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The cell envelope glycoconjugates of *Mycobacterium tuberculosis*

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Abstract

Tuberculosis (TB) remains the second most common cause of death due to a single infectious agent. The cell envelope of *Mycobacterium tuberculosis* (*Mtb*), the causative agent of the disease in humans, is a source of unique glycoconjugates and the most distinctive feature of the biology of this organism. It is the basis of much of *Mtb* pathogenesis and one of the major causes of its intrinsic resistance to chemotherapeutic agents. At the same time, the unique structures of *Mtb* cell envelope glycoconjugates, their antigenicity and essentiality for mycobacterial growth provide opportunities for drug, vaccine, diagnostic and biomarker development, as clearly illustrated by recent advances in all of these translational aspects. This review focuses on our current understanding of the structure and biogenesis of *Mtb* glycoconjugates with particular emphasis on one of most intriguing and least understood aspect of the physiology of mycobacteria: the translocation of these complex macromolecules across the different layers of the cell envelope. It further reviews the rather impressive progress made in the last ten years in the discovery and development of novel inhibitors targeting their biogenesis.

Keywords

Glycosyltransferase; phosphatidylinositol mannosides; lipoarabinomannan; arabinogalactan; acyltrehaloses; peptidoglycan; (lipo)polysaccharides; flippase

Introduction

Mycobacteria are known to produce a variety of cytosolic and cell envelope-associated (glyco)lipids and (lipo)polysaccharides of exceptional structures that play various essential roles both in their physiology and interactions with the host in the course of infection. Cytosolic glycoconjugates (e.g., glycogen, glucosylglycerate, polymethylated polysaccharides, mycothiols), for instance, are thought to be important in maintaining a reducing environment in the cytosol, protecting the cells from osmotic and nitrogen stress, regulating fatty acid synthesis and as a carbohydrate reserve (Newton *et al*., 2008; Jackson

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Declaration of interest

The authors report no declarations of interest.

and Brennan, 2009; Kaur *et al*., 2009; Behrends *et al*., 2012). The bulk of the glycoconjugates produced by mycobacteria, however, is found in their cell envelope providing shape and rigidity to the cells and contributing to their impermeability to biocides and nutrients. They also confer unique staining properties to the cells that aid in the microscopy-based diagnosis of mycobacterial diseases and ultimately direct much of the interactions of mycobacteria with the host. While the interest in mycobacterial glycoconjugates originally stemmed from their structural diversity and antigenicity, continued research in this field has been driven by their important contribution to pathogenesis as well as from the standpoint of developing drugs, vaccines, diagnostics and biomarkers. In this regard, developments in the genomics and genetics of mycobacteria in the 1990s have provided a major impetus to the study of *Mycobacterium tuberculosis* (*Mtb*) cell envelope glycoconjugates culminating in significant progress made in the last two decades in elucidating the biosynthetic pathways leading to their elongation, assembly and export. Concomitantly, the ability to generate isogenic knock-outs, knock-ins and knock-downs of *Mtb* proficient or, on the contrary, deficient in the synthesis or export of specific glycoconjugates has allowed for the definition of novel therapeutic targets and a better understanding of their roles in pathogenesis. This review focuses on the cell envelope glycoconjugates of *Mtb* with particular emphasis on recent findings concerning their structures, biogenesis and biological activities. It further discusses the common themes that are beginning to emerge with regard to the coupling of their biosynthesis and export, and finally reviews ongoing drug discovery efforts aimed at targeting their biogenesis.

The major cell envelope glycoconjugates of *Mtb*

The mycobacterial cell envelope is made up of three major entities [Fig. 1]. The innermost layer is the plasma membrane that seems typical of bacterial membranes except for the presence of *Mycobacterium*-specific (glyco)lipids, lipoglycans and (lipo)proteins. Outside the plasma membrane is the cell wall core comprised of peptidoglycan (PG) in covalent attachment via phosphoryl-*N*-acetylglucosaminosyl-rhamnosyl linkage units with the heteropolysaccharide arabinogalactan (AG), which is in turn esterified at its non-reducing ends to α-alkyl, β-hydroxy long-chain (C_{60}-C_{90}) mycolic acids. The cell wall core, also known as the mycolyl arabinogalactan-peptidoglycan (mAGP) complex, is essential for viability and the site of resistance and susceptibility to many anti-TB drugs (Barry *et al*., 2007; Jackson *et al*., 2013). The AG-bound mycolic acids form the bulk of the inner leaflet of the outer membrane, with the outer layer consisting of a variety of non-covalently attached (glyco)lipids, lipoglycans (lipomannan and lipoarabinomannan) and (lipo)proteins some of which are glycosylated. The organization and composition of this asymmetrical outer bilayer known as the ‘mycomembrane’ or ‘outer membrane’ (OM) (Hoffmann *et al*., 2008; Zuber *et al*., 2008) confer to mycobacteria a high intrinsic resistance to many therapeutic agents and host defense mechanisms (Minnikin *et al*., 1982; Jarlier and Nikaido, 1994). Finally, a loosely attached capsular-like structure outside the OM of *Mtb* was shown to mainly consist of polysaccharides and proteins with only minor amounts of lipids (Lemassu and Daffé, 1994; Ortalo-Magné *et al*., 1995; Sani *et al*., 2010). The three major polysaccharides found in the capsular material of *Mtb* consist of a high molecular weight α-D-glucan with a structure similar to that of glycogen, a D-arabino-D-mannan (AM), and a

D-mannan (Lemassu and Daffé, 1994; Ortalo-Magné *et al*., 1995; Dinadayala *et al*., 2004). Importantly, the nature and **amounts of outer membrane and capsular materials** vary with the *Mtb* isolates and this diversity in terms of surface composition is likely to significantly impact the way that *Mtb* interacts with the host (Cywes *et al*., 1997; Ehlers and Daffé, 1998; Daffé and Etienne, 1999; Torrelles and Schlesinger, 2010).

The glycoconjugates of the cell wall core

(1) Peptidoglycan

PG structure: PG is a complex glycopolymer forming a rigid layer outside the plasma membrane allowing the bacterium to maintain its shape and to resist the effects of changes in osmotic pressure. As in other bacteria, the synthesis and turnover of PG in *Mtb* are intimately coordinated with cell division. In mycobacteria, PG also serves as a scaffold for the rest of the cell envelope [Fig. 1]. The detailed structure and biosynthesis of the PG of *Mtb* have been reviewed recently (Pavelka *et al*., 2014). The PG of mycobacteria belongs to the A1γ chemotype as does that of *Escherichia coli* and a number of other bacteria. It consists of a glycan backbone of alternating units of *N*-acetylglucosamine (GlcNAc) and modified muramic acid (Mur) in a β-(1,4) linkage, with tetrapeptide side chains attached to the lactyl moiety of Mur typically consisting of L-alanyl-D-glutamine-*meso*-diaminopimelyl-D-alanyl-D-alanine that may be cross-linked [**Fig. 2**]. Most of the cross-links in *Mtb* (60-80%) consist of '3,3' linkages between the *meso*-diaminopimelate (*meso*-DAP) residues of adjacent peptides (Kumar *et al*., 2012); the second type of linkages found are known as '4,3' linkages and occur between the D-Ala at position 4 of one peptide and the *meso*-DAP at position 3 of an adjacent peptide. Contrary to the earlier impression that the proportion of 3,3 linkages increased as *Mtb* bacilli reached stationary phase (Lavollay *et al*., 2008), the percentage of 3,3 to 4,3 linkages is in fact relatively constant throughout the growth of *Mtb* (Kumar *et al*., 2012). The overall high degree of cross-linking typically found in *Mycobacterium spp.* (70-80% compared to 30-50% in *E. coli*) (Matsuhashi, 1966) provides added structural integrity to the cells. A particularity of the muramic acid residues found in the PG of mycobacteria and closely related actinobacteria is that they can be either *N*-acetylated (MurNAc) or *N*-glycolylated (MurNGlyc). *N*-glycolylation contributes to the resistance of mycobacteria to lysozyme (Raymond *et al*., 2005) and potentiates the innate immune recognition of their PG by Nod2 (Coulombe *et al*., 2009) but does not affect the pathogenicity of *Mtb* (Hansen *et al*., 2013). Other variations in the peptide chain include the amidation of free carboxyl group of D-Glu and that of the free carboxyl group of *meso*-DAP. It is thought that some of the structural particularities of mycobacterial PG are related to its role in stabilizing the mAGP complex (Mahapatra *et al*., 2005). AG is attached to PG through a phosphodiester link to position 6 of about 10-12% of the Mur residues. The specific linker unit ensuring this covalent attachment is made of a rhamnosyl residue attached to a GlcNAc-1-phosphate residue (McNeil *et al*., 1990) **[Fig. 3]**.

PG synthesis: PG synthesis requires a series of essential steps: (i) the cytoplasmic synthesis of precursor molecules; (ii) the translocation of the PG precursor known as lipid II to the periplasmic face of the plasma membrane and (iii) its incorporation into the existing PG.

The cytoplasmic synthesis of PG precursors in *Mtb* is for the most part similar to that of other bacteria (for a recent review, Pavelka *et al*., 2014). Consistently, most of the genes involved have been found primarily by homology. The formation of UDP-MurNAc from UDP-GlcNAc in a two-step reaction catalyzed by MurA (Rv1315) and MurB (Rv0482) is the first committed step in the biosynthesis of Park's nucleotide (UDP-*N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-diaminopimelyl-D-alanyl-D-alanine). UDP-GlcNAc is the product of the phosphoglucosamine mutase GlmM (Rv3441c) and the UDP-*N*-acetylglucosamine pyrophosphorylase GlmU (Rv1018c) (Zhang *et al*., 2008; Li *et al*., 2012). The next steps in the biosynthesis of Park's nucleotide consist of the stepwise additions of each amino acid of the peptide chain to the D-lactoyl group of Mur residues in reactions catalyzed by the Mur family of ligases. MurC (Rv2152c) catalyzes the addition of the first D-Ala residue; MurD (Rv2155c), the addition of a D-Glu residue; MurE (Rv2158c), the addition of *meso*-DAP; and MurF (Rv2157c), the addition of the last two D-Ala residues as a dipeptide. The latter dipeptide is the product of the D-Ala-D-Ala ligase (DdlA; Rv2981c). D-Ala and D-Glu are produced from L-Ala and L-Glu by the alanine racemase Alr (Rv3423c) and the glutamate racemase MurI (Rv1338), respectively. *meso*-DAP is produced from L-aspartate in a series of eight reactions involving the enzymes Ask (Rv3709c), Asd (Rv3708c), DapA (Rv2753c), DapB (Rv2773c), DapC (Rv0858c), DapD (Rv1201c), DapE (Rv1202) and DapF (Rv2726c).

As in other bacteria, the fully assembled sugar-peptide moiety of the Park's nucleotide is then transferred to a lipid carrier by the phospho-*N*-acetylmuramyl pentapeptide translocase MraY (Rv2156c) forming lipid I. Unlike other bacteria, however, the lipid carrier used by mycobacteria in the biosynthesis of PG and other major cell envelope glycoconjugates (e.g., polar forms of phosphatidyl-*myo*-inositol mannosides, AG, lipomannan, lipoarabinomannan, glycoproteins) is decaprenyl-phosphate (Dec-P) instead of the usual undecaprenyl phosphate. The biosynthesis of Dec-P in *Mtb* was reviewed recently (Daffé *et al*., 2014). The glycosyltransferase MurG (Rv2153c) next transfers GlcNAc from UDP-GlcNAc to lipid I yielding lipid II. It is at the level of lipid II that the peptides may undergo modifications (amidation, methylation and glycolylation) and that the *N*-acetyl groups of the MurNAc residues may be oxidized to *N*-glycolyl by the UDP-MurNAc hydroxylase NamH (Rv3818) (Raymond *et al.*, 2005). Although candidate genes for the modification of the peptide have been identified, their involvement in the process has not yet been validated experimentally. The physiological significance of the differentially modified lipid II molecules that arise from these modifications is currently not known, nor is it clear that all of these modifications occur in the mature PG.

