24A and 3.3.24B). The characterization of the two accumulated mannose-containing lipids indicates that they are mild-alkaline stable and mild-acid labile, suggesting they are in the PPM family (Figure 3.3.25) (Gurcha *et al.,* 2002). Based on its relative retardation factor and from previous work, these PPMs were assigned as C_{35}- and C_{50}-P-Man (lower and upper band respectively). We carried out the assay to confirm the specificity of Rv3779 to the chain length of the lipid donor by adding the synthetic acceptor C_{50}-P and C_{35}-P as the extra source of lipid donor beyond what is normally present in the membrane fraction. The overexpressed Rv3779 strain showed a significant increase in the incorporation of [^{14}C]-Man residues from GDP-[^{14}C]Man to both C_{35} and C_{50} lipid donors as compared to the empty vector control (Figures 3.3.26 and 3.3.27). These results are in complete agreement with previous studies, as Rv3779 shows a similar lack of specificity for polyprenyl phosphates like Ppm1 in relation to changes in the lipid moiety (Gurcha *et al.*, 2002).

A)



B)

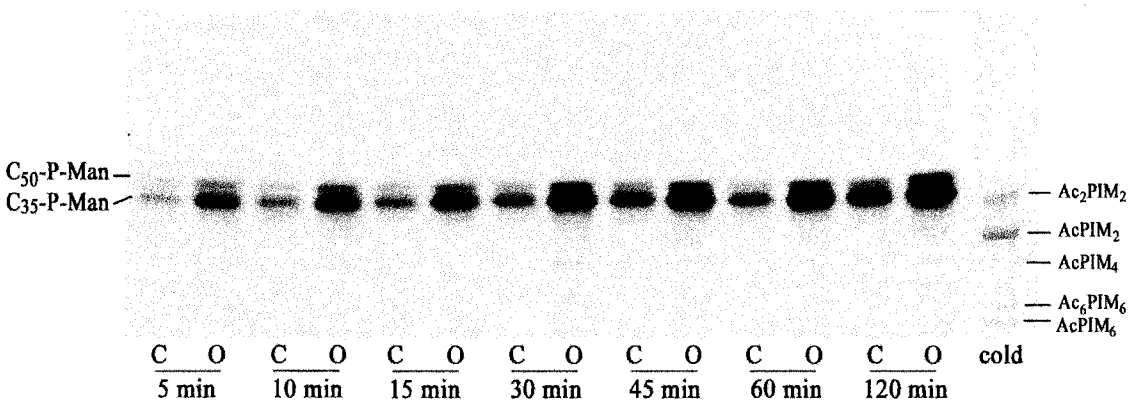


Figure 3.3.24. TLC analysis of an *in vitro* cell free assay using GDP-[^{14}C]Man and membrane extract (A) and cell lysate (B) from *M. smegmatis* pVV16 and *M. smegmatis* pVV16-*Rv3779*. Membrane fractions (250 μg protein) or cell lysate (2 mg protein) were incubated with GDP-[^{14}C]Man at 37°C. The synthesized products were extracted with chloroform/methanol (2:1, v/v) and a 10% aliquot was taken for scintillation counting and for TLC/autoradiography. The TLC was developed in chloroform/methanol/ammonium hydroxide/water (65:25:0.5:4, v/v/v/v). C is the empty vector control and O is the Rv3779 overexpressed construct.

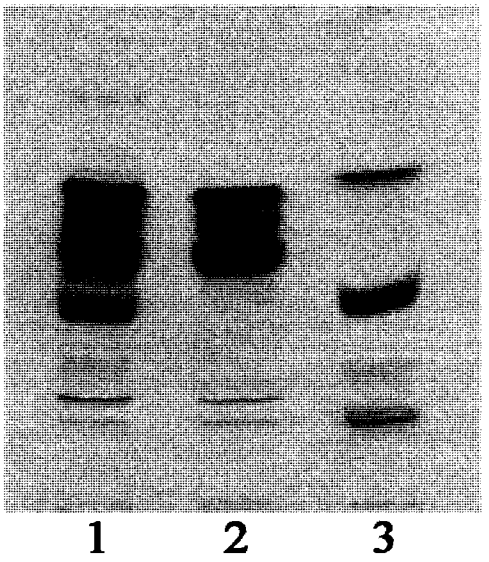


Figure 3.3.25. Characterization of the accumulated mannose-containing lipids from the *in vitro* mannosyltransferase assay by mild acid and alkaline hydrolysis. Chloroform/methanol (2:1, v/v) extract of the *in vitro* cell free assay using GDP-[^{14}C]Man and membrane extract from *M. smegmatis* pVV16-*Rv3779* shown in lane 1 (untreated). Mild alkaline and acid treatment of the chloroform/methanol (2:1, v/v) extract from the same reaction are shown in lane 2 and 3, respectively. TLC plate was developed using chloroform/methanol/ ammonium hydroxide/water (65:25:0.5:4, v/v/v/v) and autoradiographed on the X-ray film.

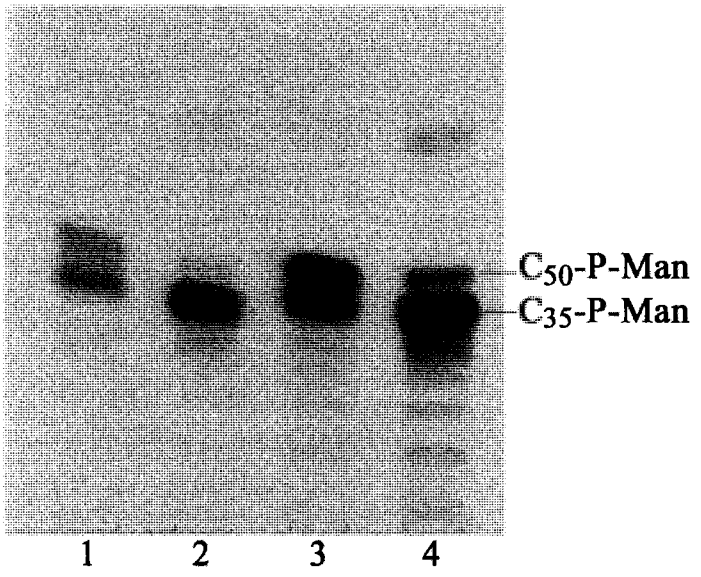


Figure 3.3.26. Incorporation of [^{14}C]Man from GDP-[^{14}C]Man into exogenous C_{35}, heptaprenyl-P using membrane extracts from *M. smegmatis* pVV16 and *M. smegmatis* pVV16-*Rv3779*. Membrane fractions were incubated with GDP-[^{14}C]Man in a total volume of 50 µl for 30 min and synthesized products isolated after a chloroform/methanol (2:1, v/v) extract. TLC/autoradiography was performed using chloroform/methanol/ ammonium hydroxide/water (65:25:0.5:4, v/v/v/v). Lane 1- *M. smegmatis* pVV16 membrane without external acceptor C_{35}-P, Lane 2 - *M. smegmatis* pVV16 membrane with 0.5 mM C_{35}-P, Lane 3 - *M. smegmatis* pVV16-*Rv3779* membrane without external acceptor C_{35}-P, Lane 4 - *M. smegmatis* pVV16-*Rv3779* membrane with 0.5 mM C_{35}-P.

