Published in final edited form as:
Adv Appl Microbiol. 2009 ; 69: 23–78. doi:10.1016/S0065-2164(09)69002-X.

Biogenesis of the cell wall and other glycoconjugates of *Mycobacterium tuberculosis*

Devinder Kaur, **Marcelo E. Guerin**, **Henrieta Škovierová**, **Patrick J. Brennan**, and **Mary Jackson***
Mycobacteria Research Laboratories, Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, CO 80523-1682, USA

Abstract

The reemergence of tuberculosis in its present-day manifectations – single, multiple and extensive drug resistant forms and as HIV-TB coinfections – has resulted in renewed research on fundamental questions such as the nature of the organism itself, *Mycobacterium tuberculosis*, the molecular basis of its pathogenesis, definition of the immunological response in animal models and humans, and development of new intervention strategies such as vaccines and drugs. Foremost among these developments has been the precise chemical definition of the complex and distinctive cell wall of *M. tuberculosis*, elucidation of the relevant pathways and underlying genetics responsible for the synthesis of the hallmark moities of the tubercle bacillus such as the mycolic acid-arabinogalactan-peptidoglycan complex, the phthiocerol- and trehalose-containing effector lipids, the phosphatidylinositol-containing mannosides, lipomannosides and lipoarabinomannosides, major immunomodulators, and others. In this review, the laboratory personnel that have been the focal point of some to these developments review recent progress towards a comprehensive understanding of the basic physiology and functions of the cell wall of *M. tuberculosis*.

I. Introduction

The latest complete data as assembled by such bodies at the World Health Organization (http://www.who.int/tb/publications/global_report/en/index.html, 2008) indicate an estimated 9.2 million new cases of TB in 2006 (139 per 100,000 population), including 4.1 million new smear-positive cases (44% of the total) and 0.7 million HIV-positive cases (8% of the total). This is an increase from 9.1 million cases in 2005, due to population growth. India, China, Indonesia, South Africa and Nigeria rank first to fifth respectively in terms of absolute numbers of cases. The African Region has the highest incidence rate per capita (363 per 100 000 population). There were an estimated 14.4 million prevalent cases of TB in 2006. There were an estimated 0.5 million cases of multidrug-resistant TB (MDR-TB) in 2006 and an estimated 1.5 million deaths from TB in HIV-negative people and 0.2 million among people infected with HIV.

What is now recognized as a human tragedy of the first order, compounded by the fear of even more wide-spread dissemination of MDR and XDR strains of *Mycobacterium tuberculosis*, has had the benefit of evoking new interest and resulting research towards a better understanding of the organism and its effects on the host, driven by the need for new diagnostic tools and new intervention strategies, whether chemotherapeutic or immunological. The cell wall of *M. tuberculosis*, its most distinctive feature and the basis of

*Corresponding author: Tel. 970-491-3582; Fax: 970-491-1815; Mary.Jackson@colostate.edu.

much of the characteristics of tuberculosis pathogenesis, is a case in point benefitting from this renewed attention.

The envelope of *M. tuberculosis* consists of two distinct parts; the plasma membrane and the cell wall as such (Brennan and Draper, 1994); the question of a true capsule is controversial (see below). Clearly, *M. tuberculosis,* especially within a host, is devoid of a classical capsule adhering to the cell with sufficient tenacity to be observed by simple techniques such as negative staining and India ink. On the other hand extracellular materials do exist – glucans, arabinans and arabinomannans and some proteins, mostly reflective of internal structures – representing more of an outer layer than a true capsule (Daffé and Draper, 1998). As for Gram positive bacteria, there probably exists an anatomically and functionally distinct periplasmic space (Matias and Beveridge, 2006). The cell wall and the membrane can readily be mechanically separated and have been studied independently (Crick *et al.*, 2008). Repeated attempts have been made to relate chemical and ultrastructural features and these attempts have been successful as far as the major components and compartments of the cell envelope are concerned, such as those of the insoluble “cell wall core”, namely peptidoglycan, arabinogalactan, and mycolic acids and the plasma membrane. Thus, the images of two electron dense layers separated by a transparent zone are in accord with a typical plasma membrane bilayer. Additional triple-layered images have been attributed to the cell wall proper and these consist of an inner layer of moderate electron density, reflective of mycobacterial peptidoglycan; a wider electron-transparent layer, attributable to the arabinogalactan-mycolic acid complex; and an outer electron opaque layer of extremely variable appearance and thickness, apparently the negatively charged so-called outer “capsular layer” containing mostly polysaccharides, glucans, arabinans and arabinomannans (Lemassu and Daffé, 1994; Ortalo-Magné *et al.* 1995).

However, the arrangement and distribution of the numerous soluble cell wall entities, such as lipoarabinomannan and lipomannan, the other phosphatidylinositol-containing glycolipids, the phthiocerol- and trehalose-containing lipids, (lipo)proteins, so important to the pathogenesis of *M. tuberculosis,* are poorly understood. An exception is the elegant topological studies of the MspA porin in the cell wall of *Mycobacterium smegmatis* and its possible extrapolations to *M. tuberculosis* (Faller *et al.* 2004) and, in that context, the clear evidence from cryo-electron tomography of an outermost morphologically symmetrical lipid bilayer, despite the presence of considerable amounts of mycolic acids with their inherently asymmetrical hydrocarbon chains (Hoffmann *et al.*, 2008; Zuber *et al.*, 2008). In this review we present the rather spectacular progress made since the re-emergence of TB as a pressing public health problem, in the chemical definition of the cell wall/envelope of *M. tuberculosis*, in its assembly and underlying genetics, and functions in the disease induction process.

II. The mycobacterial cell envelope

The cell envelope distinguishes species of the *Mycobacterium* genus from other prokaryotes. It consists of three major segments: the plasma membrane, the cell wall core and the outermost layer (Fig. 1). The cell wall core is made up of peptidoglycan (PG) in covalent attachment via phosphoryl-*N*-acetylglucosaminosylrhamnosyl linkage units with arabinogalactan (AG) which is in turn esterified to α-alkyl, β-hydroxy long-chain (C_{70}-C_{90}) fatty acids known as the mycolic acids. The outermost layer consists of a variety of noncovalently attached (glyco)lipids, polysaccharides, lipoglycans and proteins, including pore-forming proteins. In *M. tuberculosis*, the surface-exposed material of this outer layer, also called capsule, is essentially composed of polysaccharides and proteins with only minor amounts of lipids (Lemassu & Daffé, 1994;Ortalo-Magné *et al.*, 1995). Thus, *M. tuberculosis* is known to expose a hydrophilic surface.

Until recently, our view of the cell envelope architecture was essentially based on the model proposed by Minnikin in 1982. In this model, the mycolic acids are packed in a monolayer, parallel to each other, and oriented perpendicular to the plasma membrane. Interspersed somehow are the free lipids of the outer membrane (OM) with their fatty acyl chains intercalating into the mycolic acid layer. It was proposed that the covalently-bound mycolic acids of the cell wall core and the free lipids of the outermost layer form the inner and outer leaflet, respectively, of a highly impermeable asymmetrical bilayer that confers to mycobacteria their characteristic resistance to many therapeutic agents (Minnikin, 1982; Jarlier and Nikaido, 1994). A second model proposed by Rastogi (1991) suggested a similar organization except that the noncovalently-linked lipids form a monolayer that does not intercalate with the mycolic acids. Only recently have developments in microscopy techniques allowed the different layers of the mycobacterial cell envelope to be visualized in their native state (Hoffman *et al.*, 2008; Zuber *et al.*, 2008). Cryo-electron microscopy observations of vitreous sections of *M. smegmatis*, *M. bovis* BCG and of the closely related *Corynebacterium glutamicum* provided direct evidence of the existence of an outer bilayer and periplasmic space in these species. Although these studies confirmed mycolic acids as key components of the OM, the findings that the outer bilayer is apparently symmetric and much thinner than expected from the proposed models led to a significant revision of the current view of the cell envelope's architecture. In a revised model presented in Fig. 1, similar extractable lipids are present in both leaflets of the OM and the meromycolate chain of bound mycolic acids span the entire hydrophobic region (Hoffman *et al.*, 2008). Another model proposes the meromycolate chains of mycolic acids to be folded upon themselves to create a more compact structure, compatible with the observed thickness of the OM (Zuber *et al.*, 2008).

III. The capsular polysaccharides

The outermost compartment of the cell envelope of pathogenic mycobacterial species consists of a loosely-bound structure called capsule (Chapman *et al.* 1959; Hanks, 1961; Daffé and Draper, 1998). Although mycobacteria shed some of this material in the culture medium during *in vitro* growth (Ortalo-Magné *et al.* 1995), capsular components clearly coat *in vivo*-grown bacteria (Schwebach *et al.*, 2002), probably retained by the phagosomal membrane (Daffé and Draper, 1998). The capsular material primarily consists of proteins and polysaccharides (~ 97% of the total material) with only small amounts of lipids (Lemassu and Daffé, 1994; Ortalo-Magné *et al.* 1995).

Three types of polysaccharides are found in the capsular material of *M. tuberculosis*: a high molecular weight (>100,000 Da) α-D-glucan composed of a →4- α-D-Glc-1→ core branched every 5 or 6 residues by oligoglucosides, D-arabino-D-mannan (AM), and a D-mannan composed of a →6- α-D-Man-1→ core substituted at some positions 2 with a α-D-Man residue (Lemassu and Daffé, 1994, Ortalo-Magné *et al.*, 1995; Dinadayala *et al.*, 2004). All are neutral compounds, devoid of acyl substituents (Lemassu and Daffé, 1994). The structure of AM appears to be identical to that of LAM (see section IV. A), except for the loss of the phosphatidyl-*myo*-inositol anchor, suggesting that it may be formed from LAM by a specific hydrolytic enzyme. However, there is at present no evidence for this hypothesis.

α-D-glucan is the major carbohydrate constituent of the capsule of *M. tuberculosis*, representing up to 80% of the extracellular polysaccharides. Recent studies have begun to shed light on its biosynthesis. Based on the observation that α-D-glucan displays a glycogen-like structure, the orthologs of the *glg* genes involved in the biosynthesis of glycogen in *Escherichia coli* were identified in *M. tuberculosis* H37Rv and inactivated by allelic replacement. Biochemical analyses of the mutants indicated that the biosynthetic pathways

of α-D-glucan and glycogen involve common enzymes, among which the α-1,4-glucosyltransferases Rv3032 and GlgA (Rv1212c) (Table 1), the ADP-glucose pyrophosphorylase GlgC (Rv1213) and the branching enzyme GlgB (Rv1326c) (Sambou *et al.*, 2008) (see section VI. B). The disruption of *glgC* or *glgA* reduces by half the capsular α-D-glucan content of *M. tuberculosis* H37Rv. The involvement of common enzymes in the synthesis of the intracellular glycogen and capsular α-D-glucan suggests that the latter polysaccharide is synthesized inside the cytoplasm before being translocated to the cell surface by as yet unknown transporters.

Various biological functions have been associated with the polysaccharides of the capsule suggestive of a role in immunopathogenesis. Cywes *et al.* showed that capsular polysaccharides, among which the α-D-glucan, mediated the non-opsonic binding of *M. tuberculosis* H37Rv to Complement Receptor 3 (Cywes *et al.*, 1997). Given that CR3 is one of the principal phagocytic receptors of monocytes and neutrophils and that CR3-mediated phagocytosis can result in the diminution or absence of respiratory burst and suppression of IL-12 secretion, it was proposed that this route of entry may be favourable to the intracellular survival of the tubercle bacillus (Ehlers and Daffé, 1998; Fenton *et al.*, 2005). Other studies have highlighted the anti-phagocytic and immunomodulatory activities of the capsular polysaccharides of *M. tuberculosis* (Stokes *et al.*, 2004; Gagliardi *et al.* 2007). Owing to its glycogen-like structure, α-D-glucan was also proposed to be involved in *M. tuberculosis* evasion of the immune system by molecular mimicry (Lemassu and Daffé, 1994). Independently from its role in tuberculosis, α-D-glucan was reported to exhibit anti-tumor activity (Wang *et al.*, 1995; Zlotta *et al.*, 2000).