The identity of the 'flippase(s)' required to translocate lipid II to the periplasmic face of the plasma membrane and that of the transporter required to import back the decaprenyl diphosphate (or Dec-P) released upon the incorporation of the lipid II precursors into the mature AG have not yet been established. Genetic and bioinformatic evidence based on studies conducted in other bacteria suggest that FtsW- and MviN- (MurJ) like proteins may be involved (Ruiz, 2008; Mohammadi *et al*., 2011). FtsW- and MviN-like proteins seem to be present in all PG-producing eubacteria. MviN proteins are members of the multidrug/oligosaccharidyl-lipid/polysaccharide (MOP) flippase superfamily that bears sequence

similarity with the Wzx/WzxE flippases involved in the synthesis of the majority of LPS O-antigens and in that of the enterobacterial common antigen (ECA) (Hvorup *et al*., 2003; Ruiz, 2008). FtsW-like proteins belong to the shape/elongation/division and sporulation (SEDS) protein family (Henriques *et al*., 1998) which in Gram positive and Gram negative organisms have been shown to control PG synthesis during cell elongation, division and sporulation (Henriques *et al*., 1998; Pastoret *et al*., 2004; Real *et al*., 2008). Recent biochemical studies conducted on the FtsW protein of *E. coli* are consistent with this protein displaying lipid II flippase activity (Mohammadi *et al*., 2011). The *Mtb* MOP superfamily protein Rv3910 which harbors an N-terminal MviN-like domain was shown to be essential for growth. The knock-down of this gene in *M. smegmatis* causes altered cell morphology in addition to growth inhibition, and leads to the accumulation of PG precursors in the cells (Gee *et al*., 2012). Whether Rv3910 acts a lipid II flippase, however, remains to be established. Likewise, the FtsW-like protein encoded in the genome of *Mtb* H37Rv by *Rv2154c* maps in a cluster of genes dedicated to PG synthesis including *mraY* (*Rv2156c*) and *murG* (*Rv2153c*) responsible, respectively, for the formation of lipids I and II. Rv2154c was shown to physically interact with the penicillin-binding protein PbpB (Rv2163c) (see further) and FtsZ (Rv2150c) thereby likely facilitating septal PG synthesis during cell division (Datta *et al*., 2002; Datta *et al*., 2006), but its precise function in PG synthesis remains elusive. Finally, a third FtsW-like lipid II flippase candidate found in *Mtb* is encoded by *rodA* (*Rv0017c*). RodA is an essential protein in *Bacillus subtilis* required for the elongation of the lateral wall of the cells and the maintenance of their rod shape (Henriques *et al*., 1998). The recent finding that RodA of *Corynebacterium glutamicum* exclusively localizes to the cell poles and is required for optimal growth and cell length is consistent with this protein serving an analogous function in Actinobacteria even though the dispensability of this protein for viability in this species indicates that compensatory activities exist (Sieger *et al*., 2013). In other rod-shaped model organisms such as *E. coli* and *B. subtilis*, it is assumed that the requirement of the divisome and cell elongation machineries for distinct lipid II flippases accounts for the existence of more than one of these transporters in the cells (Sieger *et al*., 2013). It is thus possible that *Mtb* expresses more than one lipid II flippase and that MviN (Rv3910), FtsW (Rv2154c) and RodA (Rv0017c) all contribute to this function in the context of cell division and/or cell elongation. Clearly, the identification of *Mtb* lipid II flippase candidates is an exciting breakthrough for its potential to lead to a better understanding of cell elongation and division.

A number of transglycosylases, transpeptidases and carboxypeptidases including eight penicillin-binding proteins (PBPs), mediate the polymerization of the sugar backbone and cross-linking of the peptides of PG in *Mtb* (Pavelka *et al*., 2014). The role of some of these enzymes in PG biosynthesis, however, is not fully understood. The *Mtb* PBPs PbpA (Rv0016c) and PbpB (FtsI; Rv2163c) also play critical roles in cell division (Pavelka *et al*., 2014). A specific family of enzymes known as L,D-transpeptidases accounts for the formation of the 3,3 crosslinks in PG. The *Mtb* genome encodes five of these enzymes, four of which (LdtA [Rv0116c]; LdtB [Rv2518c]; Mt4 [Rv0192]; and Mt5 [Rv0483]) exhibit L,D-transpeptidase activity *in vitro* (Lavollay *et al*., 2008; Gupta *et al*., 2010; Cordillot *et al*., 2013). Unlike the classical D,D-transpeptidases which are penicillin-binding proteins

and can be inhibited by various classes of β-lactam antibiotics (Goffin *et al*., 2002), L,D-transpeptidases are typically resistant to most β-lactams with the exception of the carbapenem class (Lavollay *et al*., 2008; Gupta *et al*., 2010; Kumar *et al*., 2012; Cordillot *et al*., 2013; Pavelka *et al*., 2014). Interestingly, carbapenems were reported to form covalent adducts with LdtA, LdtB and Mt4 but not Mt5 (Cordillot *et al*., 2013).

PG turnover: Not much is known about PG breakdown and recycling in mycobacteria. CwlM (Rv3915) and Rv3717 are amidases of *Mtb* that cleave PG between the N-acetylmuramyl acid residues and the first L-Ala of the peptide chain (Deng *et al*., 2005; Prigozhin *et al*., 2013). In addition, the genome of *Mtb* potentially encodes seven NPL/P60 family endopeptidases, the two best-studied members of which are RipA (Rv1477) and RipB (Rv1478) that cleave PG fragments between D-Glu and *meso*-DAP (Both *et al*., 2011; Pavelka *et al*., 2014). Finally, *Mtb* has five resuscitation-promoting factor (*rpf*)-like genes whose protein products share common structural features with the so-called 'lysozyme-like' fold, suggesting that they may cleave the glycan chain of PG (Kaprelyants *et al*., 2012). Rpf proteins have partially overlapping activity *in vitro* and *in vivo*, are all highly induced during resuscitation, and are required to restore the culturability of non-replicating persistent bacilli (Kaprelyants *et al*., 2012). The mechanism through which Rpf proteins stimulate cell reactivation and growth is still unclear. While the cleavage of PG by Rfp proteins may directly account for the initiation of replication after a period of latency, it was also proposed that the muropeptides released as a result of the action of Rpf proteins may act as signaling molecules in the host or stimulate the Ser/Thr protein kinase PknB to indirectly regulate cell envelope biosynthesis and cell division (Molle and Kremer, 2010; Mir *et al*., 2011; Kaprelyants *et al*., 2012).

PG biosynthesis in the context of drug discovery: With multidrug resistance on the rise, recent years have seen a marked intensification of TB drug discovery efforts with the result that many new lead compounds are now at various stages of the drug discovery and preclinical development pipeline. These efforts not only keep pointing at cell envelope biogenesis as one of the Achille's heel of *Mtb* (for a recent review, Jackson *et al*., 2013) but are also leading the TB field to revisit earlier impressions that drug targeting the biogenesis of the cell envelope may not be synergistic with other drugs, useful against MDR-*Mtb* isolates, or active against persistent bacilli. Interestingly, PG synthesis is one of the cell envelope pathways that has undergone the greatest resurgence of interest as a promising target for new chemotherapeutic approaches. Of course, D-cycloserine has long been a useful second-line drug in the treatment of TB and nowadays in the treatment of MDR-TB despite its well known effects on the central nervous system. It competitively inhibits two enzymes in the synthesis of the peptide chain of PG, alanine racemase (Alr) which forms D-alanine from L-alanine, and D-alanine:D-alanine synthase (DdlA) in all eubacteria including mycobacteria. Towards the development of more effective inhibitors of DdlA, the crystal structures of this enzyme under its *apo* form and in complex with D-cycloserine were recently solved (Bruning *et al*., 2011). In addition, thiadiazolidinone inhibitors of Alr (IC_{50} of 0.03 to 28 μM) were found to inhibit the growth of *Mtb* at concentrations ranging from 1.6 to 100 μg/ml (Lee *et al*., 2013). Other recent efforts have focused on a series of synthetic N-methyl-2-alkenyl-4-quinolones showing IC50 values in the range of 100 μM against the

Mtb MurE ligase *in vitro* and 5 to 25 μg/ml MICs against *Mtb* in culture (Guzman *et al*., 2011). An assay suitable for the high throughput screening of inhibitors of GlmU (involved in the formation of GlcNAc) was developed, and an NIH-sponsored screening was performed (http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?aid=1376) which was later analyzed for optimization of hits (Singla *et al*., 2011). Other inhibitors of GlmU were recently described (Tran *et al*., 2013). Even though their mode of action in whole *Mtb* cells has yet to be confirmed, the synthesis of capuramycin analogs as inhibitors of bacterial phospho-*N*-acetylmuramyl pentapeptide translocases (MraY) has led to the identification of several analogs with potent activity against drug susceptible and MDR *Mtb* isolates (MIC of 2 to 4 μg/ml) as well as several other mycobacterial pathogens both *in vitro* and *in vivo* (Koga *et al*., 2004; Reddy *et al*., 2008; Nikonenko *et al*., 2009). One of these analogs, SQ641, is now at the stage of preclinical development (http://www.newtbdrugs.org). The importance of Rfps in restoring the culturability of non-replicating persistent bacilli and the structural similarity between the conserved catalytic domain of these proteins and that of cell wall lytic enzymes has prompted a search for Rpf inhibitors based on known inhibitors of the latter proteins. Nitrophenylthiocyanate derivatives were tested and found to inhibit the mycobacterial purified Rpfs. While devoid of activity against acute TB *in vivo*, some of them impair the resuscitation of dormant *Mtb* cells both *in vitro* and in mice and may thus represent a promising new scaffold for drugs targeting persistent *Mtb* bacilli (Kaprelyants *et al*., 2012). The recent success of carbapenems and clavulanate combinations in the treatment of active and latent TB (Hugonnet *et al*., 2009; England *et al*., 2012) further emphasizes the potential of PG as a target to kill persistent and MDR bacilli and has resulted in a re-visitation of the prospects of introducing β-lactam-β-lactamase inhibitors into standard TB chemotherapy. As noted earlier, the L,D-transpeptidases involved in the (3,3)-crosslinking of PG are resistant to most β-lactams with the exception of the carbapenem class. The fact that the disruption of *ldtB* negatively impacts virulence and increases the susceptibility of *Mtb* to amoxicillin-clavulanate both *in vitro* and during the chronic phase of infection (Gupta *et al*., 2010) suggests, however, that a combination of L,D-transpeptidase inhibitor, clavulanate and classical β-lactams could effectively target replicating and persistent bacilli. The structure of LdtB was recently determined and drugs targeting this enzyme are being sought (Erdemli *et al*., 2012). The discovery that synthesis of the major lipid carrier, Dec-P, in *Mtb* proceeds through the methylerythritol phosphate pathway which has no homolog in humans has provided stimulus for the identification and characterization of inhibitors of the relevant enzymes. Fosmidomycin, which is currently in clinical trials for the treatment of malaria in humans, is a competitive inhibitor of the second enzyme of the pathway, 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR, IspC). Promising results on *Mtb* were obtained with lipophilic prodrug derivatives of this compound (Uh *et al*., 2011) and the availability of several crystal structures of the *Mtb* DXR enzyme and DXR-fosmidomycin complexes has opened the way to the structure-based design of more potent analogs. Finally, another approach to targeting PG metabolism has focused on inhibiting the Ser/Thr kinases involved in the regulation of this pathway (Molle and Kremer, 2010; Mir *et al*., 2011; Gee *et al*., 2012). Accordingly, libraries of compounds were screened against PknA or PknB *in vitro* and several promising inhibitors were found (Magnet *et al*., 2010; Danilenko *et al*., 2011; Lougheed *et al*., 2011; Chapman *et al*., 2012; http://www.newtbdrugs.org). Preliminary evidence suggests, however, that the treatment of *Mtb* with some of these

inhibitors leads to the inhibition of multiple targets (Magnet *et al*., 2010; Lougheed *et al*., 2011).