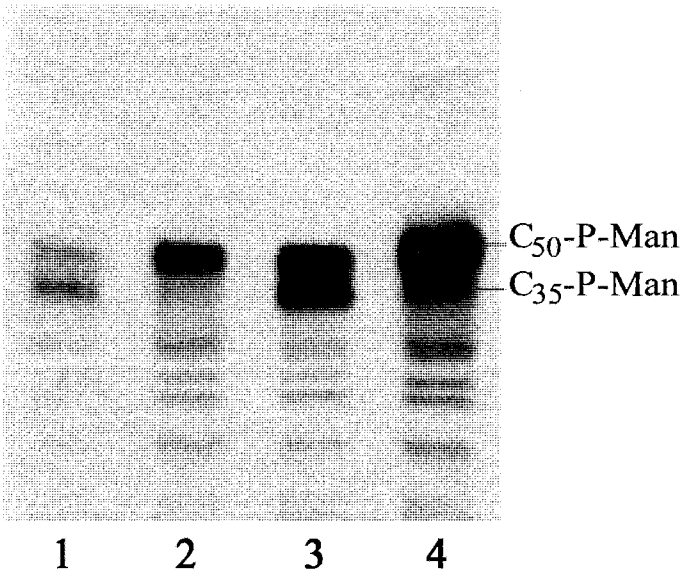


Figure 3.3.27. Incorporation of [^{14}C]Man from GDP-[^{14}C]Man into exogenous C_{50}, decaprenyl-P, using membrane extracts from *M. smegmatis* pVV16 and *M. smegmatis* pVV16-*Rv3779*. Membrane fractions were incubated with GDP-[^{14}C]Man in a total volume of 50 µl for 30 min and synthesized products isolated after chloroform/methanol (2:1, v/v) extract. TLC/autoradiography was performed using chloroform/methanol/ ammonium hydroxide/water (65:25:0.5:4, v/v/v/v). Lane 1- *M. smegmatis* pVV16 membrane without external acceptor C_{50}-P, Lane 2 - *M. smegmatis* pVV16 membrane with 0.5 mM C_{50}-P, Lane 3 - *M. smegmatis* pVV16-*Rv3779* membrane without external acceptor C_{50}-P, Lane 4 - *M. smegmatis* pVV16-*Rv3779* membrane with 0.5 mM C_{50}-P.

3.4 DISCUSSION

LAM has been implicated as the key factor in the pathogenesis of tuberculosis. Therefore, a thorough understanding of LAM biosynthesis is critical, yet incomplete at this time. It is widely accepted that the biosynthetic pathway ending in the various forms of LAM begins with the mannosylation of the 2-position of *myo*-inositol by the gene product of *pimA*, giving PIM_1 (Kordulakova *et al.*, 2002). GDP-Man is the sugar nucleotide mannosyl donor and is utilized by these distinct a-mannosyltransferases to further mannosylate PIM to the higher levels. In *M. smegmatis*, the *M. tuberculosis pimB* gene product was found to form PIM_2 by mannosylating the 6-position of the *myo*-inositol of PIM_1, while PIM_3 is the product of the *pimC* gene product (Schaeffer *et al.*, 1999; Kremer *et al.*, 2002). These first stages are thought to occur on the cytoplasmic face of the plasma membrane, whereby the PIM_3 or possibly the PIM_4 product is thought to be translocated through the plasma membrane by an uncharacterized flippase (Morita *et al.*, 2004; Berg *et al.*, 2007). PIM_4 seems to be at the branch point between the synthesis of the polar (higher) PIMs, and LM/LAM (Morita *et al.*, 2004). On the extracytoplasmic side, PIM_4 is further mannosylated to form the higher PIMs, and in a separate pathway, LM and finally LAM. It is utilized by PimE in the formation of the polar PIM_6, and as a precursor in the formation of LM (Haites *et al.*, 2005; Morita *et al.*, 2006). Unlike the early stages of PIM synthesis, GDP-Man is no longer the mannosyl donor for these reactions. Further extracytoplasmic mannosylation occurs using PPM, such as C_{50}-DPM as the mannosyl donor.

PPM in *M. tuberculosis* is formed from the donation of a Man*p* residue from GDP-Man to polyprenyl phosphate by Ppm1, a large mannosyltransferase consisting of

two distinct domains; the transmembrane region (D1), and the extracytoplasmic region (D2) (Gurcha *et al.*, 2002; Baulard *et al.*, 2003). Interestingly, this enzyme, while catalyzing such a critical step, was found to be nonessential by disruptive transposon mutagenesis and no other enzyme with this function has been described in *M. tuberculosis* (Sassetti *et al.*, 2001). Herein we propose that another enzyme, likewise found to be nonessential, catalyzes this same reaction in a redundant fashion in *M. tuberculosis*.

To characterize and elucidate the role of Rv3779, we obtained DNA and amino acid sequence information from the National Center for Biotechnology Information (NCBI), and the homologous sequences were aligned at EBI server using EBI tools. Based on BLAST analysis, Rv3779 is conserved in *M. tuberculosis* CDC1551 (MT3888), *Mycobacterium bovis* (MB3808), *Mycobacterium avium subsp. paratuberculosis* (MAP0243c), and *Mycobacterium leprae* (ML0116) as well as in *Corynebacterium glutamicum* ATCC 13032 (cg0658). We have not found any homolog of Rv3779 in *M. smegmatis* and in any fast-growing mycobacteria. Blast results also showed that *Rv3779* contains a low score of homology (scores = 37 bits and E value = 1.8) to the *M. tuberculosis* EmbA. This finding may suggest the possible role of Rv3779 in cell wall biosynthesis like the EmbA protein, which is involved in arabinogalactan biosynthesis in mycobacteria. Therefore, Rv3779 encodes a protein with multiple membrane spanning domains found only in slow growing mycobacteria such as *M. tuberculosis*, *M. bovis*, and *M. leprae*.