IV. The noncovalently bound glycoconjugates of the outer membrane

A. PHOSPHATIDYLINOSITOL MANNOSIDES, LIPOMANNAN AND LIPOARABINOMANNAN

1. Localization in the cell envelope and biological activities—One of the unique features of the mycobacterial cell envelope is its high content of mannosylated molecules, including (lipo)glycans and (lipo)glycoproteins. The mannosyl-phosphatidyl-*myo*-inositol-based glycolipids and metabolically-related lipoglycans comprising phosphatidyl-*myo*-inositol mannosides (PIMs), lipomannan (LM) and lipoarabinomannan (LAM) in particular, are found in abundant quantities in the cell envelope of all mycobacterial species. These molecules are non-covalently anchored through their phosphatidyl-*myo*-inositol (PI) moiety to the inner and outer membranes of the cell envelope (Pitarque *et al.*, 2008). The importance of these molecules in promoting the entry of *M. tuberculosis* inside phagocytic cells through mannose-specific C-type lectins, regulating phagosome maturation and modulating the host immune response *in vitro* has been well documented (for recent reviews, Briken *et al.*, 2004; Fenton *et al.*, 2005; Gilleron *et al.*, 2008). However, care should be taken in the interpretation of these studies since the majority of them have focused on the interactions of purified PIM, LM and LAM with cellular models and, thus, may not accurately reflect the relevance and individual contribution of these glycoconjugates in mycobacterial infections. In support of this assumption, a recent study on isogenic knock-out mutants of *Mycobacterium bovis* BCG and *Mycobacterium marinum* deficient in the synthesis of mannose-capped LAM (ManLAM) (see section IV.A.2) revealed only marginal differences between wild-type and mutant strains in terms of uptake by phagocytic cells, phagosome/lysosome fusion, replication *in vivo* and induction of immune responses in infected animals (Appelmelk *et al.*, 2008). On the other hand, clinical isolates of *M. tuberculosis* defective in some aspects of the synthesis and exposure of ManLAM at the cell surface were found to display important defects in phagocytosis by human primary macrophages (Torrelles *et al.*, 2008). Further studies are thus clearly required to determine the individual contribution of ManLAM to the pathogenicity of virulent species of the *Mycobacterium tuberculosis* complex. A better defined role for the most polar forms of

PIMs (containing up to six Man*p* residues) in virulence has recently emerged from the study of an *M. tuberculosis pimE* mutant deficient in the production of these lipids. The mutant was found to be greatly impaired for replication and persistence in mice and unable to replicate inside murine mouse bone-marrow macrophages (Larrouy-Maumus *et al.*, unpublished results). The impact that polar PIM production has on the permeability of the mycobacterial cell envelope is likely to account, at least in part, for the role of these lipids in intracellular survival (Larrouy-Maumus *et al.*, unpublished results).

Despite nearly two decades of studies, the role of PIM, LM and LAM in the physiology of mycobacteria is less clear. Emerging data suggest that PIMs play important roles in the permeability barrier of the cell envelope, cell membrane integrity and regulation of cell septation and division (Parish *et al.*, 1997; Korduláková *et al.*, 2002; Patterson *et al.*, 2003; Morita *et al.*, 2005; Morita *et al.*, 2006; Larrouy-Maumus *et al.*, unpublished results). The physiological functions of LM and LAM are unknown. While the non-pathogenic species *Mycobacterium smegmatis* appears to be permissive to a variety of mutations affecting PIM, LM and LAM synthesis, the inability to generate mutants of *M. tuberculosis* deficient in the acylation of PIMs or LAM synthesis (Goude *et al.*, 2008; Jackson *et al.*, unpublished results) indicates that these molecules probably play a more crucial role in the physiology of the tubercle bacillus than in that of fast-growing *Mycobacterium* species.

2. Structure of PIM, LM and LAM—PIMs, LM and LAM all share a conserved PI anchor with mannosylation extension at the C-6 position of the *myo*-inositol (*myo*-Ins) indicative of a metabolic relationship (for a recent review on the structure of PIM, LM and LAM, see Gilleron *et al*, 2008) (Fig. 1-2A). The anchor structure is heterogeneous, with variations occurring with respect to the number, location and nature of the fatty acids. The major PIM species are PI-dimannosides (Ac_1PIM_2 and Ac_2PIM_2) and PI-hexamannosides (Ac_1PIM_6 and Ac_2PIM_6). Ac_1PIM_2 and Ac_1PIM_6 contain one fatty acid linked to the Man*p* residue attached to position 2 of *myo*-Ins in addition to a diacylglycerol moiety, whereas Ac_2PIM_2 and Ac_2PIM_6 contain a fourth fatty acyl chain linked to position 3 of *myo*-Ins (Fig. 1-2A). LM and LAM possess a mannan core composed on average of 20-25 α(1→6)-linked Man*p* residues occasionally substituted at C-2 by single Man*p* units in all mycobacterial species investigated with the exception of *M. chelonae* which was reported to have branching occurring at C-3 (Guérardel *et al.*, 2002). The length of the mannan core of LM and degree of branching varies depending on the mycobacterial species. Recently, the use of MALDI-TOF mass spectrometry has allowed the precise length of the mannan domains of LM in *M. bovis* BCG, *M. smegmatis* and *M. tuberculosis* H37Rv to be defined (Gilleron *et al.*, 2006; Kaur *et al.*, 2007). A few unanswered questions remain, however, concerning the structure of this molecule. Firstly, it is at present unclear whether the linear and branched portions of the mannan core form distinct domains or whether 2,6-Man*p* and 6-Man*p* units intercalate at frequent regular intervals within the chain. Secondly, little is known about the position and number of arabinofuranose (Ara*f*) residues attached to the LM backbone. In the case of LAM, a branched arabinan polymer is further attached to the mannan core and cap motifs decorate the non-reducing termini of the arabinosyl side chains. The arabinan polymer contains about 60 Ara*f* units and consists of a linear α(1→5)-linked Ara*f* backbone punctuated with branched hexarabinofuranosides (Ara_6) and linear tetraarabinofuranosides (Ara_4). A recent study of the LAM-arabinan of *M. smegmatis* suggested the occurrence of an Ara_{18} motif resembling the internal structure of AG arabinan (Shi *et al.*, 2006) (see section V.A.1). However, the length of the terminal extensions linked at the non-reducing ends of this motif seems to vary in LAM-arabinan (Ara_{18}-Ara_{22}) (Shi *et al.*, 2006). The nature of the motifs capping the non-reducing termini of the arabinan domain of LAM differs among mycobacterial species. To date, three types of LAM have been described: mannose-capped LAM (ManLAM) found in *M. tuberculosis, M. bovis, M. bovis* BCG, *M. leprae, M. avium, M. xenopi, M. marinum* and *M. kansasii*; phospho-*myo*-inositol-capped LAM (PILAM)

found in *M. smegmatis* and *M. fortuitum*; and non-capped LAM (AraLAM) found in *M. chelonae*. LAM also differs between species in terms of the presence of recently discovered substitutions such as succinyl group and 5-deoxy-5-methylthio-*xylo*-furanose (MTX) (Delmas *et al.*, 1997; Treumann *et al.*, 2002; Guérardel *et al.*, 2003; Turnbull *et al.*, 2004; Joe *et al.*, 2006). The fundamentals of how LM and LAM are built-up is unknown, whether on a lipid carrier, growing from the reducing or non-reducing end, being built in blocks that are then put together, assembled on an enzyme complex much like polyketides or growing one glycosyl residue at a time analogous to an *N*-linked glycan.

3. Biosynthesis of PIMs

a. The early steps of PIM synthesis: The first step in PIM synthesis involves the transfer of a Man*p* residue from GDP-Man*p* to the 2-position of the *myo*-Ins ring of PI to form phosphatidyl-*myo*-inositol monomannoside, PIM_1 (Fig. 2A). We have identified PimA (Rv2610c) as the α-mannosyltransferase (ManT) responsible for this catalytic step (Korduláková *et al.*, 2002). The characterization of PimA and the acyltransferase Rv2611c, which substitutes the 6-OH group of the Man*p* residue transferred by PimA with a fatty acyl chain, provided evidence that two distinct pathways then lead to the formation of Ac_1PIM_2 from PIM_1 (Fig. 2A) (Korduláková *et al.*, 2003). PimA is essential for the growth of *M. smegmatis* and *M. tuberculosis* (Korduláková *et al.*, 2002; Jackson *et al.*, unpublished results). Recently, the crystal structure of PimA from *M. smegmatis* has been solved (Guerin *et al.*, 2007) (see below) (Table 1). PimB (Rv0557) was originally characterized as an α-ManT responsible for the synthesis of Ac_1PIM_2 from Ac_1PIM_1 and GDP-Man in *M. smegmatis* (Schaeffer *et al.*, 1999). However, the fact that the disruption of *pimB* did not affect the biosynthesis of PIMs in *M. tuberculosis* suggested that either compensatory activities existed in the cells or that PimB performed another function in *M. tuberculosis* (Kremer *et al.*, 2002;Tatituri *et al.*, 2007). Recently, the second mannosylation step in the biosynthesis of PIM in *Corynebacterium glutamicum* has been shown to be catalyzed by PimB' (NCgl2106, orthologous to Rv2188c), whereas PimB, now renamed MgtA, was implicated in the synthesis of a novel mannosylated glycolipid (1,2-di-*O*-C_{16}/$C_{18:1}$-(α-D-mannosyl)-(1→4)-(α-D-gluco-pyranosyluronic acid)-(1→3)-glycerol) and hypermannosylated "LM-like" variant produced by *C. glutamicum* (Tatituri *et al.*, 2007;Lea-Smith *et al.*, 2008). Although *Rv2188c* was able to restore Ac_1PIM_2 synthesis in the *NCgl2106* mutant of *C. glutamicum*, clear definition of the substrate specificities of Rv2188c in mycobacteria will require its purification to homogeneity and direct demonstration of *in vitro* ManT activity using purified substrates. Ac_1PIM_2 can accumulate as an end product or be further modified with an acyl chain and/or additional Man*p* residues to form higher PIM species, such as Ac_1PIM_3-Ac_1PIM_6/Ac_2PIM_3-Ac_2PIM_6 or LM and LAM. The ManT PimC from *M. tuberculosis* strain CDC1551 was shown to catalyze the formation of Ac_1PIM_3 from GDP-Man and Ac_1PIM_2 in cell-free extracts (Kremer *et al.*, 2002). However, this enzyme is absent from most *M. tuberculosis* isolates as well as *M. smegmatis* suggesting that another as yet unidentified enzyme is involved. Likewise, the ManT that catalyzes the transfer of a Man*p* residue onto PIM_3 to produce PIM_4 remains to be identified. A summary of the characterized GTs of *M. tuberculosis* is provided in Table 1.

b. The biosynthesis of polar PIMs: PimE (Rv1159) was recently identified as a ManT involved in the synthesis of the most polar forms of PIM bearing α(1→2)-linked terminal Man*p* residues. PimE belongs to the GT-C superfamily of integral membrane polyprenol-monophospho-sugar-utilizing GTs, suggesting that, in contrast to the ManTs, PimA, PimB, PimB' and PimC, it probably utilizes polyprenol-phosphomannose (PPM) as a sugar donor and catalyzes Man*p* transfer on the periplasmic side of the plasma membrane (Berg *et al.*, 2007). The disruption of *pimE* in *M. tuberculosis* H37Rv and *M. smegmatis* resulted in mutants which failed to produce PIM_6 (Morita *et al.*, 2006; Larrouy-Maumus *et al.*,

unpublished results). Using *M. smegmatis* cell lysates as an enzyme source, evidence was provided that cell-free extracts from the *M. smegmatis pimE*-deficient mutant were able to produce PIM intermediates up to PIM_4 but not PIM_5 and PIM_6 (Morita *et al.*, 2006). While this result implicated PimE in at least one α(1→2)-Man*p* transfer onto PIM_4 to form PIM_5, the use of *M. smegmatis* extracts in the assay made it impossible to distinguish whether the transfer of the sixth Man*p* residue was also the result of PimE or that of another endogenous enzyme. Using a recombinant form of *M. tuberculosis* PimE produced in *E. coli* and purified $AcPIM_4$ and PPM as acceptor and donors substrates respectively, we recently showed that PimE catalyzes the formation of one single PIM_5 product and not that of PIM_6, thereby clearly implicating this enzyme in the transfer of only one Man*p* residue onto PIM_4 to form PIM_5 (Larrouy-Maumus *et al.*, unpublished results). The α(1→2) ManT responsible for the transfer of the sixth and last Man*p* residue in the formation of PIM_6 thus remains to be identified. Like PimE, this enzyme is expected to belong to the GT-C superfamily of integral membrane PPM-utilizing GTs.

It is proposed that PIM_6 is a terminal product, as it contains two α(1→2)-linked Man*p*, a structure which is not found in LM or the mannan core of LAM (Khoo *et al.*, 1995). Thus, PIM_4 appears as the last common intermediate in the biosynthesis of polar PIMs and LM/LAM. LpqW (Rv1166), a putative lipoprotein, was shown to play a role in regulating polar PIM and LAM biosynthesis in *M. smegmatis*. Disruption of the *lpqW* gene of *M. smegmatis* yielded a mutant with wild-type apolar and polar PIM profiles but unable to make LAM, although it apparently retained the ability to produce LM (Kovacevic *et al.*, 2006). The mutant displayed, however, an unstable phenotype and rapidly recovered the ability to synthesize LAM at the expense of apolar PIM, a property accounted for by the accumulation of secondary mutations in the *pimE* gene (Crellin *et al.*, 2008). Altogether, results thus suggested that LpqW might function to channel the PIM intermediate, $AcPIM_4$, into LAM synthesis. The crystal structure of the LpqW protein of *M. smegmatis* has been reported (Marland *et al.*, 2006).

c. Topology of PIM synthesis: As evidenced by the nature of the GTs involved, the biosynthesis of PIM is topologically complex. Whereas the first three mannosylation steps of the biosynthetic pathway involve GDP-Man-dependent GT-B enzymes and occur on the cytoplasmic face of the plasma membrane (Morita *et al.*, 2004; Morita *et al.*, 2005), further steps in the pathway of polar PIMs (PIM_4-PIM_6), LM and LAM most likely rely upon integral membrane GT-C-type GTs and take place on the extra-cytoplasmic side of the plasma membrane (Besra *et al.*, 1997; Morita *et al.*, 2005; Berg *et al.*, 2007) (Fig. 2A). Such a compartmentalization implies that as yet unidentified transporters/flippases translocate PIM intermediates from the cytoplasmic to the periplasmic side of the plasma membrane. It is at present not clear whether a form of PIM_2, PIM_3 or PIM_4 is translocated (for simplicity, only Ac_1PIM_3 is shown in Fig. 2A).