(2) Arabinogalactan

Structure of AG: The most recent model of *Mtb* AG, based on the analysis of this heteropolysaccharide from *in vitro*-grown *Mtb* H37Rv, indicates that it contains on average 79 glycosyl residues distributed between a galactan domain made of 23 Gal*f* residues, two arabinan domains each containing about 26 Ara*f* residues, and a specific linker unit made of a rhamnosyl residue attached to a *N*-acetylglucosaminosyl-1-phosphate residue which serves in the covalent attachment of AG to PG (Bhamidi *et al*., 2011) **[Fig. 3]**. It was estimated that 1.3 AG molecules were present per 10 repeating units of PG in *Mtb* (Bhamidi *et al*., 2011). The characteristic non-reducing termini of the arabinan domain of AG consist of an Ara_6 motif, Ara*f*β–(1,2)-Ara*f*α–(1,5)(Ara*f*β–(1,2)Ara*f*α–(1,3))-Ara*f*α–(1,5)-Ara*f*α–(1, where both the terminal β-Ara*f* and the penultimate 2-α-Ara*f* serve as the anchoring points for the mycolic acids. Approximately two-thirds of these attachment sites are occupied with mycolate residues in *in vitro*-grown *Mtb* (McNeil *et al*., 1991; Bhamidi *et al*., 2011). 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. In addition, galactosamine (α-D-Gal*p*NH_2 thereafter referred to GalN) and succinyl substituents were found specifically attached at O-2 of a portion of the internal (3,5)-branched D-Ara*f* residues in the AG of *Mtb* (Draper *et al*., 1997; Lee *et al*., 2006; Bhamidi *et al*., 2008; Peng *et al*., 2012). α-D-GalN was estimated to occur at the level of about one residue per entire AG molecule and succinyl groups at the level of one to three residues per AG molecule (Bhamidi *et al*., 2008). Interestingly, a similar GalN residue was found to substitute the AG of *M. avium*, *M. kansasii*, *M. bovis* BCG (Draper *et al*., 1997) and *M. leprae* but not that of *M. smegmatis*, *M. neoaurum* and *M. phlei* (Draper *et al*., 1997; Lee *et al*., 2006; Bhamidi *et al*., 2008; Bhamidi *et al*., 2011), suggesting that fast-growing *Mycobacterium* spp. are devoid of GalN substituent.

AG biosynthesis: The synthesis of AG begins with the cytoplasmic formation of the linker unit on a decaprenyl monophosphate (Dec-P) carrier lipid followed by the addition of Gal*f* residues still on the cytosolic face of the plasma membrane and that of Ara*f* residues on the periplasmic side of the membrane (Mikušová *et al*., 1996; Mikušová *et al*., 2000; Yagi *et al*., 2003) [Fig. 4]. Many of the enzymes involved in this process have been identified (Kaur *et al*., 2009). A WecA-like transferase encoded by *Rv1302* in the genome of *Mb* H37Rv transfers GlcNAc-1-phosphate from UDP-GlcNAc to Dec-P to form Dec-P-P-GlcNAc (also known as GL-1) (Mikušová *et al*., 1996; Jin *et al*., 2010). The attachment of a rhamnosyl residue from the sugar nucleotide dTDP-Rha to the 3-position of GlcNAc is catalyzed by WbbL1 yielding GL-2 (Dec-P-P-GlcNAc-Rha), “the linker unit” (Mills *et al*., 2004). dTDP-Rha is synthesized from glucose-1-phosphate through a four-step reaction catalyzed by RmlA (Rv0334), RmlB (Rv3464), RmlC (Rv3465) and RmlD (Rv3266c) (Ma *et al*., 1997; Stern *et al*., 1999; Hoang *et al*., 1999; Ma *et al*., 2001). GL-2 then serves as an acceptor for the sequential cytoplasmic addition of Gal*f* residues from UDP-Gal*f* catalyzed by two bifunctional galactosyltransferases, GlfT1 (Rv3782) and GlfT2 (Rv3808c). UDP-Gal*f* is generated from UDP-Gal*p* by the UDP-Gal*p* mutase Glf (Rv3809c) (Weston *et al*., 1997;

Mikušová *et al*., 2000). GlfT1 is endowed with β-(1,4) and β-(1,5) galactosyltransferase activities and transfers the first two Gal*f* residues to GL-2 (Belanova *et al*., 2008). The remaining alternating 5- and 6-linked Gal*f* residues are added by the bifunctional galactosyltransferase GlfT2 (Kremer *et al*., 2001; Rose *et al*., 2006; Belanova *et al*., 2008; Wheatley *et al*., 2012). The identity of the transporter responsible for the translocation of the fully elaborated or nascent lipid-linked galactan chain to the periplasmic side of the plasma membrane has not yet been firmly established although an ABC-transporter has been proposed for this function (Dianiskova *et al*., 2011). The arabinosylation of AG next takes place on the periplasmic side of the plasma membrane catalyzed by membrane-associated decaprenyl-phosphate arabinose (Dec-P-Ara)-dependent arabinosyltransferases (AraTs). Dec-P-Ara is the only known arabinose donor in the building of the arabinan domains of the two essential cell envelope glycoconjugates, AG and lipoarabinomannan (LAM) (Wolucka *et al*., 1994). It is synthesized from 5-phosphoribose-1-pyrophosphate (Scherman *et al*., 1995; Scherman *et al*., 1996) - the product of the phosphoribosyl-pyrophosphate synthetase PrsA (Alderwick *et al*., 2011a) - through four reaction steps involving the Dec-P 5-phosphoribosyltransferase, UbiA (Rv3806c) (Huang *et al*., 2005; Huang *et al*., 2008; Alderwick *et al*., 2005), the phosphoribosyl-monophosphodecaprenol phosphatase Rv3807c (Jiang *et al*., 2011), and DprE1 (Rv3790) and DprE2 (Rv3791) responsible for the epimerization of decaprenyl-phosphate ribose to Dec-P-Ara (Mikušová *et al*., 2005). The AraTs involved in the formation of the arabinan domain of AG identified to date include AftA (Rv3792), responsible for the transfer of the very first Ara*f* residues to the galactan domain of AG (Alderwick *et al*., 2006a), the terminal β–(1,2)-capping AraT AftB (Rv3805c) (Seidel *et al*., 2007), AftC (Rv2673) involved in the internal α–(1,3)-branching of AG (Birch *et al*., 2008) and the EmbA (Rv3794) and EmbB (Rv3795) proteins involved in the formation of the Ara_6 motif of AG (Escuyer *et al*., 2001; Khasnobis *et al*., 2006). We successfully overexpressed and purified a soluble form of AftC from *M. smegmatis* and showed that it retains α–(1,3)-branching AraT activity *in vitro* upon reconstitution into proteoliposomes containing mycobacterial lipids (Zhang *et al*., 2011). By analogy with the Emb protein of *C. glutamicum* (NCgl0184) (Alderwick *et al*., 2005) and EmbC (Rv3793) which is required for the elongation of the arabinan domain of LAM (Berg *et al*., 2005; Shi *et al*., 2006) (see PIM, LM, LAM section), it was proposed that EmbA and/or EmbB (or an EmbA/EmbB dimer) acted as the α-(1,5) AraTs responsible for the elongation of the arabinan domain of AG (Bhamidi *et al*., 2008). However, direct evidence for this assumption is still lacking. Moreover, elongating α–(1,5) AraT activities - some of which are apparently unrelated to the Emb proteins - have been detected in cell-free assays using mycobacterial cell wall preparations and synthetic arabinan acceptors (Lee *et al*., 1997; Lee *et al*., 1998; Zhang *et al*., 2007). Finally, another functional Dec-P-Ara-dependent AraT with α–(1,3) branching activity on linear α–(1,5)-linked neoglycolipid acceptors was identified as AftD (Rv0236c) (Škovierová *et al*., 2009). *aftD* is an essential gene in *M. smegmatis*. Alterations in its level of expression caused defects in cell division, reduced growth, altered colony morphology and accumulation of trehalose dimycolates in the cell envelope. Overexpression of *aftD* in *M. smegmatis*, in contrast, induced the accumulation of arabinosylated compounds with carbohydrate backbones reminiscent of that of LAM. Collectively, these results suggest that AftD is involved in the synthesis of the arabinan

domains of AG and LAM, although its precise function in these pathways remains to be defined.

Importantly, the enzymes involved in the formation of Dec-P (Eoh *et al*., 2007), dTDP-Rha (Ma *et al*., 2002; Li *et al*., 2006), UDP-Gal*f* (Pan *et al*., 2001), GL-I (Jin *et al*., 2010; Ishizaki *et al*., 2013), GL-II (Mills *et al*., 2004), the galactan domain (Pan *et al*., 2001), Dec-P-Ara (Crellin *et al*., 2011; Kolly *et al*., 2014) and the arabinan domain of AG (Alderwick *et al*., 2005; Amin *et al*., 2008; Shi *et al*., 2008; Škovierová *et al*., 2009) are all essential for mycobacterial growth providing opportunities for new chemotherapeutic strategies against *Mtb* (see further).

By analogy to the biosynthetic pathway responsible for the modification of lipid A with a D-GalN unit in *Francisella* (Kanistanon *et al*., 2008; Wang *et al*., 2009; Song *et al*., 2009), we identified and functionally characterized two glycosyltransferases, Rv3631 and Rv3779, responsible for the synthesis and transfer of the GalN motif of AG (Škovierová *et al*., 2010) [Fig. 4]. Rv3631 displays polyprenol-phospho-GalNAc (Dec-P-GalNAc) synthase activity, generating on the cytoplasmic face of the plasma membrane Dec-P-GalNAc from Dec-P and UDP-GalNAc. Dec-P-GalNAc or its deacylated counterpart, Dec-P-Gal, then serve as the sugar donors used by the GT-C glycosyltransferase Rv3779 in the periplasmic transfer of GalN (or GalNAc) onto the arabinan domain of AG (Škovierová *et al*., 2010). The deacetylase required to generate Dec-P-GalN from Dec-P-GalNAc and the “flippase” required to translocate Dec-P-GalNAc (or Dec-P-GalN) from the cytosolic to the periplasmic side of the plasma membrane have not yet been identified. The enzyme responsible for the transfer of succinyl residues to the arabinan domain of AG is also presently not known.