Topology prediction has shown Rv3779 to be a 666 amino acid protein identified as an integral membrane protein with the number of transmembrane spanning

domains varying between 12-14, depending on the algorithm used. The TMHMM 2.0 algorithm, which has one of the highest reliability scores in predicting helix bundle membrane proteins, predicted 14 transmembrane helices, while SOSUI predicted only 12 transmembrane domains. Rv3779 contains a DLD motif at amino acid position 82, located on the second cytoplasmic loop (Figure 3.3.3). Interestingly, the DLD motif of Rv3779 lies in the cytoplasm on the second cytoplasmic loop, where it can interact with a substrate. The signature DxD motif is typically on the first extracytoplasmic loop in the GT-C superfamily, however. This strongly implies that Rv3779 utilizes the substrate GDP-Man, in the cytoplasm, unlike the other members of the GT-C superfamily. It is these findings that prompted our further investigation and biochemical characterization of the role of Rv3779.

The evidence from construction and analysis of the Rv3779 knockout mutant of *M. tuberculosis,* described herein, and the *in vitro* assay of Rv3779-overexpressed strain of *M. smegmatis* membrane enriched fraction, allowed us to conclude that Rv3779 possesses PPM synthase activity homologous to Ppm1, since *M. smegmatis* lacks the *Rv3779* gene. Our subsequent biochemical analysis and characterization with the $\triangle$*Rv3779* mutant has shown far less PIMs as compared to wild type, and subsequent mass spectrum analysis confirmed this finding. Overall, the ratio of amounts of the lower form of PIMs to the higher forms was 4.4 times higher than in the wild-type, indicating the deletion of *Rv3779* in *M. tuberculosis* has profound effects on the synthesis of the higher PIMs. This had a serious effect on the viability and morphology of the bacilli, as the knockout strains grew much slower in liquid culture than that of wild type, with cells being on average 37% shorter than that of wild type.

Changes in the MAME profile due to the knockout of Rv3779 in *M. tuberculosis* $H_{37}Rv$ were not obvious, but a profound decrease in the amounts of $AcPIM_6$ and Ac_2PIM_6 was observed. The expression of Rv3779 in *M. tuberculosis* $\triangle$*Rv3779* mutants restores full levels of these higher PIMs (PIM_6), while the empty vector controls remained deficient. Furthermore, the disruption of *Rv3779* through homologous recombination produced a lipoglycan-less mutant in which the normal phenotype was restored with the gene complementation. The dramatic loss of the higher forms of PIM due to the loss or absence of Rv3779 is evident that it is required for their synthesis. Moreover, the availability of defined mutants of *M. tuberculosis* deficient in PIM and LM/LAM biosynthesis (such as the $\triangle$*Rv3779* mutants) are unique and insightful tools for the study of the immunopathogenesis of the live tubercle bacilli. The $\triangle$*Rv3779* mutant of *M. tuberculosis*, which produces less polar PIMs and LM/LAM would be just such a useful tool.

In light of this evidence, it is evident that Rv3779 is involved in the transfer of a mannosyl residue from the GDP-Man glycosyl donor to both forms of polyprenyl phosphate (C_{35} and C_{50}) to form PPM, which is used in the further mannosylation of PIM_4, creating the higher PIMs, LM, and ultimately LAM. This conclusion helps to further elucidate the biosynthetic pathway of these important lipoglycans in *M. tuberculosis.*

3.5 CONCLUDING REMARKS AND FUTURE RESEARCH

The varying forms of LAM are used by various species of mycobacteria to modulate the immune response of the host, and in the case of TB, enhance the pathogenesis of *M. tuberculosis*. LAM, and its synthetic precursors; the higher forms of the PIMs and LM, are extracytoplasmic and therefore rely on the transfer of mannosyl residues by PPM for further hypermannosylation and maturation. The role of Rv3779 in *M. tuberculosis* was unknown and it was postulated to be a glycosyltransferase, based on bioinformatic analysis of its sequence. A detailed understanding of Rv3779 therefore provides further insight into the metabolism and pathogenesis of mycobacteria.

From the experimental analysis presented herein, it seems likely that Rv3779 functions as a PPM synthase, a mannosyltransferase involved in PIM, LM, and LAM mannosylation in the same manner as Ppm1, accepting mannose from GDP-Man to form PPM from Pol-P. While the elucidation of the role of Rv3779 in *M. tuberculosis* presented herein is compelling, further and more definitive characterization can still be done. This is made difficult however, by the fact that Rv3779 is an integral membrane protein, and obtaining a purified and enzymatically functional preparation of this protein is highly improbable. Therefore, direct substrate-level assays with a purified protein extract are as yet, unable to be performed.

It would therefore seem prudent that future studies would entail the generation of a knockout of Ppm1 (*ΔRv2051c*) in *M. smegmatis*, as *Rv3779* is not present in fast growing mycobacteria such as *M. smegmatis*. Transforming the knockout mutant with pVV16-*Rv3779* would presumably restore the ability of the *ΔRv2051c* mutant to form

PPM, however, generation of such a mutant is unlikely as the *△Rv2051c* deletion may be lethal or simply lead to non-growing transformants. Further radioisotope labeling experiments with possible *E. coli* clones with *Rv3779* may also yield valuable insights, and despite unsuccessful attempts at expressing *Rv3779* into *E. coli*, perhaps continued future attempts might be successful. Finally, there are questions of substrate specificity, such as the preference for C_{35}-P or C_{50}-P, especially in lieu of the fact that Ppm1 seems to possess dual functionality. Perhaps Rv3779 utilizes just C_{50}-P to form DPM in *M. tuberculosis* while Ppm1 may use both C_{35}-P and C_{50}-P. These are questions that can be answered upon further study, and in the end, the role of Rv3779 can be more fully ascertained.

The availability of these defined mutants of *M. tuberculosis* deficient in higher PIM, LM and LAM biosynthesis can provide a unique opportunity to further study the effects of the immunopathogenesis carried by the live tubercle bacilli. The *△Rv3779* mutant of *M. tuberculosis*, which produces less polar PIMs, LM, and LAM, as well as suffering profound changes in growth characteristics, cellular morphology, and cell envelope composition, would likewise be such a useful tool in further probing the roles of glycosyltransferases in the pathogenicity of *M. tuberculosis*.

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CHAPTER 4

STRUCTURAL CHARACTERIZATION OF THE GALACTAN INTERMEDIATES OF ARABINOGALACTAN, THE MAJOR CELL WALL COMPONENT IN MYCOBACTERIA

4.1 INTRODUCTION

The mycobacterial cell wall consists of covalently linked peptidoglycan (PG), arabinogalactan (AG), and mycolic acids, which together form the mAGP complex (Brennan and Nikaido, 1995; Brennan, 2003). As discussed in previous chapters, this macromolecule forms a unique and impermeable structural layer that protects the cell, and disruption of the synthesis of this macromolecule is known to be detrimental to the bacterium. Two of the frontline anti-tuberculosis agents, INH and ethambutol, interrupt the synthesis of some of these cell wall components (Takayama, 1974; Winder, 1982; Banerjee *et al.*, 1994; Mikusova *et al.*, 1995; Belanger *et al.*, 1996). The specifics of its assembly are still not fully elucidated, but further insight into these synthetic pathways will be useful for the development of new drugs against mycobacterial diseases such as tuberculosis and leprosy.