Little is known of the mechanisms that govern the interaction of GTs with membranes and/or lipid substrates. Members of GT-B superfamily, to which PimA, PimB, PimB' and PimC belong, bind membranes using a variety of mechanisms including transmembrane α-helices, amphipathic α-helices and protein-protein interactions (Martinez-Fleites *et al.*, 2006; Miley *et al.*, 2007; Wang *et al.*, 2008). The determination of the crystal structure of PimA from *M. smegmatis* in complex with the donor substrate GDP-Man and subsequent *in vitro* studies have shed some light on the molecular mechanisms involved in the very initial steps of PIM synthesis (Guerin *et al.*, 2007) (Fig. 2B). The notion of membrane-association through electrostatic interactions is consistent with the finding of an amphipathic α-helix and surface-exposed hydrophobic residues in the N-terminal domain of PimA. Although the sugar transfer is catalyzed between the mannosyl group of GDP-Man and the *myo*-Ins ring of the PI acceptor substrate, both fatty acyl chains of PI are absolutely required for the

transfer reaction to occur. Further, the fact that PimA was capable to bind mono-disperse PI but that its activity was stimulated by high concentrations of non-substrate anionic surfactants indicated that the reaction requires a lipid-water interface. Altogether, experimental data thus supported a model of interfacial catalysis in which PimA recognizes the fully acylated PI substrate with its polar head within the catalytic cleft and the fatty acid moieties only partially sequestered from the bulk solvent (Guerin *et al.*, 2007). Membrane attachment is likely to be mediated by an interfacial-binding surface located in the N-terminal domain of the protein. Structural changes or allosteric effects are expected to result from this interaction with the membrane and/or the lipid substrate and may be necessary for the formation of a competent enzyme-substrate complex.

A search for possible proteins responsible for flipping PIM intermediates (whether PIM_2, PIM_3 or PIM_4) across the plasma membrane has highlighted two possible candidates in the vicinity of the putative α(1→6) ManT Rv1459c, implicated in the initial steps of the elongation of the mannan core of LM/LAM from PIM in *Corynebacterium glutamicum* (Mishra *et al.*, 2008) (see Section IV.A.4). Three genes, *Rv1458c*, *Rv1457c* and *Rv1456c*, which are conserved in all *Corynebacterinaeae*, are apparently translationally coupled with *Rv1459c* (Wang *et al.*, 2006). These genes encode two ABC-transporter integral membrane proteins, with *Rv1458c* encoding the putative ATP-dependent binding protein. Rv1458c exhibits remote structural similarities to sugar-binding proteins of ABC carriers, such as the sugar-binding protein of *Pyrococcus horikoshii* (Marabotti *et al.*, 2004) or the maltose/maltodextrin-binding protein MalK of *E. coli* (Lu *et al.*, 2005). Rv1457c encodes a permease component of an ABC-2-type transporter, typically involved in the export of drugs and carbohydrates (Reizer *et al.*, 1992). As transmembrane channels of ABC-2-type transporters are either homo- or hetero-oligomers and Rv1456c also displays features of a transporter protein, it is reasonable to assume that Rv1456c forms with Rv1457c a membrane channel coupled to the GT, Rv1459c. On the basis of the altered mycolic acid profile of a *Corynebacterium matruchotii* transposon mutant carrying an insertion in the *Rv1456c-Rv1459c* cluster, Wang *et al.* (2006) proposed one or more of these genes to be involved in mycolic acid transport. However, in light of the recent finding that Rv1459c catalyzes the initial α(1→6) elongation of the mannan core of LM/LAM in *C. glutamicum*, this change in mycolylation may only have been an indirect consequence of the loss of LM/LAM in this species. Moreover, with our recent data suggesting a different function for Rv1459c in mycobacteria than in corynebacteria (see Section IV.A.4), it has become clear that the further examination of the *Rv1456c-Rv1459c* cluster directly in mycobacterial species will be required to precisely define the role of these genes in the transport of cell envelope precursors across the plasma membrane.

4. Biosynthesis of LM and LAM—Nothing was known until recently about the enzymes, intermediates and steps involved in the biosynthesis of the mannan backbone of LM/LAM. “Linear LM”, devoid of mannan core α(1→2) branching, has been recognized as a precursor but only through metabolic labeling of cell-free extracts (Besra *et al.*, 1997). Given the complexity of the mannan chain found in LM and LAM, a number of ManTs are expected to be involved in its elongation and branching.

Through genetic and biochemical studies, the membranous GT-C superfamily members relying on polyprenyl-linked sugar donors have emerged as the GTs responsible for much of its synthesis (Dinadayala *et al.*, 2006; Kaur *et al.*, 2006; Kaur *et al.*, 2007; Mishra *et al.*, 2007; Mishra *et al.*, 2008) (Table 1). The NCgl1505 protein of *C. glutamicum* (MptB; which shares ~ 35 % amino-acid identity with Rv1459c from *M. tuberculosis*) has been shown to be an α(1→6) ManT involved in the initiation of the mannan core of corynebacterial LM (Misha *et al.*, 2008). Although, Rv1459c and its ortholog in *M. smegmatis*, MSMEG3120, demonstrated similar α(1→6) ManT activity in cell-free extracts, the facts that these

enzymes were not able to restore LM production in the *C. glutamicum* mutant and that the disruption of *MSMEG3120* in *M. smegmatis* had no effect on LM/LAM synthesis, suggest that Rv1459c and MSMEG3120 may have a distinct function in whole mycobacterial cells. Our recent work on a recombinant strain of *M. smegmatis* overexpressing *Rv1459c* tends to support this hypothesis (see section V.A.2). Hence, the α(1→6) ManTs responsible for the initial polymerization steps leading to the mannan backbone of LM from PIM_4-PIM_5 in mycobacteria is (are) currently unknown.

We and others recently characterized Rv2174 (MptA) as the α(1→6) ManT involved in the latter stages of the elongation of mannan in mycobacteria and corynebacteria (Kaur *et al.*, 2007; Mishra *et al.*, 2007). We also identified Rv2181 as the α(1→2) ManT responsible for the synthesis of the α(1→2) Man*p*-linked branches, characteristic of the mannan backbone of LM and LAM (Kaur *et al.*, 2006). LM is further arabinosylated to produce LAM in a set of poorly defined reactions involving the GT-C protein, EmbC (Zhang *et al.*, 2003). EmbC is an essential enzyme of *M. tuberculosis* (Goude *et al.*, 2008). Site-directed mutagenesis of an aspartic acid in the GT-C motif of EmbC led to the abolition of LAM synthesis (Berg *et al.*, 2005). Furthermore, introduction of point mutations in the conserved proline motif of EmbC proximal to the C-terminal domain led to the synthesis of smaller arabinan domains largely devoid of linear Ara_4, resembling the arabinan structure of AG (Berg *et al.*, 2005). Altogether, these data suggest that EmbC is most likely a multifunctional protein possessing polymerization and chain length regulating activities in addition to arabinosyltransferase (AraT) activity. The presence of two to three Ara*f* residues attached to the mannan backbone of LM in an *M. smegmatis embC* knock-out strain (Zhang *et al.*, 2003) suggests the existence of additional AraTs required for arabinan priming/synthesis.

Based on the hypothesis that the Man-capping of ManLAM probably takes place on the periplasmic side of the plasma membrane and on the assumption that the enzymes responsible should be missing from those *Mycobacterium* spp. devoid of ManLAM (e.g. *M. smegmatis*), we previously identified the GT-C member MT1671 of *M. tuberculosis* CDC1551 (orthologous to *Rv1635c* of *M. tuberculosis* H37Rv) as responsible for the addition of the first Man*p* unit of the mannose capping motifs of ManLAM (Dinadayala *et al.*, 2006). However, the extension of this strategy to search for the subsequent α(1→2) ManTs responsible for the formation of the immunomodulatory di- and tri-mannoside caps of ManLAM failed to identify putative candidates. The only other putative GT-C enzyme devoid of an ortholog in *M. smegmatis* is Rv3779. However, the encoding gene belongs to an AG biosynthetic gene cluster (Berg *et al.*, 2007). An alternative hypothesis was that the mannoside caps arose from the action of a promiscuous α(1→2) ManT, such as PimE (Rv1159) or Rv2181, responsible for the transfer of α-1,2-linked Man*p* units to PIM_4 and the mannan backbone of LM and LAM, respectively (Kaur *et al.*, 2006; Morita *et al.*, 2006; Larrouy-Maumus *et al.*, unpublished). Indeed, the α(1→2)-linkages of the di- and tri-Man*p* units on the non-reducing ends of ManLAM, the terminal mono- and di-Man*p* units of PIM_5 and PIM_6, the α(1→2) Man*p* branching residues of the mannan core of LM/LAM and the di- and tri-mannoside units of the 45-47 kDa glycoprotein of *M. tuberculosis*, are all identical. Moreover, the enzymes catalyzing the addition of these Man*p* residues use lipid-linked sugar donors, belong to the GT-C superfamily of GTs, and catalyze the reactions on the extra-cytoplasmic side of the plasma membrane (vanderVen *et al.*, 2005; Kaur *et al.*, 2006; Morita *et al.*, 2006) (Table 1). To investigate this hypothesis, knock-out mutants of *pimE* and *Rv2181* were generated in *M. tuberculosis* H37Rv. Structural analyses of the LM and LAM variants produced by the *M. tuberculosis Rv2181* mutant revealed the presence of but a single Man*p* residue on the non-reducing arabinan termini of LAM in addition to a complete absence of α(1→2)-linked Man*p* branching on the mannan backbones of LM and LAM (Kaur *et al.*, 2008). A novel recombinant strain was constructed in ManLAM-deficient *Mycobacterium smegmatis* that co-expressed *Rv2181* and *Rv1635c* - the ManT responsible

for the addition of the first Man*p* capping residue of ManLAM. Analysis revealed LAM termini fully capped with di- and tri-Man*p* motifs in addition to α(1→2)Man*p* branching on the mannan backbones of LM and LAM, confirming the involvement of the α(1→2)ManT Rv2181 in the dual role of Man-capping and mannan-core branching (Kaur *et al.*, 2008). It is intriguing then that the expression of *Rv1635c* in *M. smegmatis* yielded a LAM population that had t-Ara*f* residues capped only with a single Man*p* (Dinadayala *et al.*, 2006), despite *M. smegmatis* expressing an active ortholog of *Rv2181* (*MSMEG_4247*) (Kaur *et al.*, 2006). Indeed, while MSMEG_4247 from *M. smegmatis* clearly participated in mannan-core branching, it displayed no detectable Man*p*-capping activity on ManLAM. The catalytic activity of MSMEG_4247 may be too low to mannosylate this heterologous substrate in addition to the mannan backbone. Alternatively, if LAM-capping has evolved to play a role in host cell interactions, it is tempting to speculate that Rv2181 from *M. tuberculosis* has acquired the ability to α(1→2)-mannosylate ManLAM while MSMEG_4247 from *M. smegmatis* has not. Further delineation of the structure-function aspects of Rv2181 is required to understand its role in the biosynthesis of LM and ManLAM.

Interestingly, disruption of *pimE* in *M. tuberculosis* H37Rv led to a significant and consistent decrease in the amounts of the dimannoside motif capping ManLAM in addition to the abolition of polar PIM synthesis (Larrouy-Maumus *et al.*, unpublished). Both phenotypes were restored in the complemented mutant strain. Whether PimE is directly or only indirectly involved in ManLAM-capping, however, is at present not clear given the exclusive role Rv2181 seems to be playing in this process (Kaur *et al.*, 2008). Rv2181 and PimE may act jointly in ManLAM capping, as proposed for other mycobacterial GT-Cs involved in the biosynthesis of AG (Khasnobis *et al.*, 2006), the first enzyme playing the predominant role. Clearly, the precise delineation of the functions of PimE will have to await the development of cell-free assays using purified enzyme and substrates.

The biosynthetic origins of the MTX motif linked to t-Man*p* residues of ManLAM and succinyl residues linked to the arabinan domain of LAM have not yet been determined.