Topology of the AG biosynthetic pathway and evidence for the existence of multiprotein complexes: In spite of the significant advances made in the last 15 years in understanding the biosynthesis of AG and underlying genetics, the fundamentals of how the different domains of AG are assembled, if on a lipid carrier, growing stepwise from the reducing towards the non-reducing end through the sequential addition of glycosyl residues or assemble through the polymerization of building blocks, are at present not fully understood. Based on available evidence, the sequential addition of arabinosyl and galactosyl residues is favored over the polymerization of building blocks such as described in the biosynthesis of some bacterial O-antigens, glycoproteins and capsular polysaccharides (Raetz and Whitfield, 2002; Rick *et al*., 2003; Whitfield, 2006; Alaimo *et al*., 2006; Raetz *et al*., 2007; Ruiz *et al*., 2008; Mohammadi *et al*., 2011). Experimental evidence further points to the concurrent galactosylation and arabinosylation of lipid-linked AG precursors, at least in cell-free assays (Mikušová *et al*., 2000), and possibly in intact cells (Larrouy-Maumus *et al*., 2012). Given that galactosylation and arabinosylation events are topologically split across the plasma membrane, this finding could suggest a ‘synthase-dependent’ type of pathway for AG biosynthesis wherein the nascent lipid-linked galactan chain is progressively extruded across the plasma membrane as it is elongated (Raetz and Whitfield, 2002). The transporter involved has not yet been identified although an ABC-transporter has been proposed to participate in this function (Dianiskova *et al*., 2011). The periplasmic arabinosylation of AG further raises the question of the flipping of Dec-P-Ara from the

cytosolic face of the membrane where this sugar donor is synthetized (Mikušová *et al*., 2005) to the outer leaflet of the membrane where it can then be used by GT-C superfamily arabinosyltransferases. ErmE-like Small Multidrug Resistance (SMR) transporters, typically 105-121 amino acids in size and containing four transmembrane domains (Bay *et al*., 2008), participate in the translocation of lipid-linked phosphate sugars across the plasma membrane in a variety of microorganisms. Examples include the *E. coli* transporter encoded by *arnE/ arnF* that flips undecaprenyl phosphate 4-amino-4-deoxy-L-arabinose across the plasma membrane (Yan *et al*., 2007), and the GtrA protein produced by the bacteriophage SfX of *Shigella flexneri* which was proposed to mediate the translocation of undecaprenyl phosphate glucose used in the glucosylation of O-antigen (Guan *et al*., 1999; Korres *et al*., 2005). The presence of an SMR transporter-like gene, *Rv3789*, located immediately upstream *dprE1* and *dprE2* (responsible for the formation of Dec-P-Ara) in the genome of *Mtb* was suggestive of the involvement of Rv3789 in the re-orientation of Dec-P-Ara to the periplasm [Fig. 4]. Consistently, our results have shown that the disruption of the ortholog of *Rv3789* in *M. smegmatis* resulted in a truncation of the arabinan domains of both AG and LAM that accompanied the accumulation of Dec-P-Ara in the mutant cells (Larrouy-Maumus *et al*., 2012). Further supporting the characterization of Rv3789 as a translocase, AG and LAM synthesis was restored in the mutant not only upon expression of the *Rv3789* gene from *Mtb* but also upon that of the undecaprenyl phosphate aminoarabinose flippase *arnE/F* genes from *E. coli*. Interestingly, our studies on Rv3789 also served to establish for the first time that, similar to capsular polysaccharide, lipopolysaccharide, glycoprotein and PG synthesis in other prokaryotic organisms (Whitfield, 2006; Marolda *et al*., 2006; Sperandeo *et al*., 2009; Clarke *et al*., 2009), the biogenesis of AG in mycobacteria most likely relied on multiprotein complexes made of transporters and biosynthetic enzymes for efficient elongation and export. Indeed, Rv3789 was found to physically interact with the galactosyltransferase GlfT1, likely accounting for the membrane association of this enzyme and the dramatically reduced polymerization rate of the galactan domain of AG in the *Rv3789* knock-out mutant (Larrouy-Maumus *et al*., 2012). Alternatively, assuming that lipid-linked AG precursors need to undergo concurrent galactosylation and arabinosylation in the process of elongation and extrusion across the plasma membrane, one may hypothesize that the decreased arabinosylation activity of the mutant consecutive to the disruption of *Rv3789* negatively impacts the galactosylation rate of the precursors on the cytoplasmic face of the plasma membrane. In spite of its pivotal role in the biogenesis of the arabinan domains of AG and LAM, Rv3789 is not essential for the growth of *M. smegmatis* and *Mtb* indicating that multiple transporters with overlapping Dec-P-Ara flipping activities exist in mycobacteria.

Attachment of AG to PG: The last step in the biosynthesis of AG and PG is the covalent attachment of these two macromolecules via phosphoryl-*N*-acetylglucosaminosyl-rhamnosyl linkage units **[Fig. 3]**. The ligase(s) responsible for the formation of a 1-*O*-phosphoryl linkage between the GlcNAc residue of the linker unit of the mature AG and the 6-position of a MurNAc residue of PG has(have) not yet been identified. AG-PG ligation has been demonstrated in cell-free preparations of *M. smegmatis* (Yagi *et al*., 2003) and shown to require newly synthesized PG undergoing concomitant cross-linking (Hancock *et al*., 2002). Thus, ligation likely occurs either at the lipid II level, as seen for other PG modifications, or

while the nascent PG is being formed. The observation that a Dec-P-Ara-deficient (*ubiA* knock-out) mutant of *C. glutamicum* which is unable to synthesize the arabinan domain of AG was viable and still capable of producing a simplified cell wall consisting of the galactan chain of AG attached to PG suggests that neither the arabinosylation of AG or its mycolylation are prerequisites for its attachment to PG (Alderwick *et al*., 2005; Alderwick *et al*., 2006b).

Biological significance of the minor covalent modifications of AG: The biological significance of the galactosamine and succinyl residues esterifying some of the interior branched (3,5)-Ara*f* residues of AG is at present not known. It has been proposed that the protonated GalN ($GalNH_3^+$) interacts with the negatively charged succinyl residues leading to a more rigid and tightened AG structure (Bhamidi *et al*., 2008). The apparent lack of succinylation on the mycolylated arabinan chains (Bhamidi *et al*., 2008) could further suggest that succinylation negatively controls mycolylation. This possibility, however, needs to be considered with care given that the succinyl group is rather far from the site of mycolylation and succinylation might follow mycolylation rather than precede it. The possibility has also been raised that the protonated GalN ($GalNH_3^+$) interacts with anionic substances such as phosphates of glycero(glyco)lipids and the phosphatidyl-*myo*-inositol anchor of LM and LAM (Draper *et al*., 1997) thereby potentially affecting the organization of these compounds in the OM and the way they interact with the host in the course of infection. Our recent work involving wild-type *Mtb* versus isogenic GalN-deficient mutants (Škovierová *et al*., 2010) with human peripheral blood monocyte-derived dendritic cells (PBM-DCs) provides support for this hypothesis. Indeed, these studies have shown that the presence of the GalN substituent on AG abrogates a complete maturation/activation DC phenotype (as determined by decreased CD80/86, CD40 and HLA-DR expression) and stimulates increased IL-10 secretion while showing no difference in initial interaction and phagocytosis of the bacilli (W. Wheat, R. Dhouib, S. Angala, M. Jackson, unpublished results). Since purified AG from either wild-type or GalN-deficient mutants do not alter human DC maturation, it is therefore postulated that GalN may impose a topological modulation of the *Mtb* cell surface that provides better access to DC-SIGN or perhaps other receptors such as mannose receptor (MR) on macrophages and DCs preventing maturation signaling and resulting in the down-regulation of the initial immune response. More studies aimed at testing these hypotheses are in progress.

AG biosynthesis in the context of drug discovery: Ethambutol (EMB) has been known as an effective anti-TB drug since the early days of chemotherapy and is now a component of the ‘short-course chemotherapy’ involving isoniazid, rifampicin, pyrazinamide and EMB. EMB inhibits the synthesis of the arabinan domains of LAM and AG through the inhibition of the Emb arabinosyltransferases EmbA, EmbB and EmbC (Belanger *et al*., 1996; Goude *et al*., 2009). This observation and the pivotal roles played by other glycosyltransferases of the GT-C superfamily in the biosynthesis of AG has stimulated the design of innovative assays for inhibitor screening against these enzymes (Zhang *et al*., 2010; Zhang *et al*., 2011). In the last five years, whole cell-based screening of compounds against *Mtb* has produced several inhibitors of the essential epimerase DprE1 required for the formation of Dec-P-Ara (Makarov *et al*., 2009; Christophe *et al*., 2009; Magnet *et al*., 2010; Stanley *et al*., 2012;

Wang *et al*., 2013). The molecular mechanism of action of some of these compounds has been thoroughly investigated (Trefzer *et al*., 2010; Trefzer *et al*., 2012; Neres *et al*., 2012; Batt *et al*., 2012) and several Dec-P-Ara inhibitors are now reported to be in the hit-to-lead or pre-clinical development phases (Jackson *et al*., 2013; http://www.newtbdrugs.org). Other ongoing approaches to AG inhibition consist of targeting the synthesis of Dec-P (the common lipid carrier in the biosynthesis of AG, PG and other major cell envelope glycoconjugates) as described in the previous (PG) section. Finally, recent studies have shown that the caprazamycin derivative CPZEN-45 which displays potent activity against *Mtb in vitro* (MIC of 0.2 to 1.5 μg/ml) is an inhibitor of the decaprenyl-phosphate-GlcNAc-1-phosphate transferase WecA, which catalyzes the first committed step in the biosynthesis of AG (Ishizaki *et al*., 2013).

Phosphatidylinositol mannosides and lipoglycans

Structures of PIM, LM and LAM—Mannosyl-phosphatidyl-*myo*-inositol-based glycolipids **(PIM)** and related lipoglycans comprising lipomannan (LM) and lipoarabinomannan (LAM) are found in abundant quantities in the cell envelope of mycobacteria and closely related Actinomycetes. PIMs, LM and LAM are non-covalently-linked components of the cell envelope. They are anchored in the inner and outer membranes via their phosphatidyl-D-*myo*-inositol unit (Ortalo-Magné *et al*., 1996; Pitarque *et al*., 2008). The existence of mannosylated phosphoglycolipids now known as the phosphatidylinositol mannosides (PIM) in mycobacteria has been known since the 1930s (Anderson and Roberts, 1930). Structural studies by a number of investigators have since established the complete structures of mono-, di-, tri-, tetra-, penta- and hexamannoside variants of these lipids in *Mtb*, *M. bovis* BCG, *M. smegmatis* and *M. phlei* (for reviews, Gilleron *et al*., 2008; Guerin *et al*., 2010). The basic core of PIMs consists of an acylated *sn*-glycerol-3-phospho-(1-D-*myo*-inositol) moiety (phosphatidyl-*myo*-inositol; PI) further glycosylated at the C-2 and C-6 positions of *myo*-inositol with one to six mannopyranose (α-D-Man*p*) residues [Fig. 5]. Their structures are extremely diverse with respect to the number and position of acylations they carry (C16:0, C18:0, C18:1, and C19 tuberculostearic acid are the major fatty acid forms found in PIM). The two most common forms of PIMs found in all mycobacterial species, are the tri- and tetraacylated PIM_2 and PIM_6 [Fig. 5]. Triacylated-PIMs (Ac_1PIM_2/Ac_1PIM_6) harbor two fatty acyl chains on the glycerol moiety (usually C16:0 and C19) and an additional acyl chain linked either to the C-6 position of the Man*p* residue linked to C-2 of *myo*-inositol or to C-3 position of *myo*-inositol. Tetraacylated-PIMs (Ac_2PIM_2/Ac_2PIM_6) are acylated on both sugar residues (Gilleron *et al*., 2001; Gilleron *et al*., 2003). The complete structural analysis of acylated PIMs from *M. bovis* BCG has been determined using advanced mass spectrometric approaches (Gilleron *et al*., 2006a, Hsu *et al*., 2007a, Hsu *et al*., 2007b).