The initial steps of the biosynthesis of AG occurs on a lipid carrier, polyprenyl

phosphate (Pol-P), specifically decaprenyl phosphate (C_{50}-P) in *M. tuberculosis*, with the transfer of GlcNAc-1-P and Rha*p* from UDP-GlcNAc and dTDP-Rha respectively, to the lipid carrier, decaprenyl phosphate, to form decaprenyl-P-P-GlcNAc (lipid 1 or glycolipid I (GL-1)) and later, decaprenyl-P-P-GlcNAc-Rha (lipid 2 or glycolipid (GL-2)) (Mikusova *et al.*, 1996). GL-2 is then sequentially polymerized with Gal*f* residues from the high energy donor UDP-Gal*f*, resulting in the glycolipid decaprenyl-P-P-GlcNAc-Rha-Gal_x (where x is ~25-30 residues) (Mikusova *et al.*, 2000). The transfer of Ara*f* residues from β-D-arabinofuranosyl-1-monophosphoryldecaprenol (DPA) to the growing decaprenyl-P-P-GlcNAc-Rha-galactan generates decaprenyl-P-P-GlcNAc-Rha-Gal_x-Ara_y (where y is 60-70 residues) (Mikusova *et al.*, 2000; Yagi *et al.*, 2003). The macromolecule is then mycolylated and transferred to peptidoglycan at some point (Figure 4.1.1).

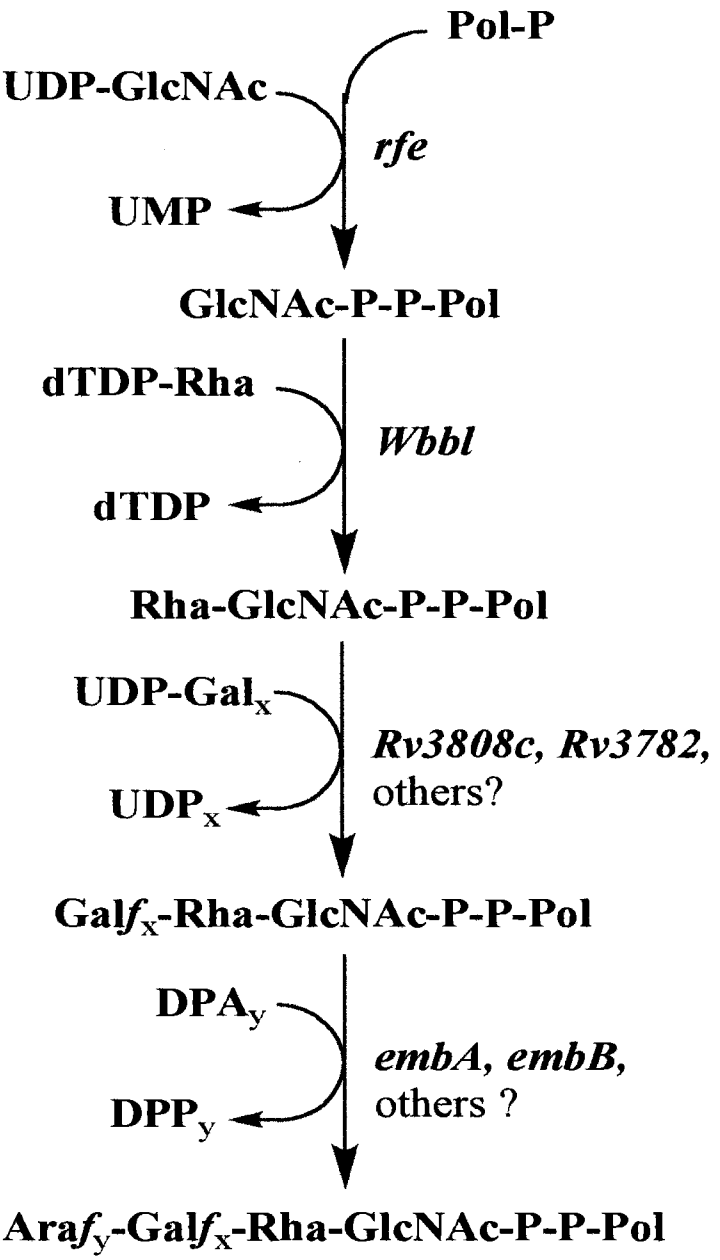

Figure 4.1.1. Pathway for the synthesis of the arabinogalactan mycobacterial cell wall core. The ethambutol resistance genes, *embA-B are thought to* encode arabinosyltransferases involved in AG synthesis. X and Y are the number of Gal*f* and Ara*f* residues, respectively. X and Y have been estimated to be approximately 30 Gal*f* and 70 Ara*f*. *Abbreviation*: DPA – Decaprenyl phosphoarabinose, DPP – Decaprenyl diphosphate, Pol-P – Polyprenyl phosphate.

Recent studies have identified several enzymes involved in mAGP assembly in mycobacteria using genetic and biochemical approaches. For example, the genes involved in the synthesis of sugar-nucleotide precursors such as dTDP-Rha (*rmlA* (*Rv0034*), *rmlB* (*Rv3464*), *rmlC* (*Rv3465*), and *rmlD* (*Rv3266c*)) and UDP-Gal*f* formation (*galE* (*Rv3634*) and *glf* (*Rv3809c*)) have been reported (Ma *et al.*, 1997; Weston *et al.*, 1998; Hoang *et al.*, 1999; Stern *et al.*, 1999; Giraud *et al.*, 2000). The synthesis of Lipid I (C_{50}-P-GlcNAc) was proposed to be carried out by the *rfe* (*Rv1302*) gene product (decaprenyl-monophosphate-α-*N*-acetylglucosaminyltransferase) based on homology to *Escherichia coli wecA*, which catalyzes the transfer of *N*-acetylglucosamine (GlcNAc) to an undecaprenyl phosphate lipid carrier in the first step in the biosynthesis of O7-specific and other O-specific polysaccharides containing GlcNAc (Meier-Dieter *et al.*, 1990; Meier-Dieter *et al.*, 1992; Alexander and Valvano, 1994; Mikusova *et al.*, 2000). *wbbL* (*Rv3265c*) was characterized as the essential gene that encodes the rhamnosyltransferase which catalyzes the transfer of Rha from dTDP-Rha to lipid I, forming lipid II (Mill *et al.*, 2004).