B. ACYLTREHALOSES

The search for virulence factors and immunodominant species-specific antigens in the envelopes of mycobacteria has led to the definition of a remarkable array of glycolipids, among which several families of trehalose-containing lipids and the phenolic glycolipids described in section IV.C.

Trehalose is a simple non-reducing disaccharide of glucose (α-D-Glc*p*-(1<-> 1)-α-D-Glc*p*) found in bacteria, yeast, fungi, plants and invertebrates, but not in mammalian cells. It is found free in the cytosol of mycobacteria or esterified to a variety of fatty acyl groups in the OM of their cell envelope. Under its free form, trehalose is thought to function as a storage carbohydrate and as a stress protectant. The acyltrehaloses found in the cell envelope of *M. tuberculosis* include sulfatides (SL), diacyltrehaloses (DAT), triacyltrehaloses (TAT), polyacyltrehaloses (PAT), trehalose monomycolate (TMM) and trehalose dimycolates (TDM) (Fig. 3). In addition, a few strains of *M. tuberculosis*, known as Canetti strains, produce highly polar species-specific lipooligosaccharides (LOS) (Daffé *et al.*, 1991) (Fig. 3).

Mycobacteria are unusual amongst microorganisms in possessing three pathways for trehalose synthesis (de Smet *et al.*, 2000). One pathway involves condensation of glucose-6-phosphate with UDP-glucose to form trehalose-6-phosphate followed by dephosphorylation to release the free disaccharide. These reactions are catalyzed by trehalose-6-phosphate synthase (OtsA, Rv3490) (Pan *et al.*, 2002) and trehalose-6-phosphate phosphatase (OtsB2, Rv3372). The second pathway generates trehalose from glycogen in a two-step mechanism

involving the maltooligosyltrehalose synthase (TreY, Rv1653c) and the maltooligosyltrehalose trehalohydrolase (TreZ, Rv1562c). The third pathway consists of the conversion of maltose to trehalose by the trehalose synthase (TreS, Rv0126). While the three pathways are functionally redundant in *M. smegmatis* (Woodruff *et al.*, 2004), the OtsAB pathway was found to be predominant in *M. tuberculosis* (Murphy *et al.*, 2005). OtsB2 was demonstrated to be strictly essential for growth in *M. tuberculosis* (Murphy *et al.*, 2005). Thus, consistent with its pleiotropic role in several other functions than stress protection alone, trehalose is essential for growth of *M. tuberculosis* (Murphy *et al.*, 2005). In view of the absence of trehalose in mammalian cells and essentiality, the OtsB2 enzyme may provide a useful target for novel drugs.

In TDM, trehalose is esterified by mycolates at positions 6 and 6'. It was proposed that mycolic acids can be transferred via a mycolyl-mannosylphosphoheptaprenol donor (Besra *et al.*, 1994) to trehalose 6-phosphate, arising from the OtsAB pathway, to yield phosphorylated trehalose monomycolate, which then can be dephosphorylated to yield TMM (bearing only one mycolyl chain) (Takayama *et al.* 2005). The subsequent synthesis of TDM, from two TMM molecules, and the transfer of mycolates to the non-reducing ends of AG have been shown to involve the antigens 85A, 85B, and 85C (Belisle *et al.* 1997; Jackson *et al.* 1999). However, the precise functions of these enzymes remain unclear, as is the process through which mycolates are translocated from the cytoplasm where they are synthesized to the cell envelope.

The reactions leading to the acylation of trehalose with short chain fatty acyl substituents and long-chain multi-methyl-branched fatty acids to form SL, DAT, TAT and PAT have not been fully elucidated either. The only two acyltransferases characterized to date are PapA2, involved in the transfer of a palmitoyl group to the 2′-position of trehalose-2-sulfate in SL biosynthesis, and PapA1, apparently responsible for the transfer of the first (hydroxy)phthioceranoyl group onto the product of PapA2 (Kumar *et al.*, 2007). Both acyltransferases are essential for the synthesis of SL-1 (Kumar *et al.*, 2007; Bhatt *et al.*, 2007). The identity and localization of the enzymes responsible for the final acylation steps have yet to be determined. Based on what is known of the biosynthesis of related polyketide-derived lipids known as the phthiocerol dimycocerosates, it is tempting to speculate that the biosynthesis of the entire acyltrehalose molecules takes place on the cytoplasmic side of the membrane and that biosynthesis and translocation are coupled (Jain and Cox, 2005). Alternatively, the accumulation within interior layers of the cell envelope of partially acylated SL intermediates by *M. tuberculosis* mutants deficient in the membrane transporter MmpL8 could be interpreted as the initial acylation reactions taking place in the cytosol and the remaining in the cell envelope (Converse *et al.*, 2003; Domenech *et al.*, 2004). At present, there is no clear experimental evidence supporting any of these two models.

Sft0 (Rv0295c) was characterized as the sulfotransferase responsible for the formation of the trehalose-2-sulfate moiety of SL (Mougous *et al.*, 2004). The topics of the synthesis of the polyketide or mycolic acid moieties of these molecules are beyond the scope of this review but have been extensively reviewed recently (Jackson *et al.*, 2007; Marrakchi *et al.*, 2008; Natarajan *et al.*, 2008; Guilhot and Daffé, 2008). Likewise, the translocation and biological activities of trehalose esters have been reviewed recently and will thus not be further detailed here (Jackson *et al.*, 2007; Glickman, 2008; Bertozzi and Schelle, 2008; Guilhot and Daffé, 2008).

Although the genetics of the species-specific LOS of *M. marinum* (Burguière *et al.*, 2005; Ren *et al.*, 2007) has begun to be explored, nothing is known of LOS biosynthesis in the Canetti strains of *M. tuberculosis*.

C. *p*-HYDROXYBENZOIC ACID DERIVATIVES AND PHENOLIC GLYCOLIPIDS

Recent interest has focused on the phenolic glycolipids (PGL) produced by some strains of *M. tuberculosis*. Their structure is presented in Fig. 4. Although their role in the pathogenesis of some W-Beijing isolates has been documented (Reed *et al.*, 2004;Tsenova *et al.* 2005), most strains of *M. tuberculosis* do not produce PGL due to a frameshift mutation in the polyketide synthase gene *pks15/1*, which is required for the assembly of the lipid moiety of the molecule (Constant *et al.*, 2002). However, all *M. tuberculosis* isolates analyzed to date have retained the ability to produce and secrete *p*-hydroxybenzoic acid derivatives (*p*-HBADs)- glycoconjugates which share with PGL the same glycosylated aromatic nucleus (Fig. 4) (Constant *et al.*, 2002). The biosynthesis and biological activities of PGLs and related *p*-HBADs have been reviewed recently and will thus not be described here (Jackson *et al.*, 2007;Berg *et al.*, 2007;Guilhot *et al.*, 2008;Malaga *et al.*, 2008).

D. MANNOSYL-β-1-PHOSPHOMYCOKETIDES

Mannosyl-β-1-phosphomycoketides consist of a mannosyl-β-1-phosphate moiety identical to that found in mannosyl-β-1-phosphodolichols from mammalian cells and a C_{30}-C_{34} fully saturated 4, 8, 12, 16, 20-pentamethylpentacosyl unit (Fig. 5) (Matsunaga *et al.*, 2004). They have been found in *M. tuberculosis*, *M. avium* and *M. bovis* BCG but not in the rapidly growing saprophytes, *M. phlei*, *M. fallax* and *M. smegmatis*. Suggestive of a role in pathogenesis, this family of lipids activates human CD1c-restricted T-cells (Matsunaga *et al.*, 2004). With the exception of the polyketide synthase Pks12 which has been found to be required for the production of these lipids (Matsunaga *et al.*, 2004;Chopra *et al.*, 2008) the biosynthetic pathway of mycoketides has not yet been elucidated.

E. GLYCOPROTEINS

The glycosylation patterns of the only two mycobacterial glycoproteins that have been biochemically characterized to date - the MBP83 antigen of *M. bovis* and the 45-47 kDa (Apa) antigen of *M. tuberculosis* - indicate that they are modified at threonine residues with one to three α(1→3)- (MBP83) or α(1→2) (Apa) -linked Man*p*, a glycosylation pattern reminiscent of eukaryotic short-chain mannoproteins (Dobos *et al.*, 1996; Michell *et al.*, 2003). The decorating mannose motifs of Apa have been implicated in various biological activities, including the ability of this protein to induce a delayed-type hypersensitivity response in guinea pigs, stimulate primed T-cells *in vitro*, and bind C-type lectins such as the surfactant protein A and, potentially, DC-SIGN (Romain *et al.*, 1999; Horn *et al.*, 1999; Pitarque *et al.*, 2005; Ragas *et al.*, 2007).Several other proteins of *M. tuberculosis* are believed to be glycosylated even though their glycosyl appendages have not been characterized (Espitia *et al.*, 1989; Garbe *et al.*, 1993).

The glycosyltransferases involved in protein glycosylation in *M. tuberculosis* remain largely unknown. Bioinformatic approaches identified a single *M. tuberculosis* protein, Rv1002c, sharing amino acid identity with the *O*-mannosyltransferases of *Saccharomyces cerevisiae* and a similar hydropathy profile (VanderVen *et al.*, 2005). This integral membrane protein, which belongs to the GT-C superfamily of glycosyltransferases, was shown to catalyze the initial step of the mannosylation of Apa. Moreover, evidence was provided that, analogous to eukaryotic systems, Sec-translocation is required for protein mannosylation in *M. tuberculosis*. The glycosyltransferases involved in the further elongation of the α(1→2) or α(1→3)-linked oligomannoside motifs have not yet been identified. Our recent work on a knock-out mutant of *M. tuberculosis* H37Rv deficient in the α(1→2)-mannosyltransferase Rv1159 (PimE) suggests that this enzyme is not involved in the glycosylation of Apa (Larrouy-Maumus *et al.*, unpublished).

V. The glycoconjugate polymers of the cell wall core

A. ARABINOGALACTAN

1. Structure of AG—AG was recognized as the major cell wall polysaccharide of mycobacteria as early as the 1950s. Its apparent function as a whole is the tethering of the mycolic acid layer to the PG. The function of the galactan region beyond where arabinan attaches is unknown, but it was proposed to produce a viscous hydrophilic region between the PG and mycolic acid layers. The most recent model of AG indicates that it contains 125 glycosyl residues in total distributed between a galactan domain made of 30 Gal*f* residues, three arabinan domains each containing 31 Ara*f* residues, and a specific linker unit ensuring its covalent attachment to PG made of a rhamnosyl residue attached to a *N*-acetylglucosaminosyl-1-phosphate residue (Bhamidi *et al.*, 2008). The galactan of AG is made of a disaccharide repeating unit, $[\rightarrow 6\text{-D-Gal}f\beta 1\rightarrow 5\text{-D-Gal}f\beta]_{15}$. Arabinan chains are attached to *O*-5 of Gal*f* residues 8, 10 and 12 of galactan. The characteristic non-reducing termini of the arabinan domain of AG consist of an Ara_6 motif, Ara*f*β1→2Ara*f*α1→5(Ara*f*β1→2Ara*f*α1→3)-Ara*f*α1→5Ara*f*α1→, where position 5 of both the terminal β-Ara*f* and the penultimate 2-α-Ara*f* serve as the anchoring points for the mycolic acids. Under physiological conditions, approximately two-thirds of the Ara_6 motifs are mycolylated (McNeil *et al.*, 1991). The inner core of the arabinan domain is essentially made of stretches of α–1,5-linked Ara*f* residues with a critically positioned α–3,5-branch site (Fig. 1). Some of the interior α–3,5-Ara*f* residues are substituted at position 2 with either a non-*N*-acetylated galactosamine residue or a succinyl residue (Lee *et al.*, 2006; Bhamidi *et al.*, 2008). The inner core of the D-arabinan portion of AG is very similar to that of LAM in that the same linkages of Ara*f* units are found and both structures share an internal Ara_{18} motif extending from the α–3,5-Ara*f* interior residues (Fig. 1). The interior regions of LAM arabinan, however, have been shown to be more variable in terms of the length of this Ara*f* motif (Ara_{18}- Ara_{22}) (Shi *et al.*, 2006; Bhamidi *et al.*, 2008).