Suggestive of a metabolic relationship with PIMs, the reducing end of LM and LAM consists of PI wherein the *myo*-inositol residue is mannosylated at positions C-2 and C-6 and the glycerol moiety, *myo*-inositol and Man*p* residue linked to C-2 of *myo*-inositol are esterified with similar fatty acyl chains as in PIMs (Chatterjee *et al*., 1992a; Hunter and Brennan, 1990; Khoo *et al*., 1995a; Gilleron *et al*., 2006b; Nigou *et al*., 1997) [Fig. 6]. LM and LAM share a common linear α-(1,6)-linked mannan backbone made up of 20-25 Man*p*

residues elaborated by α-(1,2)-monomannose side chains. Our most recent structural data indicate that a stretch of uninterrupted linear α-6-linked mannosyl units attached to the inositol unit precedes the occurrence of contiguously occurring α-(1,2)-monomannose branches on the main chain (D. Kaur *et al*., manuscript in preparation). The major LAM glycoforms contain about 110 glycosyl residues (approximately 60 Ara*f* and 50 Man*p* units) and consist of what appears to be a single D-arabinan chain attached to the α-(1,6) D-mannan backbone through an α-(1,2) linkage in *Mtb* (Chatterjee *et al*., 1993; D. Kaur *et al*., manuscript in preparation; S. K. Angala, manuscript in preparation). Our recent structural analyses confirmed this α-(1,2) linkage in LAM purified from *M. smegmatis* (S. K. Angala *et al*., unpublished results). The D-arabinan portion of LAM is very similar to that of AG in that the same linkages of Ara*f* units are found and both structures share an Ara_{18} motif extending from the α–(3,5)-Ara*f* interior residues (Shi *et al*., 2006; Bhamidi *et al*., 2008) [Fig. 6]. However, the D-arabinan structure of LAM has been found to be more variable than that of AG in terms of the length of this particular motif (Ara_{18}- Ara_{22}) (Shi *et al*., 2006). Further, in contrast to the presence of two arabinan chains per molecule of AG (Bhamidi *et al*., 2011), LAM seems to carry a single arabinan domain (Kaur D. *et al*., manuscript in preparation). Other distinctive features of the D-arabinan of LAM are found in its non-reducing termini which, in addition to the branched Ara_6 motif found in AG, may consist of linear Ara_4 [Fig. 6]. LAM further displays considerable structural micro-heterogeneity at its non-reducing arabinan termini. While in slow-growing mycobacterial species such as Mtb, M. leprae, M. avium, M. bovis, M. kansasii, M. xenopi, M. marinum and *M. bovis* BCG, these termini are capped with one to three α-(1,2)-Man*p*-linked residues giving rise to mannosylated LAM (known as ManLAM) (Gilleron *et al*., 2008), the LAM of fast-growing species may either be capped with phospho-inositol (yielding PILAM) as in *M. smegmatis* and *M. fortuitum* (Khoo *et al*., 1995b) or not carry any capping motifs (AraLAM) as in *M. chelonae* (Guérardel *et al*., 2002) [Fig. 6]. More recently, some of the Man*p* caps of *Mtb* ManLAM were found to be decorated with an α-(1,4)-linked methyl-thio-D-xylose (MTX) residue (Treumann *et al*., 2002, Ludwiczak *et al*., 2002; Turnbull *et al*., 2004, Joe *et al*., 2006; Turnbull and Stalford, 2012). Interestingly, the same MTX motif was found in *M. kansasii* ManLAM but attached to the mannan backbone rather than to the Man*p* caps (Guérardel *et al*., 2003). Finally, the arabinan chains of ManLAM from *Mtb* and *M. bovis* BCG may be substituted with lactate or succinate residues at the C-2 position of the α–(3,5)-Ara*f* interior residues (Hunter *et al*., 1986, Delmas *et al*., 1997).

Biosynthesis of PIM, LM and LAM

(a) PIM biosynthesis: Since the publication of the first genome sequence of *Mtb* in 1998 (Cole *et al*., 1998), major efforts have been committed to defining the molecular bases of the biosynthesis of apolar PIMs. Using a combination of biochemical assays, recombinant genetic approaches and structural biology, the gene products of *pimA* (Rv2610c in *Mtb* H37Rv), *pimB'* (Rv2188c) and *Rv2611c* were defined as the enzymes involved in the cytoplasmic synthesis of apolar forms of PIMs (PIM_1, PIM_2, Ac_1PIM_1, Ac_1PIM_2, Ac_2PIM_1, Ac_2PIM_2) (for a review, Guerin *et al*., 2010) [Fig. 7]. Work from our laboratory defined the first mannosylation step involved in the biosynthesis of PIMs; we showed that the mannosyltransferase (ManT) PimA transfers a Man*p* residue from GDP-Man*p* to the C-2 position of the *myo*-inositol ring of PI to form PIM_1 on the cytosolic face of the plasma

membrane (Korduláková *et al*., 2002; Guerin *et al*., 2009a). PimA is an essential enzyme in *M. smegmatis* and *Mtb*. **Several structures** of PimA under its *apo* form and in complex with GDP and GDP-Man*p* have been reported and its peripheral interactions with the plasma membrane and conformational changes undergone during catalysis deciphered (Guerin *et al*., 2007; Guerin *et al*., 2009b; Giganti *et al*., 2013; Abesa-Jove *et al*., 2014). The second ManT of the pathway is a GDP-Man*p*-dependent ManT named PimB' (Rv2188c in *Mtb* H37Rv) that transfers a single Man*p* residue to the C-6 position of the *myo*-inositol ring of PIM_1 to form PIM_2 (Lea-Smith *et al*., 2008; Guerin *et al*., 2009a). Unlike *M. smegmatis pimB'* knock-out mutants, *pimB'* null mutants of *C. glutamicum* were found to be viable despite their loss of ability to produce PIM_2, LM and LAM (Lea-Smith *et al*., 2008, Mishra *et al*., 2008a; Guerin *et al*., 2009a). Crystal structures of PimB' from *C. glutamicum* in complex with GDP and GDP-Man were reported (Batt *et al*., 2010). The attachment of an acyl chain to the C-6 position of the Man*p* residue linked to C-2 of *myo*-inositol in PIM_1 and PIM_2 is catalyzed by an acyltransferase encoded by *Rv2611c* in *Mtb* H37Rv (Korduláková *et al*., 2003). Rv2611c is an essential enzyme in *Mtb* (Barilone *et al*., unpublished results). Its disruption is achievable in *M. smegmatis* but leads to severe growth defects (Korduláková *et al*., 2003). Assays using purified PimA and PimB' proteins indicated that Rv2611c favors PIM_2 over PIM_1 as a substrate (Guerin *et al*., 2009a). PimC (RvD2-*ORF1* in *Mtb* CDC1551), a non-essential GDP-Man*p*-dependent ManT present in only some *Mtb* isolates, catalyzes the formation of Ac_1PIM_3 from Ac_1PIM_2 (Kremer *et al*., 2002). The identity of the analogous PimC enzyme in *Mtb* isolates lacking an ortholog of RvD2-*ORF1* is at present not known and nor is that of the acyltransferase catalyzing the transfer of an acyl group to C-3 of the *myo*-inositol ring. Assuming that the third conserved and possibly essential ManT of the PIM pathway is also a GDP-Man*p*-utilizing ManT, it is likely that the synthesis of Ac_1PIM_3 (and perhaps Ac_2PIM_3) is completed on the cytoplasmic face of the plasma membrane. Once synthesized, these PIM products are thought to be flipped to the periplasmic face of the plasma membrane by an as yet unknown transporter in order to serve as substrates for further integral membrane ManTs of the GT-C superfamily reliant on polyprenyl (C_{35}/C_{50})-monophospho-mannose rather than GDP-Man*p* as the Man*p* donor (Berg *et al*., 2007). Heptaprenyl- and decaprenyl-monophospho-mannose (Dec-P-Man) are synthesized from GDP-Man*p* and polyprenyl phosphates by the polyprenol monophospho-mannose synthase encoded by *ppm1* (*Rv2051c*) in *Mtb* (Gurcha *et al*., 2002). Ppm1 is an essential enzyme in both *Mtb* and *M. smegmatis* (Zhang *et al*., 2012; Rana *et al*., 2012). The ManT (PimD) responsible for the formation of tetra-mannosylated forms of PIMs from Ac_1PIM_3/Ac_2PIM_3 is not known. The addition of two α-(1,2)-linked Man*p* residues to Ac_1PIM_4/Ac_2PIM_4 leads to the synthesis of higher order forms of PIMs commonly referred to as "polar PIMs" [Fig. 5]. PimE was identified as the Dec-P-Man-dependent α-(1,2) ManT responsible for the formation of Ac_1PIM_5/Ac_2PIM_5 from Ac_1PIM_4/Ac_2PIM_4 (Morita *et al*., 2006) [Fig. 7]. Whether this enzyme can also transfer a second α-(1,2)-linked Man*p* to Ac_1PIM_5/Ac_2PIM_5 to form Ac_1PIM_6/Ac_2PIM_6, the end products of the PIM pathway, or whether another enzyme participates in this process is currently not known but our preliminary enzyme assays with purified PimE favor the second hypothesis (Larrouy-Maumus *et al*., unpublished results).

(b) Biosynthesis of LM and LAM: Ac_1PIM_4/Ac_2PIM_4 appear to be the last common intermediates in the biosynthesis of PIM, LM and LAM. Extension of this subpopulation of PIMs with chains of α-(1,6)-linked Man*p* and further modification with α-(1,2)-monomannose side chains lead to the formation of LM [Fig. 7]. LpqW (Rv1166) is a putative lipoprotein involved in regulating access of Ac_1PIM_4/Ac_2PIM_4 to either PimE (to form polar PIMs) or the α-(1,6) ManTs responsible for the elongation of LM. An *M. smegmatis lpqW* knock-out mutant produced wild-type forms of PIMs but had a reduced capacity to synthesize LM and LAM (Kovacevic *et al*., 2006). This mutant was found to be unstable and to accumulate secondary mutations in *pimE* that resulted in a block in the synthesis of polar PIMs and restored synthesis of LM and LAM (Crellin *et al*., 2008). Determination of the three dimensional structure of LpqW from *M. smegmatis* revealed the existence of a putative Ac_1PIM_4 binding site (Marland *et al*., 2006) suggesting that this protein may function as a glycolipid chaperone, regulating the access of ManTs to this substrate. Recent genetic and enzymatic studies conducted on an *lpqW* (*NCgl1054*) knock-out mutant of *C. glutamicum* now suggest that LpqW may in fact regulate the activity of the α-(1,6) ManT, Cg-MptB, involved in the initial steps of the elongation of LM from tetramannosylated PIMs (Rainczuk *et al*., 2012).

The extension of Ac_1PIM_4/Ac_2PIM_4 by Dec-P-Man-dependent α-(1,6) ManTs on the periplasmic face of the plasma membrane leads to the biosynthesis of LM. The first committed enzyme in this process was identified in *C. glutamicum* as Cg-MptB (NClg1505) (Mishra *et al*., 2008b) but the corresponding enzyme in *Mtb* has not yet been identified. Indeed, the closest *Mtb* H37Rv ortholog, Rv1459c (which shares about 35% identity with Cg-MptB), failed to complement a *C. glutamicum mptB* knock-out mutant and disruption of the orthologous gene in *M. smegmatis* (*MSMEG_3120*) had no effect on LM and LAM biosynthesis. The GT-C glycosyltransferase Rv1459c thus appears to have a distinct, albeit as yet unknown, function in *Mtb*. MptA (Rv2174 in *Mtb* H37Rv) was characterized as a GT-C superfamily Dec-P-Man-dependent α-(1,6) ManT responsible for the elongation of the mannan backbone of LM in mycobacteria and corynebacteria (Kaur *et al*., 2007; Mishra *et al*., 2007). Disruption of *mptA* (*MSMG_4241*) in *M. smegmatis* leads to a phenotype marked by a virtual absence of LM and LAM and a build-up of truncated forms of LM in the mutant strain with only 5 to 20 Man*p* residues as compared to wild-type LM consisting of 21-34 Man*p* residues, but with only few changes in the branching pattern (Kaur *et al.*, 2007). The Dec-P-Man-dependent α-(1,2) ManT involved in the branching of LM was identified as Rv2181 (MSMEG_4247 in *M. smegmatis*) (Kaur *et al*., 2006; Kaur *et al*., 2008). Interestingly, while the disruption of *Rv2181* in *Mtb* abrogates the monomannose branching of the mannan backbone of LM and LAM (Kaur *et al*., 2008), knocking-out the orthologous gene in *M. smegmatis* leads to a mutant unable to synthesize LM and producing a shorter LAM devoid of α-(1,2) branches (Kaur *et al*., 2006). By modulating the level of expression of *MSMEG_4241* (*mptA*) and *MSMEG_4247* in *M. smegmatis*, it was shown that the elongation and branching of the mannan backbone of LM and LAM are tightly coordinated, with the overexpression of *MSMEG_4247* leading to the synthesis of dwarfed LM and LAM presenting a shorter mannan backbone as well as a significantly smaller arabinan domain in the case of LAM (Sena *et al*., 2010). Our recent structural analyses of the dwarfed LAM produced by a *M. smegmatis MSMEG_4247* knock-out strain (Kaur *et al*., 2006) revealed a