Based on analysis by gas chromatography mass spectrometry (GC-MS) and fast atom bombardment mass spectrometry (FAB-MS), the results suggests that the arabinogalactan structure consists of a linear alternating galactofuranose (Gal*f*) polymer of around 30 residues possessing alternating beta-(1→5) and beta-(1→6) linked Gal*f* residues with 2-3 arabinan chains attached to C-5 of some of the beta(1→6) Gal*f* residues (Daffe *et al.*, 1990; McNeil *et al.*, 1990; Besra *et al.*, 1995; McNeil, 1999). The presence of various types of glycosyl linkages within the galactan chain suggests two or more galactosyltransferases may be involved in its synthesis. Two galactofuranosyl

transferases, Rv3808c (GlfT2) and Rv3782, are responsible for the polymerization of galactan (Belanova *et al.*, 2008). The Rv3782 bifunctional transferase enzyme is capable of adding beta-(1→4) and beta-(1→5) linked Gal*f* residues to GL-2 and is therefore involved in the initial steps of galactan formation. Its product, decaprenyl-P-P-GlcNAc-Rha-Gal*f*-Gal*f*, serves as the substrate for the continual polymerization of galactan, catalyzed by GlfT2 (Belanova *et al.*, 2008; Mikusova *et al.*, 2006). These steps are outlined in Figure 4.1.2.

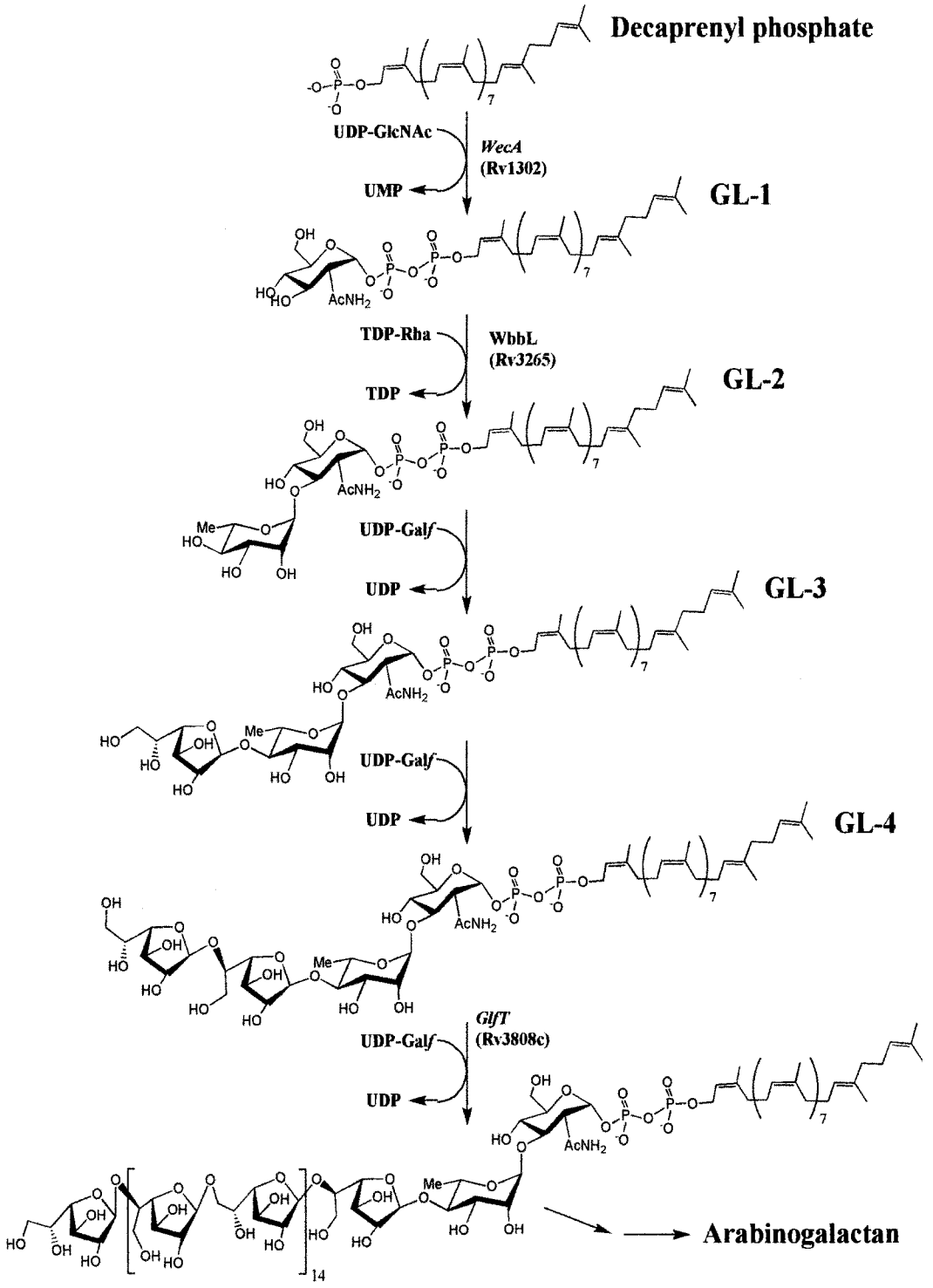

Figure 4.1.2. Pathway for the synthesis of the galactan intermediates that serve as the framework for arabinogalactan. Suggested enzymes involved are presented (Figure adapted from: Mikusova *et al.*, 2006).

GlfT2 is reported to be a bifunctional transferase enzyme capable of adding alternating beta-(1→5) and beta-(1→6) linked Gal*f* residues (Mikusova *et al.,* 2000; Kremer *et al.,* 2001; Alderwick *et al.*, 2008). Whether or not the Gal*f* residues are added one at a time at this point remains unclear and is therefore the focus of this study.

The structure of AG, as well as how the galactan and arabinan components are assembled is still not fully understood, despite tremendous progress over the last 2 decades. The numerous enzymes involved in its synthesis represent possible drug targets. For example, based on several studies, WecA, an enzyme that is responsible for AG synthesis, is sensitive to antibiotics such as tunicamycin (Ward, 1977; Ward and Curtis, 1982). Other enzymes (some of which are essential for growth of mycobacteria), such as WbbL and RmlD, represent novel candidate targets (Mills *et al.*, 2004; Ma *et al.*, 2002). Therefore, it has been suggested that the intermediate steps in mAGP synthesis, especially these stages of polymerization of galactan, would represent promising and novel drug targets (Kremer *et al.*, 2001, Ma *et al.*, 2001). In this report, we describe the development of methods for the isolation and structural characterization of the galactan intermediates by mild acid hydrolysis and thin layer chromatography. The work demonstrates how the galactan intermediates are assembled by the transfer of Gal*f* from their sugar donor, UDP- Gal*f,* one residue at a time. This study will provide initial insights into understanding the complexity of how the galactan chain is assembled in mycobacterium to allow for better characterization of the structure of AG, and possibly the future development of new chemotherapeutic approaches against tuberculosis.