2. AG biosynthesis—The galactan and arabinan domains of AG are synthesized as a unit on a decaprenyl phosphate (Dec-P) carrier lipid before being transferred onto PG by an as yet unidentified ligase (Yagi *et al.*, 2003). The synthesis of AG is thus initiated on a Dec-P molecule with formation of the linker unit, followed by what appears to be the simultaneous addition of Gal*f* and Ara*f* residues (Mikušová *et al.*, 1996; Mikušová *et al.*, 2000; Yagi *et al.*, 2003) (Fig. 6). Many of the enzymes involved in this complex process have been identified. Detailed reviews of the biosynthesis of polyprenyl phosphate have been published (Crick *et al.*, 2001; Barry *et al.*, 2007; Crick and Brennan, 2008; Wolucka *et al.*, 2008). It is predicted that a transferase encoded by *Rv1302* (a putative ortholog of WecA) in the genome of *M. tuberculosis* H37Rv transfers GlcNAc-1-phosphate to Dec-P to form Dec-P-P-GlcNAc (GL-1) (Mikušová *et al.*, 1996; Dal Nogare *et al.*, 1998; McNeil, 1999). This step is followed by the attachment of a rhamnosyl residue to the 3 position of GlcNAc in a reaction catalyzed by WbbL (Rv3265c) to form GL-2, “the linker unit” (Mills *et al.*, 2004). dTDP-Rha, which serves as the Rha donor in this reaction, is synthesized from glucose-1-phosphate through a four-step reaction catalyzed by the α-D-glucose-1-phosphate thymidylyl transferase RmlA (Rv0334) (Ma *et al.*, 1997), the dTDP-D-glucose 4,6-dehydratase RmlB (Rv3464) (Ma *et al.*, 2001), the dTDP-4-keto-6-deoxy-D-glucose 3,5 epimerase RmlC (Rv3465) (Stern *et al.*, 1999) and the dTDP-rhamnose synthetase RmlD (Rv3266c) (Hoang *et al.*, 1999).

Gal*f* residues are then added to the linker unit from UDP-Gal*f* which originates in UDP-Gal*p* in a reaction catalyzed by UDP-Gal*p* mutase (Glf; Rv3809c) (Weston *et al.*, 1997; Mikušová *et al.*, 2000). UDP-Gal*p* is synthesized from UDP-glucose by UDP-Gal*p* epimerase, likely encoded by *Rv3634*. The galactosyltransferase Rv3782 (GlfT1) catalyzes the first stages of

galactan synthesis transferring the two first Gal*f* residues onto the linker unit (Mikušová *et al.*, 2006; Belánová *et al.*, 2008) while Rv3808c (GlfT2) continues with the subsequent polymerization events (Mikušová *et al.*, 2000; Kremer *et al.*, 2001; Rose *et al.*, 2006; Belánová *et al.*, 2008). GlfT2 is a bifunctional galactosyltransferase that catalyzes the synthesis of the alternating 5- and 6-linked, linear, galactofuran in a processive manner. It is thought to be responsible for the polymerization of the bulk of the galactofuran.

As in the case of LAM, knowledge of the enzymes involved in the elongation of the D-arabinan structures of AG is more limited. Polyprenol-monophosphoryl-β-D-arabinose (DPA) is the only known Ara*f* donor in mycobacteria (Wolucka *et al.*, 1994). It is synthesized from 5-phosphoribose-pyrophosphate (Scherman *et al.*, 1995; Scherman *et al.*, 1996) following four catalytic steps which have been characterized (Huang *et al.*, 2005; Mikušová *et al.*, 2005; Huang *et al.*, 2008). Since DPA is the only known Ara*f* donor, it is expected that the arabinosylation of AG and LAM is catalyzed by membrane-associated polyprenyl-dependent glycosyltransferases (GTs) on the periplasmic side of the plasma membrane (Berg *et al.*, 2007). We and others have recently reported of the potential existence of seventeen such enzymes in *M. tuberculosis* H37Rv, the implication of twelve of which in the glycosylation of various proteins, glycolipids or polysaccharides has now been established (for a review, Berg *et al.*, 2007; Seidel *et al.*, 2007; Kaur *et al.*, 2007; Mishra *et al.*, 2008; Birch *et al.*, 2008). Arabinofuranosyltransferases characterized to date include AtfA (Rv3792), involved in the transfer of the very first Ara*f* residues to the galactan domain of AG (Alderwick *et al.*, 2006; Shi *et al.*, 2008), the terminal β1,2-capping arabinosyltransferase AtfB (Rv3805c) (Seidel *et al.*, 2007), Rv2673 (AtfC) involved in the α–1,3-branching of the arabinan domain of AG (Birch *et al.*, 2008) and the EmbA and EmbB proteins involved in the formation of the Ara_6 motif (Escuyer *et al.*, 2001; Khasnobis *et al.*, 2006). Although EmbA and EmbB, acting alone or as heterodimers, have also been proposed to participate in the α–1,5-elongation of the linear portion of arabinan, experimental evidence for this assumption is still lacking (Bhamidi *et al.*, 2008). Our most recent results suggest that Rv0236c, the largest putative GT-C identified in the genome of *M. tuberculosis* displays, like Rv2673, α–1,3-branching arabinofuranosyltransferase activity, catalyzing *in vitro* the transfer of an Ara*f* residue from DPA to a synthetic linear α–1,5-linked Ara_5 acceptor. We found the ortholog of *Rv0236c* in *M. smegmatis* mc^2155 to be an essential enzyme and its activity to be rate-limiting for the synthesis of AG as a whole (Skovierová *et al.*, unpublished results). As proposed earlier for the Emb proteins (Berg *et al.*, 2005; Berg *et al.*, 2007), the large size of Rv0236c (1400 amino acids) suggests that it might have additional functions – e.g., controlling the length of the various interior or exterior segments of the arabinan polymer, acting a scaffold for a multi-enzyme machinery involved in arabinosylation - in addition to the direct transfer of Ara*f* units from a Dec-P-Ara*f* donor. Interestingly, our recent results also indicate that Rv1459c, another GT-C enzyme whose distantly-related ortholog in *Corynebacterium glutamicum* was shown to be an α–1,6-mannosyltransferase involved in the elongation of PIM_2 (Mishra *et al.*, 2008), displays *in vitro* the same α–1,3-branching arabinosyltransferase activity as Rv2673 and Rv0236c on a synthetic linear α–1,5-linked Ara_5 acceptor. In contrast to what has been reported (Mishra *et al.*, 2008), we found the ortholog of *Rv1459c* in *M. smegmatis* mc^2155 to be essential for growth (Korduláková, Škovierová *et al.*, unpublished). The reason for this discrepancy between the results of these two studies is unknown. The reason for the apparent existence of three non-redundant α–1,3-arabinosyltransferases, Rv2673, Rv1459c and Rv0236c, is at present unclear but could be related to the existence of a multi-protein complex participating in the formation of the arabinan domain or to their different involvement in the branching of the interior or outermost domains of arabinan in AG and/or LAM (Fig. 1). Alternatively, one cannot exclude that under physiological conditions, these enzymes participate in the α–1,5 elongation rather than in the branching of the arabinan domains of AG and LAM. Indeed, although elongating α–1,5 arabinofuranosyltransferase

activities - some of which apparently unrelated to the Emb proteins - have been detected in cell-free assays using mycobacterial cell wall preparations and synthetic linear or branched arabinan acceptors (Lee *et al.*, 1997; Lee *et al.*, 1998; Zhang *et al.*, 2007), the identity of the responsible enzymes remains to be determined. Likewise, the enzymes responsible for the transfer of the galactosamine and succinyl residues onto some of the interior α–3,5-Ara*f* residues of arabinan are not yet known.

The fact that the addition of Gal*f* and Ara*f* residues appears to occur simultaneously makes it unclear whether polymerization events leading to the formation of AG take place inside or outside the plasma membrane. While the involvement of GT-C enzymes in the polymerization of the arabinan domain clearly points to the periplasmic localization of arabinan formation, the donor of Gal*f* residues in galactan synthesis is a nucleotide sugar expected to be cytoplasmic. The fact that the data leading to the conclusion of simultaneous galactosylation and arabinosylation events were generated using cell-free assays in which the natural topology of the membrane was disrupted may be the basis of this apparent discrepancy. In that case, the transporters involved in the translocation of the galactan polymer synthesized inside the cytosol to the periplasm remain to be identified.

Interestingly, many of the genes involved in the biogenesis of the galactan and arabinan domains of AG and LAM, the formation of mycolic acids and the transfer of mycolates onto AG or trehalose are clustered in a region of the *M. tuberculosis* chromosome often refered to as the “cell wall biosynthetic cluster” (Fig. 7). This cluster also carries a number of as yet uncharacterized sugar-modifying enzymes and transporters.

3. AG biosynthesis and drug discovery—The inhibitory activity of ethambutol, a first line anti-TB drug shown to target the Emb proteins, first illustrated the essentiality of AG in *M. tuberculosis* and other mycobacteria (Lee *et al.*, 1995; Belanger *et al.*, 1996). Other studies have since provided genetic evidence of the essentiality of multiple steps of the biogenesis of AG (Barry *et al.*, 2007). Genes found to be essential in *M. tuberculosis* or *M. smegmatis* include those involved in the biogenesis of Dec-P (Eoh *et al.*, 2007), the formation of dTDP-Rha (Ma *et al.*, 2002; Li *et al*, 2006), the linker unit (Mills *et al.*, 2004), the UDP-Gal*f* mutase gene *Rv3809c* (Pan *et al.*, 2001), the galactosyltransferase genes *Rv3782* and *Rv3808c* (Pan *et al.*, 2001), and the arabinosyltransferase or putative arabinosyltransferase genes *embA, embC, Rv3792* (*atfA*), *Rv1459c* and *Rv0236c* (Shi *et al.*, 2008, Amin *et al.*, 2008; our unpublished results).

In view of the absence of most of the biosynthetic pathway of AG in mammalian cells and essentiality, there is considerable interest in exploiting several of the enzymes of this pathway as therapeutic targets. The few drug discovery enzyme assays that have been designed and the target enzymes whose crystal structures have been determined have recently been reviewed (Brennan and Crick, 2007; Barry *et al.*, 2007; Wolucka, 2008).

B. PEPTIDOGLYCAN

Two opposing models of the secondary structure of the mycolyl-AG-peptidoglycan complex (mAGP) have been proposed and reviewed (Crick *et al.*, 2008). One predicts that the PG and galactan are parallel to the plasma membrane (McNeil and Brennan, 1991). This orientation is consistent with traditional models of the PG structure; however, other modeling studies suggest that the PG and AG strands may be coiled and perpendicular to the plane of the plasma membrane (Dmitriev *et al.*, 1999, 2000). Recently, the relevant studies on the secondary structure of PG in Gram-negative bacteria were reviewed (Vollmer and Holtje**,** 2004) and the preponderance of data appears to favour the parallel model in these organisms, but the perpendicular model recently gained support from the three dimensional solution structure of a synthetic fragment of the cell-wall as determined by NMR. These

results indicate that the glycan backbone of the synthetic PG fragment forms a right-handed helix with a periodicity of six sugar residues, leading the authors to conclude that the glycan strand of PG is orthogonal to the plane of the membrane *in vivo* as opposed to the parallel hypothesis (Meroueh et al., 2004). Thus, the overall structure and topology of the mAGP complex in *Mycobacterium* spp. remains open to debate as does the three dimensional structure of PG in other eubacteria.

The detailed primary structure of the PG of *Mycobacterium* spp. has been reviewed many times, as recently as 2008 (see Crick *et al.*, 2008) (Fig. 8). PG is a complex polymer forming a rigid layer outside the plasma membrane providing cellular shape and the strength to withstand osmotic pressure, as well as a scaffold for the structures described above (Fig. 1). PG from *M. tuberculosis* has been classified as A1γ as has that of *Escherichia coli* and *Bacillus* spp. according to the classification system of Schleifer and Kandler (1972) and is composed of linear chains of *N*-acetyl-α-D- glucosamine (GlcNAc) and modified muramic acid (Mur) substituted with peptide side-chains that are heavily cross-linked providing added structural integrity to the bacterium; the overall degree of cross-linking is 70-80% in *Mycobacterium* spp. (Matsuhashi, 1966) compared to 50% in *E. coli* (Vollmer and Holtje, 2004). The tetrapeptide side chains of PG consist of L-alanyl-D-isoglutaminyl-meso-diaminopimelyl-D-alanine (L-Ala-D-Glu-A_2pm-D-Ala) with the Glu and A_2pm being further amidated (Adam *et al.*, 1969; Kotani *et al.*, 1970; Lederer *et al.*, 1975; Petit *et al.*, 1969*;* Wietzerbin-Falszpan *et al.*, 1970). About two thirds of the peptide cross-links found in *M. tuberculosis* PG are between the carboxyl group of a terminal D-Ala and the amino group at the D-center of A_2pm resulting a D,D bond (Wietzerbin *et al.*, 1974) and approximately one third of the peptide cross-links occur between the carboxyl group of the L-center of one A_2pm residue and the amino group of the D-center of another A_2pm residue forming a L,D-cross-link (Wietzerbin *et al.*, 1974). L,D-cross-links are relatively rare, but recently have also been reported in the PG of *Streptomyces* spp., *Clostridium perfringens* (Leyh-Bouille *et al.*, 1970) and stationary phase *E. coli* (Goffin and Ghuysen, 2002; Templin *et al.*, 1999). In addition, in mycobacterial PG some of the Mur residues are *N*-acylated with glycolic acid (MurNGlyc) rather than *N*-acetylated (MurNAc) (Mahapatra *et al.*, 2000, 2005a; Raymond *et al.*, 2005) and the hydroxyl moiety of C-6 of some of the Mur residues forms phosphodiester bonds to C-1 of MurNGlyc which in turn is (1→3) linked to a α-L-rhamnose (Rha) residue providing the “linker unit” between the galactan of AG and PG (McNeil *et al.*, 1990).