single arabinosylation site on the mannan backbone (D. Kaur *et al*., manuscript in preparation). The priming arabinosyltransferase (AraT) responsible for the transfer of the first Ara*f* residue of LAM has not yet been identified although we were able to detect a matching enzymatic activity in cell-free extracts prepared from *M. smegmatis* (S. K. Angala *et al*., manuscript in preparation). Dec-P-Ara being the only known Ara*f* donor in mycobacteria (Wolucka *et al*., 1994), it is expected that all of the arabinosylation of LAM, like that of AG, takes place on the periplasmic side of the plasma membrane catalyzed by integral membrane AraTs of the GT-C superfamily of glycosyltransferases (Berg *et al*., 2007) [Fig. 7]. One of these enzymes, known as EmbC, was found to be critical in this process (Zhang *et al*., 2003; Berg *et al*., 2005; Shi *et al*., 2006). EmbC is an essential enzyme in *Mtb* (Goude *et al*., 2008) where it serves as one of the targets of the TB drug EMB (Goude *et al*., 2009). The knock-out of *embC*, however, is achievable in *M. smegmatis* resulting in a mutant deficient in LAM synthesis (Zhang *et al*., 2003). EmbC is predicted to carry 13 transmembrane spanning helices followed by an hydrophilic extracytoplasmic carbohydrate-binding C-terminal domain. Like EmbA and EmbB involved in AG biosynthesis, EmbC harbors a proline-rich motif homologous to that of bacterial polysaccharide co-polymerases (Berg *et al*., 2007). Consistently, biochemical analyses of *M. smegmatis* recombinant strains expressing truncated and point-mutated variants of EmbC indicated that this protein is most likely multi-functional possessing polymerization and chain length regulating functions in addition to AraT activity (Berg *et al*., 2005; Shi *et al*., 2006). The C-terminal domain of EmbC was recently co-crystallized with a synthetic di-arabinoside acceptor substrate (Alderwick *et al*., 2011b). Another critical AraT in the biosynthesis of LAM is the α-(1,3)-branching AraT AftC. Disruption of *aftC* in *M. smegmatis* results in a mutant producing a truncated form of LAM whose arabinan domain is devoid of (3,5)-Ara*f*-branching residues (Birch *et al*., 2010). Thus, AftC participates in the branching of the arabinan domains of both AG and LAM (Birch *et al*., 2008). Evidence based on the analysis of the cell envelope content of *M. smegmatis* recombinant strains expressing different levels of *aftD* (*Rv0236c*) suggests that this essential GT-C enzyme, which displays α-(1,3)-branching AraT activity on synthetic α-(1,5) arabinosyl acceptors *in vitro*, may also participate in the synthesis of the arabinan domains of both AG and LAM although its precise function remains to be determined (Škovierová *et al*., 2009). The presence of two to three Ara*f* residues attached to the mannan backbone of LM in the *embC* knock-out mutant of *M. smegmatis* supports the existence of a “priming” AraT activity independent of EmbC (Zhang *et al*., 2003); the corresponding enzyme has not yet been identified.

The ManT responsible for transferring the first Man*p* residue of the mannoside caps of ManLAM to the non-reducing termini of the arabinan domain was identified as Rv1635c (also known as CapA) (Dinadayala *et al*., 2006). The further elongation of the mannoside cap with at least one Man*p* residue requires the promiscuous α-(1,2) ManT Rv2181 which is also responsible for the monomannoside branching of LM (Kaur *et al*., 2008) [Fig. 7]. The enzymes required for the biosynthesis and transfer of a MTX motif to the *t*-Man*p* residue of the mannoside caps of ManLAM have not yet been identified although biosynthetic models have recently been proposed (Turnbull and Stalford, 2012). Likewise, the biosynthetic origin of the succinyl residues linked to the arabinan domain of LAM remains to be determined.

Regulatory mechanisms: As illustrated above, the biosynthetic steps leading to the formation of PIMs, LM and LAM are highly complex and tightly coordinated to ensure the production of appropriate levels of fully elaborated molecules. Although our current understanding of PIM/LM/LAM biosynthesis suggests the existence of multiple points of controls of these pathways, our knowledge of the regulatory mechanisms involved is still limited. EmbR, a protein homologous to the OmpR class of transcriptional regulators, was implicated in the positive regulation of the *embCAB* operon and LM/LAM biosynthesis (Belanger *et al*., 1996; Sharma *et al*., 2006a; Alderwick *et al*., 2006c). EmbR is phosphorylated by the Ser/Thr kinase PknH, enhancing its binding to the promoter region of *embCAB* (Molle *et al*., 2003). EmbR is also phosphorylated by the Ser/Thr kinases PknA, PknB and PknJ and is dephosphorylated by the Ser/Thr phosphatase PstP (Sharma *et al*., 2006b; Molle & Kremer, 2010; Jang *et al*., 2010). Beyond these transcriptional and post-translational aspects, the compartmentalization of the PIM, LM and LAM pathways is expected to play an important role in regulating the access of enzymes to their substrates (Morita *et al*., 2011). Finally, elegant work on LpqW has highlighted the critical role of this protein in regulating the activity of the ManTs acting at the juncture of the polar PIM and LM elongation pathways (Kovacevic *et al*., 2006; Crellin *et al*., 2008; Rainczuk *et al*., 2012).

Topology of the PIM/LM/LAM biosynthetic pathway: As in the case of AG, the fundamentals of how the different domains of LAM are assembled and exported are at present not fully understood. As outlined in the preceding sections, the early steps of PIM biosynthesis take place on the cytosolic face of the plasma membrane until di- or tri-mannosylated forms of PIMs are translocated across the plasma membrane to serve as substrates for further mannosylation reactions catalyzed by PimE and other GT-C polyprenyl-phosphate mannose-dependent glycosyltransferases [Fig. 7]. Since the unassisted transbilayer movement of polar (glyco)lipids across the plasma membrane is energetically unfavorable (Daleke *et al*., 2007; Sanyal *et al*., 2008) such a compartmentalization implies that an as yet unknown translocase (or “flippase”) translocates PIM intermediates from the cytoplasmic to the periplasmic side of the plasma membrane. Evidence to date then points to LM and LAM elongation proceeding through the sequential addition of mannosyl and arabinosyl residues to a PIM_4 substrate from the reducing towards the non-reducing end on the periplasmic face of the plasma membrane. Our recent studies have established the SMR-like transporter Rv3789 as a likely Dec-P-Ara translocase required for the optimal arabinosylation of AG and LAM (Larrouy-Maumus *et al*., 2012) (see Arabinogalactan section). Finally, the export of PIM, LM and LAM from the inner membrane to the outer membrane and cell surface most likely requires dedicated translocation machineries but none of the components of this(ese) machineries have yet been formally identified. Evidence based on physical interactions and co-crystallography suggests that the lipoprotein LprG (Rv1411c) which shares structural resemblance to LppX, a lipoprotein thought to carry phthiocerol dimycocerosates (PDIM) across the periplasm (Sulzenbacher *et al*., 2006), may participate in the transport of PIM, LM and LAM to the cell surface (Drage *et al*., 2010) [Fig. 7]. Further biochemical studies are required, however, to confirm the involvement of LprG in this process and precisely delineate its substrate specificity.

Physiological functions and biological activities of PIM, LM and LAM: PI and PIMs make up as much as 56% of all phospholipids in the cell wall and 37% of those in the plasma membrane of *M. bovis* BCG (Goren, 1984) and are thus important structural components of the mycobacterial cell envelope. Emerging data indicate that PIM not only play important roles in the permeability of the cell envelope but also in inner 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). The dramatic changes in β-lactam susceptibility and acid-fast staining properties of mycobacterial cells that accompany structural defects in LM and ManLAM indicate that these lipoglycans play equally important roles in cell envelope integrity, also impacting the pathogenicity of *Mtb* (Fukuda *et al*., 2013). The amount of higher order PIMs (PIM_5-PIM_6) recovered from *M. smegmatis* cells increases with the age of the culture, apparently at the expense of the apolar forms (PIM_1 –PIM_4) and LAM, the synthesis of which was shown to decrease in *M. smegmatis* as the bacilli approached stationary phase (Penumarti and Khuller, 1983; Morita *et al*., 2005; Dhiman *et al*., 2011). Important changes affecting the amounts and structures of PIMs and LAM were also reported to occur in *Mtb* during *in vitro* growth (Yang *et al*., 2013). The physiological significance of these changes is not known.

In addition to their physiological and structural roles, a substantial number of biological activities have been associated with PIMs, LM and LAM. These have been the object of several reviews (Briken *et al*., 2004; Gilleron *et al*., 2008; Torrelles and Schlesinger, 2010; Mishra *et al*., 2011; Neyrolles and Guilhot, 2011) and we will only summarize here some of the most significant findings that have occurred in the field in the last couple of years. The contribution of PIMs, LM and LAM to the infection process accompanies virtually every step of the lifecycle of *Mtb* inside the host. The fact that even subtle variations in the structures of these molecules (including their degree of acylation and mannan branching, the lengths of the mannan and arabinan chains, and the nature of the substituents capping the non-reducing end of the arabinan domain) dramatically impact their biological activities (Gilleron *et al*., 2001; Gilleron *et al*., 2006; Gilleron *et al*., 2008; Nigou *et al*., 2008; Torrelles and Schlesinger, 2010; Mishra *et al*., 2011; Stoop *et al*., 2013) suggests that they are probably important modulators of host-pathogen interactions in the course of infection. The mannoside caps of ManLAM bind to the mannose receptor (MR) thereby contributing to the phagocytosis of *Mtb* by human macrophages. The mannoside caps of ManLAM also bind to the C-type lectin DC-SIGN present on dendritic cells (DCs) resulting in anti-inflammatory effects that have been proposed to contribute to the immune evasion of *Mtb*. LM, in contrast, associates with DC-SIGN but not MR. Contrary to earlier impressions that ManLAM dominated the interactions of *Mtb* with antigen-presenting cells, however, *Mtb* and *M. bovis* BCG mutants deficient in the mannose-capping of LAM showed no impairment in DC-SIGN binding, interactions with macrophages *in vitro*, virulence in mice and immunogenicity (Appelmelk *et al*., 2008; Afonso-Barroso *et al*., 2012). The presence of multiple other MR and DC-SIGN ligands at the cell surface of *Mtb*, including glycoproteins (see Glycoproteins section), polar forms of PIMs (PIM_5-PIM_6) and capsular polysaccharides (arabinomannan, mannan) (see Capsular Polysaccharides section) sharing with ManLAM terminal α–(1,2)-linked oligomannoside appendages, is thought to account for this absence of phenotype. Polar forms of PIMs in particular have been shown to participate in DC-SIGN

binding (Torrelles *et al*., 2006; Driessen *et al*., 2009) and phagocytosis events through the MR limiting phagosome-lysosome fusion. In addition to C-type lectins, *Mtb* interacts with Toll-like receptors (TLR); LM and, to a lesser extent, ManLAM have been shown to be potent TLR-2 ligands (Nigou *et al*., 2008). Once inside phagocytic cells, *Mtb* resides in a phagosome that fails to fuse with lysosomes. The ability of ManLAM and/or derived products to intercalate within host cell membranes was proposed as a possible mechanism through which these molecules may impair phagosome maturation (Welin *et al*., 2008). Inside the cells, *Mtb* releases significant amounts of cell envelope components among which PIMs, LM and ManLAM that traffic within the cells and may be released through exocytosis (Russell, 2011). These molecules may not only be taken up by bystander antigen-presenting cells, they also act as modulators of the functions of the host cell and surrounding tissue required for granuloma formation and protection. ManLAM for instance is known to negatively modulate the production of nitric oxide, oxygen radicals and inflammatory cytokines by macrophages and DCs, inhibit *Mtb*-induced apoptosis, and interfere with signaling pathways of T-lymphocytes affecting cytokine production (Shabaana *et al*., 2005) and cell migration (Richmond *et al*., 2012). Thus, whereas PI-LAM is generally considered to induce pro-inflammatory responses, ManLAM has anti-inflammatory effects. LM, on the other hand, induces apoptosis and a pro-inflammatory response through TLR-2. Preliminary studies employing purified MTX and MTX-Man disaccharide have begun to investigate the possible biological roles of the α-D-methylthioxylofuranosyl (MTX) substituent of ManLAM. It was found that MTX-Man has immunomodulatory properties inhibiting the production of TNF-α and IL-12p70 by activated human THP-1 monocytes (Joe *et al*., 2006; Turnbull and Stalford, 2012). MTX and its sulfinyl analog (α-D-methyl-sulfinyl-xylofuranosyl; MSX), on the other hand, have the ability to sequester hydroxyl radicals thereby potentially promoting the intracellular survival of *Mtb* (Turnbull and Stalford, 2012). The extent to which these properties associated to the MTX and MSX substituents of LAM impact the pathogenesis of *Mtb* remains to be determined; such experiments will have to await the construction of isogenic mutants deficient in the biosynthesis of these motifs. As in the case of AG, the biological significance of the succinyl residues esterifying some of the interior branched (3,5)-Ara*f* residues of LAM (Delmas *et al.*, 1997) is not known. Finally, in addition to their involvement in innate immune mechanisms, PIMs, LM and ManLAM are also recognized as antigens by the adaptative immune system upon presentation to T-lymphocytes by MHC-I-like molecules of the CD1 family. Biochemical and structural studies have begun to elucidate the molecular basis of their processing and presentation (Porcelli and Modlin, 1999; Fischer *et al*., 2004; de la Salle *et al*., 2005; Torrelles *et al*., 2004; Torrelles *et al*., 2011; Garcia-Alles *et al*., 2011; Torrelles *et al*., 2012; Cala-De Paepe *et al*., 2012).