4.2 MATERIALS AND METHODS

4.2.1 Bacterial strains and other materials

Mycobacterium smegmatis mc^2155 strain TM102 was propagated in nutrient broth at 37°C with constant agitation. The growth of bacterial culture was monitored by measuring the optical density (OD) at 600 nm using a spectrophotometer (Bausch & Lomb). Most chemicals used were at least analytical grade and purchased from Sigma Aldrich (St. Louis, MO). Organic solvents were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Radiolabeled UDP-[U-^{14}C]-galactose was obtained from PerkinElmer (Waltham, MA).

4.2.2 Preparation of membrane and cell wall enzyme fractions

M. smegmatis was grown to mid-log ($OD_{600\ nm}$ 0.7) as described in 3.2.1, harvested, washed once with phosphate-buffered saline pH 7.4, and stored at -20°C until required. Then 10 g wet weight of the mycobacterial cells were washed and resuspended in 30 ml of buffer A (which consists of 50 mM MOPS adjusted to pH 8.0 with KOH, 5 mM mercaptoethanol, and 10 mM $MgCl_2$) at 4°C and subjected to probe sonication using a Sanyo model Soniprep 150. The cells were sonicated at 4°C at 50% of maximum power rating for the sonicator tip for ten 60 second cycles at 100% duty cycle using a 90 second cooling period between each cycle. The sonicated lysates were then centrifuged at 27,000 × *g* for 60 min at 4°C. The resulting pellets were fully resuspended in buffer A by grinding with a tissue homogenizer. Percoll (Amersham Pharmacia Biotech) was added to the suspension to achieve a final concentration of 60% and centrifuged at 27,000 × *g* for 1 h at 4°C. The resulting white upper layer, which consists of particulate, diffuse enzymatically active cell wall material, is called the P60 fraction. This was collected and the Percoll was removed by washing three times with buffer A. The final cell wall pellet was resuspended in buffer A at a final

protein concentration of 10 mg/ml (per Mikusova *et al.*, 2006). Membrane fractions were obtained by ultracentrifugation of the 27,000 x *g* supernatant at 100,000 x *g* for 1 h at 4°C. The resulting supernatant was carefully removed and the resulting cell membrane enriched pellets were resuspended in buffer A at a protein concentration of 20 mg/ml. Protein concentrations were determined using the bicinchoninic acid (BCA) protein assay (Smith *et al.*, 1985).

4.2.3 Preparation of UDP-Gal*p* mutase

Escherichia coli BL21(DE3) (Stratagene, La Jolla, CA) cells transformed with plasmid pORF6 containing *glf* (*Rv3809c*) was provided kindly by Dr. Michael R. McNeil, Colorado State University, Fort Collins, CO. UDP-Gal*p* mutase was prepared and assayed as described previously (Lee *et al.*, 1996).

4.2.4 Preparation of dTDP-Rha.

dTDP-Rha was prepared as described previously (Mikusova *et al.*, 2000) with modification. Briefly, 12 nmol of dTDP-glucose (sodium salt, Sigma) was incubated with 25 µl of the 100,000 x *g* supernatant of sonicated *M. smegmatis* (approximately 70 µg of cytosolic protein) at 37°C for 1 h. We used the disrupted *M. smegmatis* instead of purified enzymes since they contain all the endogenous cofactors and the dTDP-Rha synthetic enzymes (Ma *et al.*, 1997). The reaction mixture was stopped by the addition of 100 µl of ice-cold ethanol, stored at –20°C for 1 h and centrifuged at 14,000 x g for 30 min. The supernatant was carefully removed and evaporated under a stream of nitrogen. The dried material was resuspended in 25 µl of sterile deionized water and stored at –20°C until use.

4.2.5 Galactosyltransferase assay

The reaction mixtures for measuring [^{14}C]Gal incorporation were composed of 1 μCi UDP-[U-^{14}C]Gal (at a specific activity of 327 mCi/mmol and preincubated with 0.13 mg of UDP-Gal*p* mutase at 37°C for 15 min), 25 μl TDP-Rha (prepared as previously described), 25 μM UDP-GlcNAc, 62.5 μM ATP, 1 mg of membrane enriched fraction, and 1.5 mg cell wall fraction (P60) in a final reaction volume of 320 μl. The reaction mixtures were then incubated at 37 °C for 1 h and 6 ml of chloroform/methanol (2:1, v/v) was then added to stop the reaction. The mixture was extracted by further incubation at room temperature for 15 min and centrifuged at 3,000 x g. The supernatant was recovered and 680 μl of deionized water was added to obtain a biphasic layer. The upper phase (water) was removed and discarded, and the lower organic phase was Folch-washed with 2 ml of chloroform/methanol/water (3:47:48, v/v/v) (Folch *et al.*, 1957). The aqueous phase was removed and discarded and the bottom phase was dried under a stream of nitrogen at room temperature. The final product was resuspended in 200 μl of chloroform/methanol/water/concentrated ammonium hydroxide (65:25:3.6:0.5, v/v/v/v).

To solubilize the polar lipid-lipid linked polymers (lipid-linked galactan polymer) from the pellet resulting from the 3,000 x g centrifugation step, the pellet was washed with 2 ml of the following solvent mixture; methanol/0.9% NaCl (1:1, v/v), methanol/water (1:1, v/v) and methanol (100%), respectively, to remove any un-incorporated radiolabel. The washed pellet was then extracted for polar lipid-linked polymers with 1 ml chloroform/methanol/water (10:10:3, v/v/v) (Rush *et al.*, 1993). Finally, 1 ml of water/ethanol/diethyl ether/pyridine/concentrated ammonium hydroxide

(15:15:5:1:0.017, v/v/v/v/v) (called "E-soak") was added to obtain the [^{14}C]Gal-labeled lipid-linked products of even greater polarity (Angus and Lester, 1972).

The total cpm of radiolabeled product in chloroform/methanol (2:1, v/v), chloroform/methanol/water (10:10:3, v/v/v) and E-soak fractions were measured by scintillation counting on a Beckman LS6500 Scintillation counter (Beckman, Fullerton, CA) using 7 ml of Ultima Gold^{TM} liquid scintillation cocktail (PerkinElmer). The radiolabeled material (10% aliquot) was subjected to thin-layer chromatography (TLC) in chloroform/methanol/water/concentrated ammonium hydroxide/1 M ammonium acetate (180:140:23:9:9, v/v/v/v/v) on aluminum-backed Silica Gel 60 plates ((EMD Chemicals). Autoradiography was performed by exposing the chromatograms to X-ray film (Kodak Biomax MR) for 4-5 days at -80°C.

4.2.6 Analytical procedures

To perform size exclusion chromatography of the polyprenyl phosphate-linked polymers, the chloroform/methanol/water (10:10:3, v/v/v) and E-soak fractions were hydrolyzed in mild acid, which selectively cleaved the polyprenyl phosphate apparently without destroying the sugar polymer as follows (Lucas *et al.,* 1975). Samples (~10,000 cpm) were dried under a stream of nitrogen and resuspended in 50 µl of 1-propanol by bath sonication. Then 100 µl of 0.02 N HCl was added and incubated for 30 min at 60 °C (Lucas *et al.*, 1975; Turco *et al.*, 1984). The reaction mixture was carefully neutralized with 10 µl of 0.2 N NaOH.