In terms of PG biosynthesis, the arrangement of genes responsible for PG synthesis in *M. tuberculosis* is similar to that in other bacteria (Mahapatra *et al.*, 2000), and in all probability, so is the biochemistry (Mahapatra *et al.*, 2005b). Hence, the excellent reviews on bacterial PG synthesis, in general (Van Heijenoort 1994, 1996, 1998, 2001a, 2001b) are applicable. However, there are some unusual aspects related to PG synthesis in mycobacteria. As noted above, the glycan chains are composed of alternating units of β 1→4 linked GlcNAc, MurNAc and MurNGlyc, whereas, most other bacteria contain *N*-acetylmuramic acid. The oxidation of the *N*-acetyl group to *N*-glycolyl is catalyzed by a recently identified enzyme designated NamH (Raymond *et al.*, 2005). Although there is no indication that this modification is essential for *M. tuberculosis* survival, *M. smegmatis* mutants devoid of *namH* show increased sensitivity to β-lactams and lysozyme (Raymond *et al.*, 2005). In addition, the free carboxylic acid groups of the A_2pm or D-isoglutamic acid residues of mycobacterial PG may be amidated, and some of the free carboxylic groups of the D-isoglutamic acid residues may also be modified by the addition of a glycine residue in peptide linkage (Adam *et al.*, 1969). The importance of these modifications to the survival of the bacillus awaits identification of the enzymes responsible.

VI. Cytosolic glycoconjugates

A. POLYMETHYLATED POLYSACCHARIDES

Polymethylated polysaccharides (PMPS) are unusual carbohydrates unique to the order *Actinomycetales*. Although all mycobacteria were originally thought to produce two classes of them, the 3-*O*-methylmannose polysaccharides (MMPs) and the 6-*O*-methylglucose lipopolysaccharides (MGLPs), our preliminary evidence suggests that MMPs may be restricted to fast-growing mycobacterial *spp*. and thus, absent from *M. tuberculosis* (Stadthagen *et al.*, 2007). MGLPs and MMPs were first isolated from *M. phlei, M. smegmatis* and *M. tuberculosis* in Dr. Clinton Ballou's laboratory (Lee and Ballou, 1964; Lee, 1966; Gray and Ballou, 1971) and much of the information we have about these molecules comes from early work from this group. A remarkable property associated with PMPS is their ability to form stable 1:1 complexes with long-chain fatty acids and acyl-coenzyme A derivatives and to regulate the activity of Fatty Acid Synthase I *in vitro* (reviewed in Bloch *et al.*, 1977). These findings have led to the suggestion that they may be important regulators of lipid metabolism in mycobacteria. Physiological evidence for this assumption is however lacking.

The MGLPs of *M. tuberculosis* are cytoplasmic lipopolysaccharides of intermediate size containing up to 20 sugar units, many of which are partially *O*-methylated. The structure of MGLPs from the closely related species *M. bovis* BCG has been characterized in detail (Tuffal *et al.*, 1998a) (Fig. 9A). The fact that both MMPs and MGLPs are composed of hexose units predominantly or exclusively in α-(1→4)-linkage confers on these molecules a proclivity to assume the helical conformation characteristic of amylose. The fact that many of these hexoses are partially *O*-methylated further confers on the molecules a slight hydrophobicity. These structural features in turn confer on PMPS the ability to form complexes with fatty acyl chains (Machida and Bloch, 1973; Yabusaki and Ballou, 1978; Yabusaki *et al.*, 1979; Maggio, 1980; Hindsgaul and Ballou, 1984; Tuffal *et al.*, 1998b), accounting for their unique regulatory roles on fatty acid metabolism *in vitro* (Bloch, 1977; Yabusaki and Ballou, 1979; Forsberg *et al.*, 1982). Only in recent years has the genetics of the biosynthesis of MGLPs in *M. tuberculosis* begun to be explored. Two gene clusters have been described which seem to carry much of the genes required for the glucosylation, acylation and methylation steps of these molecules (Stadthagen *et al.*, 2007; Sambou *et al.*, 2008; Kaur *et al.*, unpublished results). The glucosyl-3-phosphoglycerate synthase from *M. tuberculosis* (Rv1208) and ortholog in *M. avium* subsp. *paratuberculosis* (MAP2569c), which likely catalyze the first glucosyl transfer in the pathway (Empadinhas *et al.*, 2008), have been crystallized and the structure of the latter enzyme solved without substrate and in complex with the donor substrate, UDP-Glc (Fulton *et al.*, 2008a, Fulton *et al.*, 2008b; Gest *et al.*, 2008). The structure of the protein displays the typical organization of GT-A fold enzymes with a conserved DxD motif that coordinates a Mg^{2+} ion (Fig. 9B). Current knowledge of the biosynthesis and genetics of MGLPs in *M. tuberculosis* has recently been reviewed and we would like to refer the reader to this publication (Jackson and Brennan, 2008).

B. GLYCOGEN

Glycogen is an intracellular polysaccharide which serves as a major carbohydrate reserve in most bacteria. Glycogen content in mycobacteria, including *M. tuberculosis*, was shown to vary with growth phase and nitrogen concentration in the medium (Antoine and Tepper, 1969a,b; Elbein and Mitchell, 1973). Furthermore, the constant recycling of this molecule during exponential phase seems to be essential for growth of *M. smegmatis* (Belanger and Hatfull, 1999).

The structure of glycogen is almost undistinguishable from that of the capsular α-D-glucan described earlier (see section III). Recently, the application of analytical centrifugation and dynamic light scattering to α-D-glucan and glycogen purified from *M. bovis* BCG indicated, however, that the α-D-glucan possessed a slightly higher molecular mass (13×10^6 versus 7.5×10^6 Da) and was more compact than glycogen (Dinadayala *et al.*, 2008).

Not surprisingly, the biosynthetic pathways of glycogen and α-D-glucan share common enzymes (see section III) (Sambou *et al.*, 2008). Two partially redundant α-1,4-glucosyltransferases, GlgA (Rv1212c) and Rv3032, apparently participate in the elongation of glycogen in *M. tuberculosis* although in whole cells Rv3032 seems to be the main enzyme affected to this task (Sambou *et al.*, 2008). The molecular mechanisms underlying the preferred involvement of GlgA and Rv3032 in one or the other pathway is at present not known. Rv3032 is also involved in the elongation of the MGLPs described above (Stadthagen *et al.*, 2007). GlgB (Rv1326c) was identified as the branching enzyme and reported to be essential for growth (Garg *et al.*, 2007; Sambou *et al.*, 2008). To our knowledge, this is the first glycogen-branching enzyme reported to be essential in a prokaryotic organism. The reason of its essentiality in the tubercle bacillus is unknown. As GlgB seems to be the only branching enzyme committed in the synthesis of α-D-glucan and glycogen, its essentiality may be related to the physiological requirement of *M. tuberculosis* to produce at least one of these two polysaccharides. This assumption is also supported by the inability to generate a double *glgA/Rv3032* knock-out of *M. tuberculosis* (Sambou *et al.*, 2008). By analogy with *glgB* mutants of *E. coli* (Lares *et al.*, 1974), one may also speculate that a deficiency in GlgB could lead to the accumulation of poorly water-soluble linear polymers of 4-linked α-glucosyl residues, either in the cytosol or in the periplasmic space of the cell envelope, causing the death of the bacterium. The fact that significant amounts of α-D-glucan and glycogen were still produced by an ADP-Glc pyrophosphorylase *glgC* mutant of *M. tuberculosis* H37Rv indicates that the GlgC-dependent ADP-Glc pathway is not the only route to glycogen/glucan synthesis in *M. tuberculosis* H37Rv. Other as yet unidentified enzymes with low sequence similarity to the usual prokaryotic ADP-Glc pyrophosphorylases may exist in *M. tuberculosis*. It is also possible that one of the two α-1,4-glucosyltransferases, Rv3032 or GlgA, utilizes UDP-Glc instead of ADP-Glc as the sugar donor in the elongation reactions. A recent study has implicated the trehalose synthase, TreS, of *M. smegmatis* in the (reversible) utilization of trehalose for the production of glycogen, in addition to its role in the interconversion of trehalose and maltose (Pan *et al.*, 2008). The TreS enzyme of *M. tuberculosis* is likely to function similarly. Finally, the presence of a putative amylomaltase gene (*Rv1781c, malQ*) in the *M. tuberculosis* genome suggests that this bacterium might have the ability to synthesize α-1,4-glucans when grown on maltose or maltodextrin (Preiss and Romeo, 1989).

C. MYCOTHIOL

Mycothiol is a low-molecular-weight thiol produced by Actinobacteria whose primary role is to maintain a reducing environment in the cells which is necessary for standard metabolic activities to occur. It also represents a stabilized form of cysteine which is required for protein and CoA synthesis in the cells. Mycothiol has been shown to protect mycobacteria against oxidative stress, alkylating stress, acid stress and a broad range of antibiotics (Fig. 10A) (for a review, Newton *et al.*, 2008;Rawat *et al.*, 2002;Buchmeier *et al.*, 2003; Buchmeier *et al.*, 2006a; Rawat *et al.*, 2007;Miller *et al.*, 2007). Like glutathione in Gram-negative bacteria and eukaryotes, mycothiol also serves as an enzyme cofactor (Rawat and Av-Gay, 2007;Newton *et al.*, 2008) and it is thus thought that either mycothiol or mycothiol-dependent proteins account for the protection of mycobacteria against oxidants and other toxic agents (Rawat and Av-Gay, 2007).

Mycothiol biosynthesis proceeds through a five-step pathway. *N*-acetylglucosaminylinositol phosphate is the product of the UDP-GlcNAc:1L-*myo*-Ins-1-P α-N-acetylglucoaminyltransferase MshA (Rv0486), a nucleotide-sugar utilizing GT-B glycosyltransferase (Newton *et al.*, 2003; Newton *et al.*, 2006a). An unidentified phosphatase dephosphorylates this molecule to yield *N*-acetylglucosaminylinositol (GlcNAc-Ins) (Newton *et al.*, 2006a). Deacetylation of GlcNAc-Ins by the deacetylase MshB (Rv1170) yields 1-D-*myo*-inosityl-2-deoxy-α-D-glucopyranoside (GlcN-Ins) (Newton *et al.*, 2000; Newton *et al.*, 2006b) which is then linked to cysteine by the ATP-dependent ligase MshC (Rv2130c) to yield Cys-GlcN-Ins (Sareen *et al*, 2002). The final step of the pathway is the conversion of Cys-GlcN-Ins to acetyl Cys-GlcN-Ins (mycothiol) by the acetyltransferase MshD (Rv0819) (Koledin *et al.*, 2002).

Gene disruption studies initially showed that *mshA* and *mshC* are essential for the growth of *M. tuberculosis*, thereby implicating mycothiol as vital components of the tubercle bacillus (Sareen *et al*, 2003; Buchmeier and Fahey, 2006). This assumption was recently questioned when spontaneous isoniazid and ethionamide-resistant mutants of *M. tuberculosis* carrying mutations in *mshA* and deficient in mycothiol production were found to display a wild-type growth phenotype *in vitro* and in mice (Vilchèze *et al.*, 2008). The reason for this discrepancy is unknown but could be due to the different *M. tuberculosis* strains used in the two studies or to the ability of the spontaneous mutants to produce undetectable but sufficient amounts of mycothiol to sustain growth. *mshB* and *mshD* are clearly not essential in *M. tuberculosis* as knock-out mutants still produced significant amounts of mycothiol and biosynthetically-related thiol compounds (Buchmeier *et al.*, 2003; Buchmeier *et al.*, 2006). In view of the absence of mycothiol in mammalian cells and potential essentiality, the MshA and MshC proteins may represent attractive targets for novel anti-TB drugs. Consistently, the crystal structures of MshB and MshD from *M. tuberculosis* and MshA from the closely related *Corynebacterium glutamicum* have been determined and inhibitors of MshB and MshC have been reported (Newton *et al.*, 2006c; Metaferia *et al.*, 2007).

The catalytic mechanism of ‘retaining’ GTs that leads to retention of the anomeric sugar binding is still a matter of debate and controversy. A double-displacement mechanism via the formation of a covalent glycosyl-enzyme intermediate was first proposed. However, in the absence of direct evidence of a viable covalent intermediate, an alternative mechanism was suggested, the S_Ni ‘internal return’ (Lairson *et al.*, 2008). The crystal structure of MshA (*Cg*MshA, pdb code 3C4V), solved in the absence of substrates and in complex with UDP and Ins-P, has shed some light into the catalytic mechanism of ‘retaining’ GTs (Fig. 10B) (Vetting *et al*, 2008). Vetting and co-workers (2008) proposed a substrate-assisted mechanism, in which the β-PO_4 of UDP-N-acetylglucosamine promotes the nucleophilic attack of the 3-hydroxyl group of Ins-P and assists in the cleavage of the sugar-nucleotide bond.