In spite of their astonishing biological activities *in vitro*, the precise contribution of PIMs, LM and LAM to TB pathogenesis when carried by whole *Mtb* bacilli is far from being clear. The essentiality of much of the PIM/LM/LAM pathway for *Mtb* growth limits the number of informative isogenic mutants that can be generated for cellular and *in vivo* studies. Moreover, with the exception of one mutant dramatically affected in its cell envelope integrity (Fukuda *et al*., 2013), the few *Mtb* PIM/LM/LAM recombinant strains that have constructed thus far failed to significant differ from their wild-type parent strain in terms of

interactions with host cells, virulence or immunogenicity (Driessen *et al*., 2009; Afonso-Barroso *et al*., 2012). The presence of multiple glycoconjugates with partially overlapping activities at the surface of *Mtb*, the genetic diversity of *Mtb* isolates and the existence of regulatory mechanisms affecting the production of these compounds render the precise delineation of the roles of PIMs, LM and LAM in host-pathogen interactions extremely complex (Pitarque *et al*., 2005; Appelmelk *et al*., 2008; Driessen *et al*., 2009; Torrelles and Schelsinger, 2010; Afonso-Barroso *et al*., 2012).

PIM, LM and LAM biosynthesis in the context of drug discovery and biomarker development: The essential character of PIMs, LM and LAM, their restricted distribution to mycobacteria and closely-related Actinomycetes, and demonstrated impact on the structure and permeability of the cell envelope of *Mtb* make the biosynthetic enzymes of these molecules attractive candidates for the development of specific *Mtb* inhibitors with the potential to synergize with or potentiate the activity of other drugs used in combination. Accordingly, target-to-drug approaches are pursuing various essential enzymes acting at early (e.g., PimA, PimB') or late (e.g., Emb proteins and other lipid-linked sugar-utilizing glycosyltransferases) stages of the pathway (Zhang *et al*., 2010; Zhang *et al*., 2011). The most advanced LAM inhibitors to date (apart from EMB) are those targeting Dec-P-Ara synthesis as described earlier (see AG section). Beyond these therapeutic applications, LAM is also actively being pursued as a potential biomarker to monitor TB infection as well as the efficacy of treatments and vaccination (Wallis, 2013). Finally, the potent bioactivity of the α–(1,2)-linked oligomannoside appendages found in ManLAM, mannoproteins, polar forms of PIMs and capsular polysaccharides has stimulated innovative approaches toward the development of synthetic immunomodulators for the treatment of lung inflammatory diseases (Blattes *et al*., 2013).

Acyltrehaloses

Trehalose and acyltrehaloses in Mtb—Trehalose is a non-reducing disaccharide of glucose (1-*O*-α-D-glucopyranosyl-α-D-glucopyranoside) found in bacteria, yeast, fungi, plants and invertebrates, but not in mammalian cells. In mycobacteria, it serves as biosynthetic precursor for a range of glycolipids that populate both the inner and outer membranes of the cell envelope. The acyltrehaloses found in the cell envelope of *Mtb* include trehalose monomycolates (TMM), trehalose dimycolates (TDM), sulfolipids (SL), diacyltrehaloses (DAT), triacyltrehaloses (TAT) and polyacyltrehaloses (PAT) [Fig. 8]. In addition, *M. canettii*, a representative of smooth tubercle bacilli that seems to have originated from the same pool of ancestors as *Mtb* but rarely causes human disease (Supply *et al*., 2013) produces trehalose-based lipooligosaccharides (LOS) (Daffé *et al.*, 1991). A characteristic feature of DAT, TAT, PAT and LOS is the presence of long-chain multi-methyl branched fatty acids esterifying the trehalose moiety [Fig. 8]. These long chain methyl-branched fatty acids are produced by multifunctional polyketide synthases similar to the type I multienzyme fatty acid synthase (FAS-I) of eukaryotes. However, unlike FAS-I, these polyketide synthases preferentially use methyl-malonyl-CoA instead of malonyl-CoA for fatty acid elongation, thereby introducing methyl branches into fatty acyl chains.

Trehalose biosynthesis—Three different pathways have been described for the biosynthesis of trehalose. Most prokaryotes rely on the OtsA-OtsB pathway wherein OtsA is a **trehalose-6-phosphate** synthase catalyzing the condensation of glucose-6-phosphate and UDP-glucose to form trehalose-6-phosphate, and OtsB is a dephosphorylase releasing free trehalose from trehalose-6-phosphate (Kaasen *et al*., 1992). An alternative pathway that generates trehalose from glycogen has been identified in *Arthrobacter*, *Rhizobium* and *Sulfolobus acidocaldarius*. This pathway involves the TreY-TreZ enzymes in which the terminal α–(1,4)-linked residue of the glucose polymer is converted to an α–(1,1) linkage by the maltooligosyltrehalose synthase TreY. The terminal disaccharide is then cleaved by the hydrolase enzyme TreZ releasing trehalose (Maruta *et al*., 1996a, Maruta *et al.*, 1996b, Maruta *et al*., 1996c). Finally, a third pathway was found in *Pimelobacter* and *Arthrobacter* in which the trehalose synthase TreS **catalyzes the reversible isomerization of** the α-(1,4) linkage of maltose to the α-(1,1) linkage of trehalose (Nishimoto *et al*., 1996, Nakada *et al*., 1995). The *Mycobacterium* genus is unique in possessing all three pathways for the synthesis of trehalose (De Smet *et al*., 2000). While the three pathways are functionally redundant in *M. smegmatis* (Woodruff *et al*., 2004), the OtsAB pathway was found to be predominant in *Mtb* (Murphy *et al*., 2005). Disruption of *otsA* (*Rv3490*) in *Mtb* resulted in growth defects both *in vitro* and *in vivo* and *otsB2* (*Rv3372*) was demonstrated to be an essential gene of *Mtb* (Murphy *et al*., 2005). Furthermore, recent genetic and biochemical evidence (Kalscheuer *et al*., 2010; Miah *et al*., 2013) supported by structural data (Caner *et al*., 2013) indicates that TreS predominantly functions in the reverse orientation in *M. smegmatis* and *Mtb*, catalyzing the formation of maltose from trehalose which is then used in the biosynthesis of α-(1,4)-glucans (Kalscheuer *et al*., 2010) (see Capsular Polysaccharides section).

Trehalose monomycolates (TMM) and trehalose dimycolates (TDM)—These two glycolipids are produced by all mycobacterial species examined to date. In TMM, trehalose is esterified at the 6-position by a mycolic acid chain while TDM, also known as “cord factor”, is esterified at the 6- and 6’-positions by two mycolic acid chains [Fig. 8]. As indicated above, mycolic acids are long-chain (C_{60}-C_{90}) α-alkyl-β-hydroxy- fatty acids and are essential components of the mycobacterial outer membrane [Fig. 1]. The structure and biosynthesis of mycolic acids has been reviewed elsewhere (Barry *et al*., 1998; Takayama *et al*., 2005; Marrakchi *et al*., 2008). Upon elongation, modification and assembling in the cytosol, the completed mycolic acid chains are transferred to the 6- and 6’-positions of trehalose through an as yet unknown mechanism, generating **TDM**. TMM was recently shown to be the form under which mycolic acids are exported to the cell envelope in a process involving the integral membrane Resistance-Nodulation and Division (RND) superfamily transporter, MmpL3 (Grzegorzewicz *et al*., 2012). The genetic or chemical inactivation of MmpL3 causes the arrest of TMM translocation to the cell surface and cell death. Intriguingly, MmpL3 was identified as the target of a number of small molecules inhibitors with activity against *Mtb* bacilli in culture, including the TB drug candidate SQ109, pointing to the chemical vulnerability of this critical step of the formation of their OM (Grzegorzewicz *et al*., 2012; Stanley *et al*., 2012; La Rosa *et al*., 2012; Remuinan *et al.*, 2013; Ioerger *et al*., 2013; Poce *et al*., 2013; Konddredi *et al*., 2013; Onajole *et al*., 2013; Rao *et al*., 2013). The precise role of MmpL3 in TMM export, whether required to

translocate TMM across the plasma membrane ("flippase" activity) or to carry TMM from the outer leaflet of the plasma membrane to the periplasmic space or OM (intermembrane transport) remains to be determined. Based on what is known of the transport mechanism of RND transporters in Gram-negative bacteria (Paulsen *et al*., 1996; Tseng *et al*., 1999; Murakami, 2008), the hypothesis of an intermembrane translocation is favored. Either way, the complexity of translocating TMM across the different layers of the cell envelope suggests that MmpL3 probably functions with other membrane proteins, periplasmic adapters, lipoproteins and/or OM proteins to deliver TMM in the vicinity of the OM where the mycolic acyl chain carried by this glycolipid can then be transferred to another molecule of TMM to form TDM, or to AG. Identifying these other components of the TMM translocation machinery and understanding the substrate specificity and mechanism of transport of MmpL3 are just some of the gaps in our knowledge of the building of the mycobacterial OM that need to be addressed. The transfer of mycolic acids from TMM onto the non-reducing termini of the arabinan chains of AG or onto TDM is catalyzed by the mycolyltransferases of the Ag85 family (Ag85A, Ag85B and Ag85C). These enzymes appear to have partially redundant functions and, consistently, their individual genetic inactivation has no impact on the viability of *Mtb* (Jackson *et al*., 1999; Armitige *et al*., 2000; Puech *et al*., 2002). The reactions catalyzed by the Ag85 family of enzymes result in the periplasmic release of free trehalose as a by-product. The ABC transporter LpqY-SugABC recycles trehalose back to the cytoplasm in *Mtb* (Kalscheuer *et al*., 2011).