Another condition of mild acid treatment was also applied and both compared in terms of suitability for maintaining the full structure of the oligosaccharide segments.

The dry lipid-linked polymer was hydrolyzed with 1% acetic acid at 90°C for 1 h and neutralized with 10% ammonia until pH was ~7.0. The hydrolysates were evaporated using a rotavap and resuspended in 50 mM Tris-HCl pH 9.0. Alkaline phosphatase (10 units, Sigma Chemical, St Louis, MO) was added and the reaction mixture was incubated at 37°C for 30 min to remove any terminal phosphate residues that may remain after cleavage of the TB polyprenyl unit. The resulting product was applied to a DE52 anionic exchange column and then purified by running on a 1 x 200 cm Biogel P-6 sizing column, which was equilibrated and eluted with 20 mM ammonium acetate pH 7.0. The oligosaccharide fractions were pooled and analyzed on a HPTLC silica gel plate. The plate was developed in the following solvents in order: 1-propanol/ nitromethane/water (5:2.3:2.7, v/v/v), followed by 1-propanol/nitromethane/water (5:2:3, v/v/v), and 1-propanol/nitromethane/water (5:1.7:3.3, v/v/v).

SDS-PAGE analysis of enzymatically radiolabeled products was conducted using Novex® 10–20% Tricine gels (Invitrogen, Carlsbad, CA) under conditions recommended by the manufacturer. After electrophoresis, samples were blotted to nitrocellulose membranes, which were dried at room temperature, and subjected to autoradiography.

4.3 RESULTS

4.3.1 Synthesis of Polyprenyl-P-linked Intermediates

M. smegmatis membrane and P60 cell wall fraction were utilized in a cell-free assay for the synthesis of decaprenyl-P-P-GlcNAc-Rha-(Gal*f*)$_{1-4}$ and other more glycosylated intermediates in AG biosynthesis (Mikusova *et al.*, 1996). The less and

more glycosylated products were differentiated based on their solubility in organic solvents and susceptibility to mild acid hydrolysis and the presence of the appropriate radiolabeled sugar (Mikusova *et al.*, 2000).

This cell-free assay was essentially repeated, however, to demonstrate the sequential addition of Gal*f* residues in the synthesis of the larger galactan intermediates beyond decaprenyl-P-P-GlcNAc-Rha-(Gal*f*)$_{1-4}$. Endogenous glycosyltransferases and decaprenylphosphate were supplied by the cell wall-membrane enriched fraction of a *M. smegmatis* cell-free extract and was supplemented with UDP-GlcNAc, dTDP-Rha (the precursors of the Rha-GlcNAc linker unit), UDP-[^{14}C]Gal*p* and UDP-Gal*p* mutase enzyme extract. After incubation, the reaction products were sequentially extracted with the following organic solvent cocktails; chloroform/methanol (2:1, v/v), chloroform/methanol/water (10:10:3, v/v/v), and finally E-soak. The subsequent incorporation of [^{14}C]Gal*f* residues (ultimately from UDP-[^{14}C]Gal*p*) into these three extract fractions is shown in table 4.3.1.

Table 4.3.1. The [^{14}C]-galactosyltransferase assay. This demonstrates the buildup of [^{14}C] lipid-linked galactan polymer beyond decaprenyl-P-P-GlcNAc-Rha-(Gal*f*)$_{1-4}$ by quantification of the incorporation of [^{14}C]Gal into organic extraction fractions. (Mikusova *et al.,* 1996). *M. smegmatis* membrane and P60 cell wall fraction were utilized in a cell-free assay, and reactions 1, 2 and 3 are identical.

Reaction	Total [^{14}C]- incorporated into each fraction (cpm)		
	C/M (2:1. v/v)	C/M/W (10:10:3, v/v/v)	E-Soak
1	16,800	85,800	358,200
2	17,080	128,200	463,700
3	12,860	147,100	396,200

Further analysis of the chloroform/methanol (2:1) soluble extracts was performed by TLC chromatogram developed in chloroform/methanol/ammonium hydroxide/1M ammonium acetate/water (180:140:9:9:23, v/v/v/v/v). This TLC is shown in Figure 4.3.1. Likewise, Tricine-SDS PAGE was performed on the chloroform/methanol/water (10:10:3, v/v/v) and E-soak fractions and is shown in Figure 4.3.2.

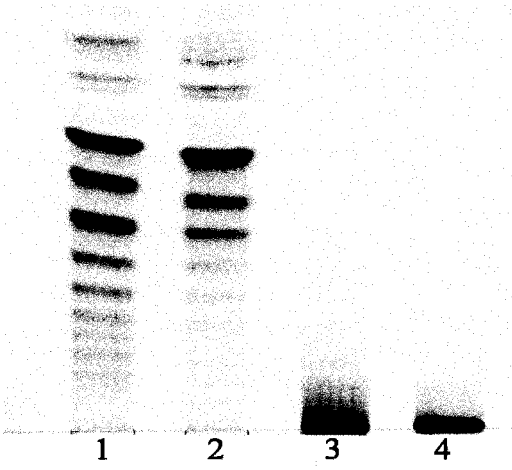

Figure 4.3.1. TLC chromatography of chloroform/methanol (2:1) soluble extracts from the cell-free galactosyltransferase assay. Solvent system: chloroform/methanol/water/ammonium hydroxide/1 M ammonium acetate 180:140:23:9:9 Lane 1- chloroform/methanol (2:1, v/v) fraction, Lane 2 – mild alkaline-treated chloroform/methanol (2:1, v/v) fraction, Lane 3 – chloroform/methanol/water (10:10:3, v/v/v) fraction, and Lane 4 – E-soak fraction.

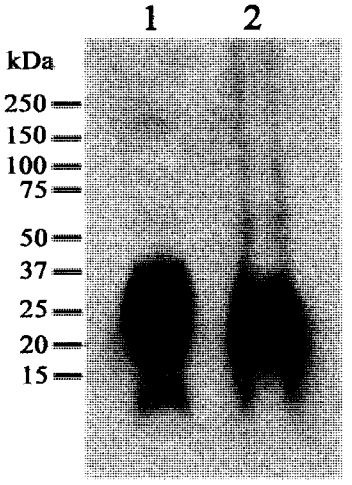

Figure 4.3.2. Tricine SDS-PAGE gel of the highly glycosylated lipid-linked polymers in the chloroform/methanol/water (10:10:3, v/v/v) extract (lane 1), and E-soak extract (lane 2).