VII. Conclusions and future prospects

From this comprehensive review one can see that the primary biosynthetic pathways responsible for the synthesis of the individual entities of the cell wall of *M. tuberculosis* have been defined or are nearing so and the roles of the individual enzymes and genes in the processes are known. Certainly this general statement applies to the syntheis of peptidoglycan, much of the arabinogalactan, the mycolic acids, LAM, the phthiocerol and trehalose-containing lipids and glycolipids. In these respects the next great challenges are the definition of the mechanisms of assembly of the final mAGP complex, precisely the enzymology and genetics of the ligation of peptidoglycan to the arabinogalactan and recognition of the juncture at which mycolic acids are attached to the complex. Beyond these challenges are the issues of the assembly of the whole, a topic still to be faced in the

case of cell wall assembly of the better studied Gram positive and Gram negative bacteria. Nevertheless, the elegant studies on the assembly of Gram negative LPS provides a paradigm for such future studies (Raetz and Whitfield, 2002). For instance completion of the Smooth-LPS molecule involves the ligation of the O-polysaccahride to the nascent Lipid A-Core and these events are located on the periplasmic face of the inner membrane, and the *waaL* gene product may be solely responsible for this ligation. Moreover the central role of MsbA in the delivery of the Lipid A-Core to the periplasmic face is now well recognized. Already searches in the *M. tuberculosis* genomes for homologs of such as the *msbA* gene, an ABC transporter, are under way. Likewise from the literature on LPS deposition (Raetz and Whitfield, 2002), we know of the roles of, for instance, WecA, WbbE and F, in the assembly of O polysaccharides in a synthase-dependent pathway, and the necessity that the O-polysaccharide be still in its polyprenyl-linked format. Thus, we speculate that the AG complex of *M. tuberculosis* is exported to the cell wall in an equivalent form perhaps mediated by WecA, WbbE and F homologs. Thus, despite impressive progress over the past 20 years in our understanding of the biogenesis of the cell wall of *M. tuberculosis*, the challenges remaining are substantial. Yet, this avenue of research is particularly pressing in light of the present day reality of drug resistant TB and the need for new antidotes. Enzymes located beyond the cytoplasmic membrane involved in unique assembly mechanisms offer great promise as new therapeutic target with novel mechanisms of action.

Acknowledgments

The authors wish to thank Dr. Jana Korduláková (Comenius University, Bratislava, Slovakia) for sharing unpublished results. Figure 1 was adapted from a figure generously provided by M. Scherman and Dr. M. McNeil (Colorado State University). Research conducted in the authors' laboratory was supported by grants from the NIH-NIAID (AI064798 and AI018357) and Colorado State University's Infectious Disease SuperCluster.

Abbreviations

PG	peptidoglycan
PPM	polyprenol-monophospho-mannose
PI	phosphatidyl-*myo*-inositol
PIMs	phosphatidyl-*myo*-inositol mannosides
LM	lipomannan
LAM	lipoarabinomannan
Gal*p*	galacto-pyranose
Gal*f*	galacto-furanose
***myo*-Ins**	*myo*-Inositol
Man*p*	manno-pyranose
Ara*f*	arabino-furanose.

PIM is used to describe the global family of PIM that carries one to six Man*p* residues. In Ac_XPIM_Y, x refers to the number of acyl groups esterified to available hydroxyls on the Man*p* or *myo*-inositol residues, y refers to the number of Man*p* residues; *e.g.* Ac_1PIM_6 corresponds to the phosphatidylinositol hexa-mannoside PIM_6 carrying two acyl groups attached to the glycerol (the diacylglycerol substituent) and one acyl group esterified to the Man*p* residue.

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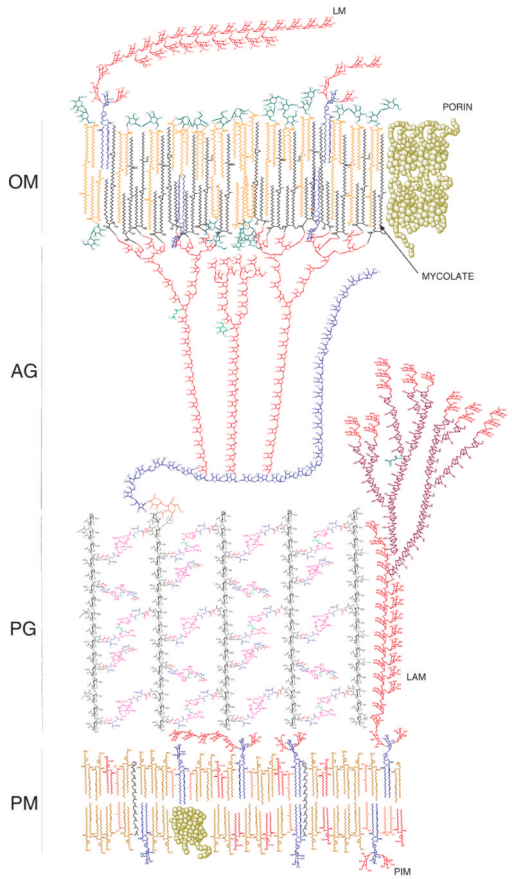
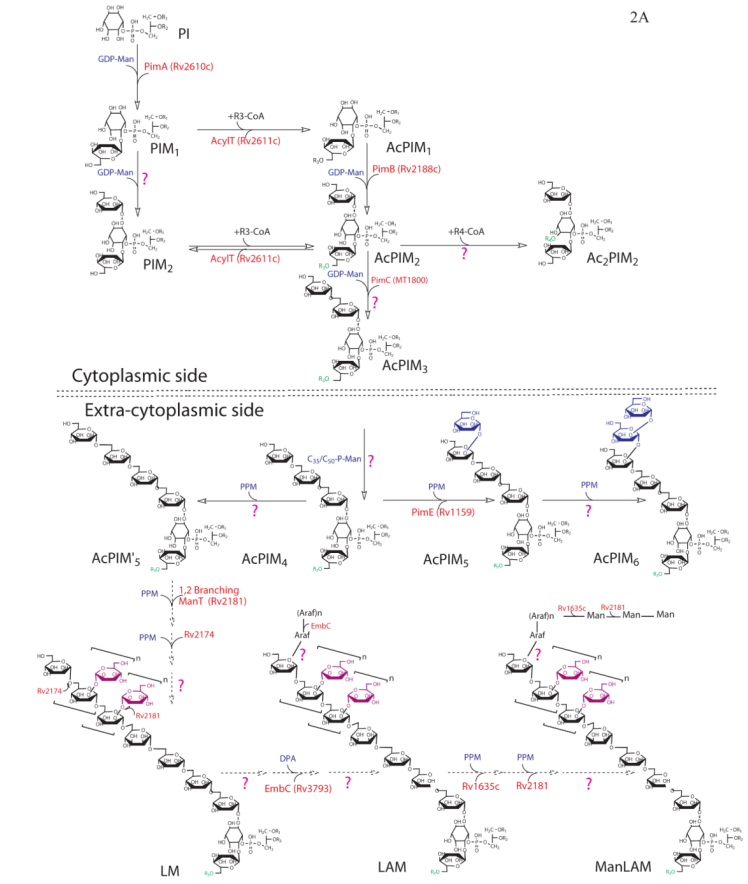
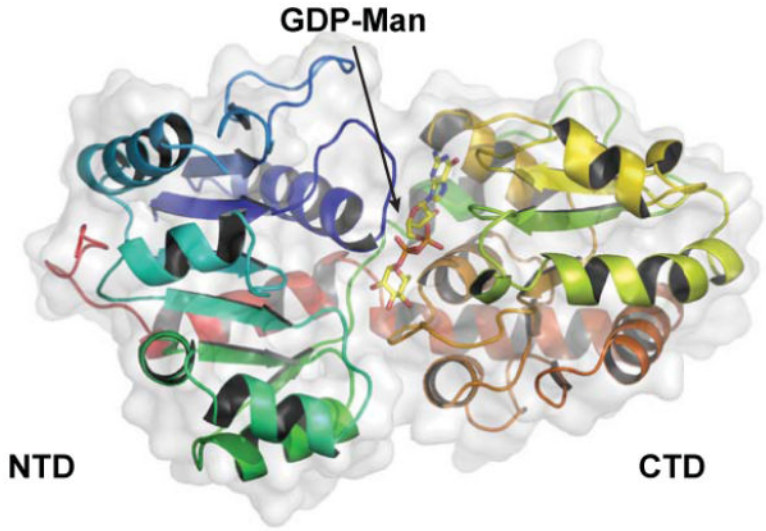


Figure 1.

A current perspective of the cell envelope of *Mycobacterium tuberculosis*. Represented is a theoretical model of the outer membrane (OM) wherein similar extractable lipids are present in both leaflets and the meromycolates of bound mycolic acids span the entire hydrophobic region. Another model proposes the meromycolate chains to be folded upon themselves to create a more compact structure, compatible with the observed thickness of the OM (see text for details). The capsular material is not represented here. Extractable lipids represented in the outer membrane include PIMs and acyltrehaloses whereas those of the plasma membrane include phospholipids and PIMs. PM, plasma membrane; PG, peptidoglycan; AG, arabinogalactan; OM, outer membrane. The Gal*f* and Ara*f* residues of AG are represented in blue and red, respectively. The succinyl and galactosamine residues of AG are in green. Man*p* residues in PIM, LM and LAM are in red; Ara*f* residues are purple.





2B

Figure 2.

Biogenesis of PIM, LM and LAM.

A) Schematic representation of the current understanding of the PIM, LM and LAM biosynthetic pathways in *M. tuberculosis*. B) Three-dimensional structure of a PimA-GDP-Man complex. Cartoon representation of the monomeric form of PimA from *M. smegmatis*. The structure is “color-ramped” from the N-terminus (NTD) (blue) to the C-terminus (CTD) (red).

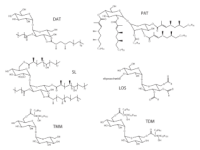


Figure 3.

Structures of some trehalose-derived molecules from *Mycobacterium tuberculosis*.

The major sulfolipid SL-I (2,3,6,6′-tetraacyl α-α’-trehalose-2′-sulfate) is represented. In SL-I, trehalose is sulfated at the 2′ position and esterified with palmitic acid and the multimethyl-branched phthioceranic and hydroxyphthioceranic acids. In DAT (2,3-di-*O*-acyltrehalose), trehalose is esterified with stearic acid and the multimethyl-branched mycosanoic acid. In PAT (polyacyltrehalose), trehalose is esterified with stearic acid and the multimethyl-branched mycolipenic acids. In TMM and TDM, trehalose is esterified with mycolic acids. The oligosaccharide of the LOS of *M. tuberculosis* Canetti strains consists of 2-*O*-methyl-α-L-Fuc*p*-(1→3)-β–D-Glc*p*-(1→3)-2-*O*-methyl-α-L-Rha*p*-(1→3)-2-*O*-methyl-α-L-Rha*p*-(1→3)-β-D-Glc*p*-(1→3)-4-*O*-methyl-α-L-Rha*p*-(1→3)-6-*O*-methyl-α-D-Glc-(1→1)-α-D-Glc. R are 2,4-dimethylhexadecanoic acid and 2,4,6,8-tetramethyloctadecanoic acid residues.

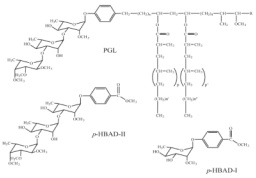


Figure 4.

Structures of phenolic glycolipids and *p*-hydroxybenzoic acid derivatives from *M. tuberculosis*.

The lipid core of PGL from *M. tuberculosis* is composed of phenolphthiocerol esterified by mycocerosic acids (m = 15-17; n = 20-22, n', n" = 16, 18; p, p' = 2-5; R = -CH_2-CH_3 or –CH_3). The trisaccharide substituent of PGL and *p*-HBAD-II consists of 2,3,4-tri-*O*-methyl-α-L-Fuc*p*-(1→3)-α–L-Rha*p*-(1→3)-2-*O*-methyl-α-L-Rha*p*. The monosaccharide substituent found in *p*-HBAD-I consists of 2-*O*-methyl-α-L-Rha*p*.

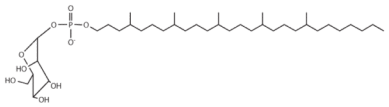


Figure 5.
Structure of the predominant mannosyl-β-1-phosphomycoketides from *M. tuberculosis* H37Rv.

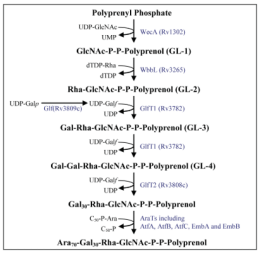


Figure 6.

Proposed pathway for the biosynthesis of mycobacterial arabinogalactan.