4.3.2. Properties of the Lipid-linked [^{14}C]Gal-labeled Polymer

Previous studies have shown that gel filtration of the chloroform/methanol/water (10:10:3, v/v/v) and E-soak extracts on a BioGel P-100 sizing chromatography column resulted in poor recovery of material in the void volume (Ciucanu and Kerek, 1984). Based on this work, a mild acid hydrolysis of the extracts in 1% acetic acid incubated at 90°C for different time points was attempted to find the best conditions for pretreating the extracts for sizing chromatography. Then the extracts were treated with alkaline phosphatase to remove charged phosphate groups before running on TLC. The hydrolyzed product was run on TLC to see whether the product was adequately hydrolyzed, giving release of the sugar polymer. As such, the TLC was developed in the solvent system 1-propanol/nitromethane/water (5:2.3:2.7, v/v/v). If the galactan intermediates were completely hydrolyzed (and not overly hydrolyzed), it would not move under these conditions. The result is shown in the Figure 4.3.3 and it was concluded that 30 minutes or more using 1% acetic acid at 90°C gave the best conditions for an adequately hydrolyzed product. This was further analyzed by loading onto a Biogel P-6 chromatography column. Fractions were collected to follow the elution profile of the intermediates by liquid scintillation counting (Figure 4.3.4A). Fractions were pooled according to size (I-VI). Then, each pooled fraction was spotted on a silica TLC-plate and developed in the solvent system 1-propanol/nitromethane/water (5:2.3:2.7, v/v/v), followed by running in 1-propanol/nitromethane/water (5:2:3, v/v/v), and finally running in 1-propanol/nitromethane/water (5:1.7:3.3, v/v/v) (Figure 4.3.4B).

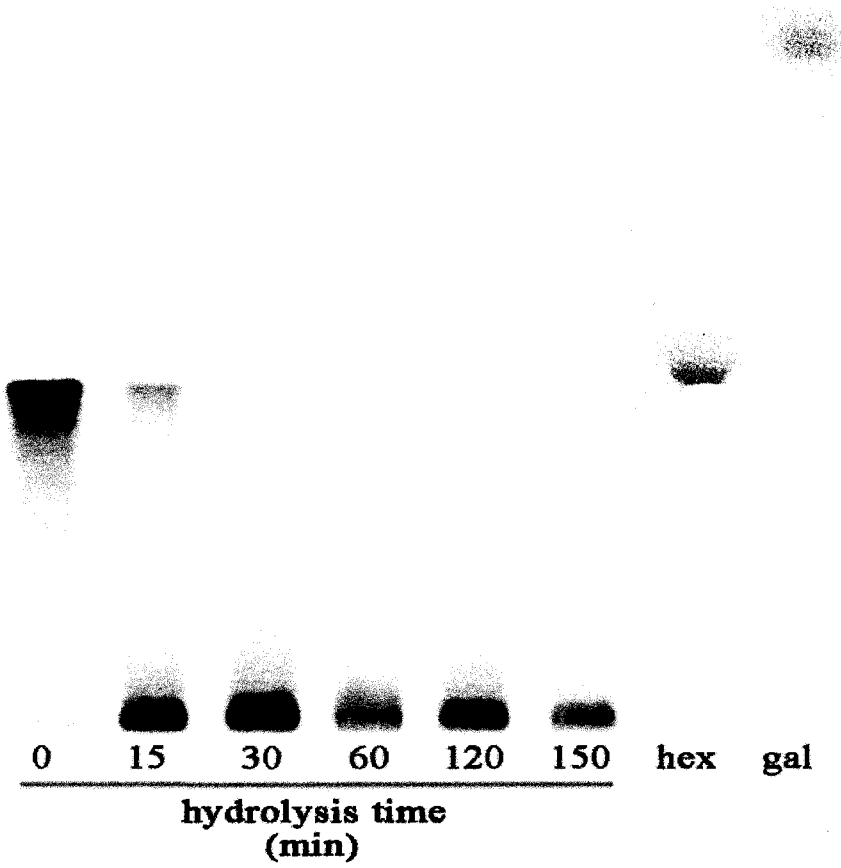

Figure 4.3.3. Partial hydrolysis of [^{14}C]Gal-labelled E-soak fraction with 1% acetic acid at 90°C for different time points. The TLC was developed in a solvent of 1-propanol/nitromethane/water (5:2.3:2.7, v/v/v) and autoradiographed. Standard sugars (hex = hexomaltose and gal = galactose) were detected by spraying with α-naphthol/sulfuric acid and heating at 100°C.

chloroform/methanol/water (10:10:3, v/v/v) and E-soak extracts on the Tricine-SDS PAGE as shown in Figure 4.3.2 is evident that these galactan intermediates are indeed large in size.

Previous studies have shown that gel filtration of the chloroform/methanol/water (10:10:3, v/v/v) and E-soak extracts on a BioGel P-100 sizing chromatography column resulted in poor recovery of material in the void volume (Ciucanu and Kerek, 1984). This suggests these extracts consist of such a large lipid-linked galactan polymer(s) that is embedded in lipid micelles, thereby making sizing chromatography unreliable. Therefore, to prepare these extracts for sizing chromatography, mild acid hydrolysis of the extracts was carried out. Previously, mild acid treatment of the extracts in 0.2N HCl at 60°C for 30 min released oligosaccharides of lipid-linked Gal*f* that ran as included on the P-100 column at the expected molecular weights (Ciucanu and Kerek, 1984). The size of the mature arabinogalactan released from the mycobacterial cell wall is on the order of 15 kDa, far larger than these oligosaccharides and thus, the decaprenyl-P-P-linked galactan polymer generated by the *in vitro* system is apparently not fully glycosylated. Moreover, the chloroform/methanol/water (10:10:3, v/v/v) extract is an incompletely glycosylated version of the E-soak fraction (Daffe *et al.*, 1990). Though this mild acid hydrolysis with HCl gave the end products of the desired size (which ran in the included region on the P-100 sizing column), they seem to be somewhat over-hydrolyzed into smaller fragments as well, thereby giving a broad peak of eluted material. Thus, we tried another mild acid treatment using 1% acetic acid instead, and incubating at 90°C for

different time points (Figure 4.3.3). These results are more encouraging, and suggest treatment for at least 30 minutes at 90°C is sufficient.

Sizing chromatography was markedly improved, and the extracts eluted in a sharper and narrower peak for the mid acid treated E-soak fraction (Figure 4.3.4A). Again, a ladder demonstrating a range of sizes of galactan intermediate polymer is evident, particularly in the more included fractions (4 and 5). When compared to the oligosaccharide standards (I-V), this suggests that each band of the ladder differs by one Gal*f* residue, much like what is seen in the chloroform/methanol/water (10:10:3, v/v/v) and E-soak fractions on TLC (Figure 4.3.4B). Based on these data, it is evident the galactan polymer is polymerized one Gal*f* unit at a time.

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