AG synthesis is initiated by a transfer of GlcNAc-1-phosphate onto a polyprenyl-phosphate and continues with the sequential addition of glycosyl residues to this lipid carrier. The enzymes that have been proposed to be involved in this process are indicated on the figure.

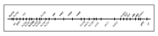


Figure 7.

A schematic representation of the *M. tuberculosis* H37Rv cell wall biosynthetic gene cluster (*Rv3779-Rv3809c*) encompassing genes involved (or likely to be) in the biosynthesis of mycolic acids (*accD4, pks13, fadD32, fbpD, fbpA*), arabinogalactan (glfT1, Rv3790, Rv3791, atfA, embA, embB, atfB, ubiA, glfT2, glf), and LAM (*embA, embB, embC, atfB,, ubiA*). Genes annotated or suggested as glycosyltransferases are marked in bold.

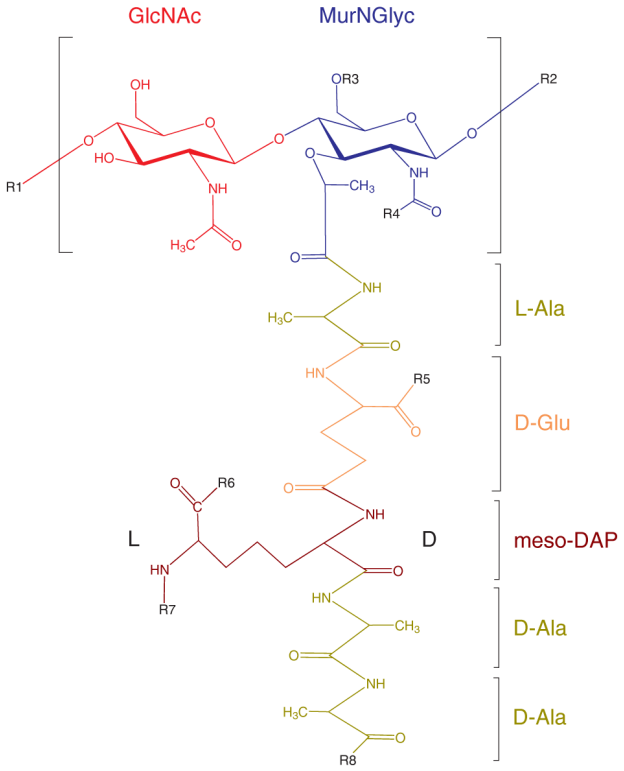


Figure 8.

Structure of a representative monomer of mycobacterial PG prior to peptide trimming. R_1, *N*-glycolylmuramic acid residue of another monomer; R_2, *N*-acetylglucosamine residue of another monomer; R_3, H or the linker unit of AG; R_4, H, $COCH_3$ (*N*-acetyl) or $COCH_2OH$ (*N*-glycolyl); R_5, R_6, R_8, OH, NH_2 or OCH_3; R_7, H, or cross-linked to penultimate D-Ala or to the D-center of another *meso*-DAP residue.

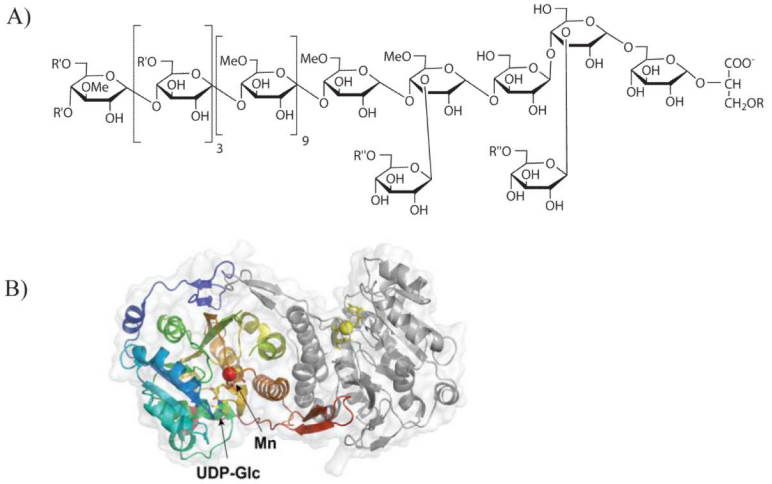


Figure 9.

Methylglucose lipolysaccharides.

A) Structure of the MGLPs from *M. bovis* BCG. The non-reducing end of the polymer is acylated by a combination of acetate, propionate and isobutyrate (R'), whereas octanoate (R) esterifies the position 1 of glyceric acid and zero to three succinate groups (R") esterify the Glc residues of the reducing end. MGLPs occur as a mixture of four main components that differ in their content of esterified succinic acid. B) Three-dimensional structure of the glucosyl-3-phosphoglycerate synthase from *M. avium* subsp. *paratuberculosis* (MAP2569c) in complex with UDP-Glc. Cartoon representation of the dimeric form of the MAP2569c. One monomer is "color-ramped" from the N-terminus (blue) to the C-terminus (red). The second monomer is in grey and UDP-Glc is shown in yellow.

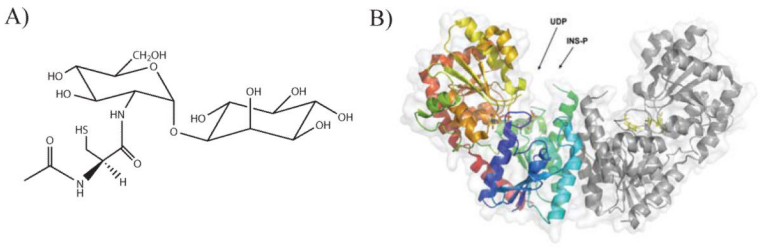


Figure 10.
Mycothiol.
A) Structure of mycothiol; B) Three-dimensional structure of a *Cg*MshA-UDP-Ins-P complex. Cartoon representation of the dimeric form of *Cg*MshA. One monomer is "color-ramped" from the N-terminus (blue) to the C-terminus (red). The second monomer is in grey and the UDP and Ins-P substrates are shown in yellow. The dimer interface is entirely composed of residues from the N-terminal domain.

Table 1

The glycosyltransferases of *M. tuberculosis*

Protein		Function	GT fold	CAZy Family	PDB code	References
PIM, LM and LAM						
Rv2610c	PimA	α-D-mannose-α-(1,2)-phosphatidyl *myo*-inositol mannosyltransferase	GT-B	GT4	**2GEJ (79)**	Korduláková *et al.*, 2002
Rv0557	PimB	mannosyltransferase	GT-B	GT4	2gej (23), 3c4v (20), 2jjm (19), 2iv3 (18), 2r66 (14), 2iw1 (13)	Schaeffer *et al.*, 1999 Tatituri *et al.*, 2007
Rv2188c	PimB'	α-D-mannose-α-(1,6)-phosphatidyl *myo*-inositol mannosyltransferase	GT-B	GT4	2gej (24), 2jjm/3c4v (23), 2iw1 (19), 2r66 (15), 2iv3 (16)	Lea-Smith *et al.*, 2008
Rv2051c	Ppm1	polyprenol phosphomannose synthase	GT-A	GT2	1qgq (10), 3bcv (17)*	Gurcha *et al.*, 2002
Rv1159	PimE	polyprenol-P-Man α-(1,2)-mannosyltransferase	GT-C	GT87	N.D.	Morita *et al.*, 2006
Rv2174	MptA	polyprenol-P-Man α-(1,6)-mannosyltransferase	GT-C	NC	N.D.	Kaur *et al.*, 2007 Mishra *et al.*, 2007
Rv1459c	MptB	polyprenol-P-Man α-(1,6)-mannosyltransferase	GT-C	NC	N.D.	Mishra *et al.*, 2008
Rv2181		polyprenol-P-Man lipo(arabino)mannan α-(1,2)-mannosyltransferase	GT-C	GT87	N.D.	Kaur *et al.*, 2006 Kaur *et al.*, 2008
Rv3793	EmbC	Arabinosyltransferase	GT-C	GT53	N.D.	Zhang *et al.*, 2003
Rv1635c		polyprenol-P-Man ManLAM-capping α-(1,2)-mannosyltransferase	GT-C	GT83	N.D.	Dinadayala *et al.*, 2006
Arabinogalactan						
Rv3265c	WbbL	dTDP-Rha: α-D-GlcNAc-PP-C_{50} α-(1,3)-L-rhamnosyltransferase	GT-A	GT2	1qgq (12), 3bcv (14)	Mills *et al.*, 2004
Rv3782	GlfT1	UDP-Gal*f* : galactan β-(1,4)-/β-(1,5)-galactofuranosyltransferase	GT-A	GT2	1qgq (13), 3bcv (14)	Mikušová *et al.*, 2006 Belanová *et al.*, 2008
Rv3808c	GlfT2	UDP-Gal*f* : galactan β-(1,5)-/β-(1,6)-galactofuranosyltransferase	GT-A	GT2	1qgq (9), 3bcv (11)*	Mikušová *et al.*, 2000 Kremer *et al.*, 2001 Belanová *et al.*, 2008
Rv3792	AftA	Arabinosyltransferase	GT-C	GT85	N.D.	Alderwick *et al.*, 2006
Rv3794	EmbA	Arabinosyltransferase	GT-C	GT53	N.D.	Escuyer *et al.*, 2001
Rv3795	EmbB	Arabinosyltransferase	GT-C	GT53	N.D.	Escuyer *et al.*, 2001
Rv3805c	AftB	β-(1,2) arabinosyltransferase	GT-C	GT89	N.D.	Seidel *et al.*, 2007
Rv2673	AftC	α-(1,3) arabinosyltransferase	GT-C	NC	N.D.	Birch *et al.*, 2008

Protein		Function	GT fold	CAZy Family	PDB code	References
Glycogen, Glucan and MGLPs						
Rv3032		α-(1,4)-glucosyltransferase	GT-B	GT4	3c4v (26), 2gej (24), 2jjm/2iw1 (19), 2r66 (16), 2iv3 (17)	Stadthagen *et al.*, 2007 Sambou *et al.*, 2008
Rv1212c	GlgA	α-(1,4)-glucosyltransferase	GT-B	GT4	3c4v (26), 2gej (21), 2jjm (20), 2iv3 (17), 2r66 (16), 2iw1 (14)	Sambou *et al.*, 2008
Rv1208		glucosyl-3-phosphoglycerate synthase	GT-A	GT81	**3CKQ (83)**	Empadinhas *et al.*, 2008
Acyltrehaloses, *p*HBADs and Phenolic Glycolipids						
Rv3490	OtsA	trehalose-6-phosphate synthase	GT-B	GT20	1uqu (32) 1rrv (20), 1iir (18),	Pan *et al.*, 2002
Rv2958c		PGL/*p*HBAD Rhamnosyltransferase	GT-B	GT1	2d0r/2iyf/2p6p/2vg8/ 2c9z (17), 1pnv (16), 2iya/2acw/2pq6 (15)	Pérez *et al.*, 2004
Rv2962c		PGL/*p*HBAD Rhamnosyltransferase	GT-B	GT1	2d0r/1rrv/1pnv/1iir (17), 2iyf/2c9z/2iya (16), 2p6p (15), 2pq6 (14), 2vg8/2acw (13),	Pérez *et al.*, 2004
Rv2957		PGL/*p*HBAD Fucosyltransferase	GT-A	GT2	1qgq (15), 3bcv (16)	Pérez *et al.*, 2004
Mycothiol						
Rv0486	MshA	UDP-GlcNAc: 1L-*myo*-inositol-1-P α-N-acetylglucoaminyltransferase	GT-B	GT4	**3C4V (51)**	Newton *et al.*, 2003
Glycoproteins						
Rv1002c		Protein-*O*-mannosyltransferase	GT-C	GT39	N.D.	VanderVen *et al.*, 2005
LOS						
Rv1500	LosA	?	GT-A	GT2	1qgq (11), 3bcv (16)	Burguiére *et al.*, 2005

Uncharacterized GTs: GT-A fold; Rv0539 (GT2), Rv0696 (GT2), Rv1514c (GT2), Rv1516c (GT2), Rv1518 (GT2), Rv1520 (GT2), Rv1525 (GT2), Rv3631 (GT2) and Rv3786c (GT2); **GT-B fold**, Rv1524 (GT1), Rv1526c (GT1), Rv2739c (GT1), Rv0225 (GT4), Rv2153c (GT28) and Rv1328 (GT35); **GT-C fold**, Rv0051 (N.C.), Rv0236c (N.C.), Rv0541c (N.C.), Rv1508c (N.C.) and Rv3779 (N.C.).

N.C., Non classified in CAZy (www.cazy.org); N.D., no structures determined.

PDB codes indicate representative protein structures for each GT family (see www.pdb.org). The percentage identity to the *M. tuberculosis* protein is indicated between parentheses. PDB codes in bold indicate percentages of identity to the *M. tuberculosis* protein greater than 50%.

*Only the GT domain of the mycobacterial enzyme was used for sequence alignment.