TDM has long been associated with the virulence of *Mtb.* It was first noticed in the 1950s that *Mtb* isolates subjected to surface lipid extraction, while retaining viability, became avirulent and unable to form cords (Bloch, 1950). This fraction was named "cord factor" and later shown to be composed primarily of TDM (Noll *et al*., 1956). A number of biological activities have been attributed to TDM over the years, as this glycolipid seems to be a major contributor of the inflammation seen in the course of mycobacterial infections. Purified TDM can by itself induce lesions characterized by chronic granulomatous in?ammation in mice and rabbits (Hamasaki *et al*., 2000; Sakaguchi *et al*., 2000). TDM also contributes to the protection of *Mtb* from killing by macrophages, is a potent modulator of the activation of macrophages and increases the resistance of mycobacteria to antibiotics (Silva *et al*., 1985; Katti *et al*., 2008; Axelrod *et al*., 2008; Indrigo *et al*., 2002, Indrigo *et al*., 2003). The study of defined *Mtb* knock-out mutants deficient in the modification of mycolic acids with cyclopropane rings and oxygenated functions has provided evidence of the impact of the fine structures of the mycolyl substituents of TDM on the biological activities of this glycolipid (Rao *et al*., 2005; Linares *et al*., 2012). Only recently have TDM receptors been identified at the surface of macrophages. The inducible C-type lectin Mincle (also called Clec4e) recognizes TDM but not other mycobacterial glycolipids such as PIM, LM or LAM **(Ishikawa *et al*., 2009)**; moreover, the binding of TDM to Mincle is required for activation of macrophages and granuloma formation in mice (Ishikawa *et al*., 2009; Lang, 2013). Mincle was shown to be essential for inflammation *in vivo* when purified TDM or heat-killed *Mtb* was administered to mice (Ishikawa *et al*., 2009; Schoenen *et al*., 2010). Nevertheless, *Mtb*-infected Mincle-deficient mice did not differ from control mice in their inflammatory response or ability to control the infection (Heitmann *et al*., 2013) suggestive of the existence of (an)other TDM receptor(s) at the surface of antigen-presenting cells. The

C-type lectin MCL (also called Clec4d) which is thought to have arisen from gene duplication of Mincle (Miyake *et al*., 2013) was recently identified as another TDM receptor. In contrast to Mincle, MCL is constitutively expressed in myeloid cells and is required for the development of TDM-induced acquired immune responses in mouse. TDM-induced granuloma formation is also severely impaired in MCL-deficient mice. Importantly, MCL appears to play a critical role in the induction of Mincle following TDM stimulation (Miyake *et al*., 2013).

Sulfolipids (SL)—Sulfolipids are a family of polyacylated trehalose-2-sulfate glycolipids esterified with two to four acyl chains (for reviews, Goren and Brennan, 1979; Goren, 1990; Bertozzi and Schelle, 2008). The major SL form found in *Mtb* is sulfolipid-1 (SL-1), a tetra-acylated glycolipid with a middle-chain saturated fatty acyl chain (palmitic or stearic acid) on the 2'-position of trehalose and different combinations of the hepta- and octa-methyl-branched phthioceranic or hydroxyphthioceranic acids (C_{31} to C_{46}) on 3'-, 6- and 6'-positions [Fig. 8]. Monomethyl-branched unsaturated C_{16} to C_{20} fatty acids have also been found as minor constituents of SL (Dubey *et al*., 2003). This family of lipids is specific to *Mtb.*

The first committed step in SL biosynthesis is the transfer of a sulfate group to the 2-position of trehalose in a reaction catalyzed by the sulfotransferase Stf0 (Mougous *et al*., 2004). The acyltransferase PapA2 then transfers a palmitoyl or stearoyl chain from palmitoyl- or stearoyl-CoA on the 2'-position of trehalose yielding a monoacylated SL (Kumar *et al*., 2007; Bhatt *et al*., 2007). The polyketide synthase Pks2 is responsible for the synthesis of phthioceranates and hydroxyphthioceranates using an activated fatty acid starter unit provided by the fatty acyl-AMP-ligase FadD23 (Sirakova *et al*., 2001; Lynett and Stokes, 2007). Upon elongation by Pks2, a (hydroxy)phthioceranyl chain is then transferred to the 3'-position of trehalose by the acyltransferase PapA1, yielding a diacylated SL also known as SL_{1278} (Kumar *et al*., 2007; Bhatt *et al*., 2007). It is thought that PapA1 directly transfers (hydroxy)phthioceranoyl groups from Pks2 and that the acylated Pks2 acyl carrier protein domain is its substrate (Kumar *et al*., 2007). The last two acylations on the 6- and 6'-positions of trehalose are catalyzed by Chp1, a cutinase-like protein anchored in the plasma membrane with its catalytic domain facing the cytoplasm (Seeliger *et al*., 2012). Unlike PapA1 and PapA2, Chp1 does not use an activated thioester acyl donor, but rather catalyzes regioselective transacylations between two SL_{1278} molecules to generate the mature tetra-acylated SL-1. This mechanism is reminiscent of the one utilized by the Ag85 mycoloyltransferases for the synthesis of TDM from two molecules of TMM. Finally, gene knock-out studies indicated that the polyketide synthase encoded by *pks8+pks17* is responsible for the production of the monomethyl-branched unsaturated C_{16} to C_{20} fatty acids found in some forms of SL (Dubey *et al*., 2003).

The *fadD23*, *papA1*, *papA2* and *chp1* genes cluster on the *Mtb* chromosome [Fig. 9]. Interspersed between these genes are two more ORFs encoding integral membrane proteins, *mmpL8* and *sap*. MmpL8, like MmpL3 involved in the translocation of TMM (see above), is an RND superfamily transporter. Sap (Sulfolipid-1-Addressing Protein) appears to facilitate the translocation of SL-1 to the cell surface. Its disruption in *Mtb* causes the intracellular build-up of SL_{1278} although the mutant is still capable of synthesizing small amounts of

SL-1 (Seeliger *et al*., 2012). Similar to the *sap* mutant, an *mmpL8* knock-out strain was shown to accumulate SL_{1278} intracellularly and to fail to export SL-I or SL_{1278} the cell surface (Seeliger *et al*., 2012). The accumulation of SL_{1278} precursor in the *mmpL8* mutant suggests that the presence of MmpL8 in the membrane is required for Chp1 to complete the acylation of SL-I. It was proposed that MmpL8 may serve as a scaffold for the coupled synthesis and transport of SL (Seeliger *et al*., 2012) [Fig. 10]. The recent isolation of a protein complex made of Pks2, MmpL8, PapA1 and FadD23 from membrane preparations of *M. bovis* BCG provides strong support for this model (Zheng *et al*., 2011). While it is expected that the translocation of SL-1 to the cell surface, similar to the situation with TMM, requires additional inner membrane, periplasmic and/or OM transporters, the identity of these protein is at presently not known.

The restriction of SL to *Mtb* has led to think that these lipids might play a role in pathogenesis. Studies by Goren more than 40 years ago, established a correlation between the presence of SL in *Mtb* isolates and their virulence in guinea pigs (Goren, 1974a). Several studies were carried out since then to establish the roles of SL during infection. *In vitro* studies using purified SL-1 have implicated this glycolipid in the prevention of phagosome-lysosome fusion, the activation of human neutrophils and the modulation of cytokine production by leukocytes (Goren *et al*., 1976; Pabst *et al*., 1988; Zhang *et al*., 1988; Zhang *et al*., 1991; Brozna *et al*., 1991; Goren, 1990). Despite these observations, *in vivo* and *ex vivo* studies with *Mtb* mutants defective in various aspects of SL biosynthesis failed to reveal any significant virulence or pathogenicity defects associated with these mutations in mice, guinea pigs and cultured macrophages (Rousseau *et al*., 2003a; Kumar *et al*., 2007). On the other hand, four studies have shown that *mmpL8* knock-out mutants of *Mtb* which accumulates the diacylated precursor sulfolipid SL_{1278} are attenuated for virulence in mouse models of infection (Converse *et al*., 2003; Domenech *et al*., 2004; Domenech *et al*., 2005; Lamichhane *et al*., 2005). SL_{1278} and other diacylated forms of SL were recognized as CD1b-restricted T-cell antigens and studies using a panel of synthetic analogs have begun to explore their structure-function relationship (Gilleron *et al*., 2004; Guiard *et al*., 2009). Recently, Gilmore *et al*. (2012) provided evidence that a *sft0* null mutant of *Mtb*, which fails to synthesize any forms of SL, survived better than its wild-type parent in human but not murine macrophages, possibly as a result of the increased resistance of this strain to human cationic antimicrobial peptides (defensins). These results suggest that SL may only have a clear impact on infection in the human host. Recent studies have highlighted the role of methyl-branched fatty acid-containing lipids such as SL, PDIM, DAT and PAT in alleviating the propionate-mediated stress undergone by *Mtb* when the bacterium switches to host cholesterol as a major carbon source during infection (Singh *et al*., 2009; Lee *et al*., 2013). The propionyl-CoA generated upon β-oxidation of cholesterol is converted to methylmalonyl-CoA by the propionyl-CoA carboxylase which is subsequently used by dedicated polyketide synthases such as Pks2, Mas and Pks3/4 in the elongation of the methyl-branched fatty acids found in SL, PDIM and DAT/PAT, respectively (see further sections). The regulator facilitating this metabolic switching to fatty acids was identified as WhiB3 (Singh *et al*., 2009).

The synthesis of SL appears to be tightly regulated during the course of infection (Graham *et al*., 1999; Rodriguez *et al*., 2013). The two-component system regulator PhoP-PhoR is required for the production of SL, DAT and PAT and a point mutation in the *phoP* gene of *Mtb* H37Ra accounts for the absence of these glycolipids from this strain (Gonzalo Asensio *et al*., 2006; Walters *et al*., 2006; Chesne-Seck *et al*., 2008). Consistent with these results, PhoP was found to activate the transcription of several *Mtb* genes including those involved in the production and transport of SL such as *mmpL8*, *papA1* and *pks2* (Walters *et al*., 2006; Goyal *et al*., 2011; Cimino *et al*., 2012). The regulatory protein WhiB3 also regulates the synthesis of SL, DAT, PAT and PDIM as indicated above. A *whiB3* mutant of *Mtb* produces 3 to 10-fold less SL, DAT and PAT compared to the wild-type parental strain (Singh *et al*., 2009). Collectively, these studies highlight the complexity of the regulation of acyltrehalose biosynthesis in *Mtb* and the pleiotropic roles that these glycolipids are likely to play at various stages of the infection.

Diacyltrehaloses (DAT) and Polyacyltrehaloses (PAT)—The 2,3-di-*O*-acyltrehaloses (DAT) consist of trehalose esterified at the 2-position by a middle-chain saturated fatty acid (palmitic, stearic or tuberculostearic acid) and at the 3-position by a long-chain methyl-branched fatty acid [Fig. 8]. The methyl-branched fatty acids found in DAT consist of di-methyl-branched mycosanoic acids (C_{21}-C_{25}) and, less commonly, of tri-methyl-branched C_{25}-C_{27} mycolipenic (phthienoic) and mono-hydroxylated tri-methyl-branched C_{24}-C_{28} mycolipanolic acids (Minnikin *et al*., 1985; Lemassu *et al*., 1991; Besra *et al*., 1992). Polyacyltrehaloses (PAT) comprise a family of penta-acylated trehaloses where the trehalose moiety is esterified at the 2-position with a middle-chain saturated fatty acyl chain (palmitic, stearic or tuberculostearic acid) and at the 3-, 6-, 4’ and 6’-positions with four methyl-branched mycolipenic acids, although minor amounts of mycolipanolic acids can also be found (Minnikin *et al*., 1985; Daffé *et al*., 1988). In addition, the presence of 2,3,6-triacyltrehaloses (TAT) has been reported in *Mtb*. In TAT, trehalose is esterified by two middle-chain saturated fatty acids and one mycolipenic or mycolipanolic acid (Muñoz *et al*., 1997). DAT, TAT and PAT with this type of fatty acid composition are exclusively found in species of the *Mtb* complex. Monomethyl-branched unsaturated C_{16} to C_{20} fatty acids have also been found as minor constituents esterifying PAT and DAT (Dubey *et al*., 2003).

Little is known about the biosynthesis of DAT and PAT although a dedicated biosynthetic gene cluster has been identified and found to resemble that of the better studied SL-1 (Hatzios *et al*., 2009). Of the genes present in this cluster, only *pks3/4* and *papA3* have been confirmed to participate in the biosynthesis of DAT and PAT thus far. *pks3/4* encodes the polyketide synthase responsible for the elongation of mycosanoic and mycolipenic acids. Accordingly, an *Mtb pks3/4* knock-out mutant was reported to be deficient in PAT and DAT production (Dubey *et al*., 2002; Rousseau *et al*., 2003b). *In vitro* studies with the purified PapA3 protein have shown that this acyltransferase is capable of sequentially transferring two palmitoyl groups onto positions 2 and 3 of trehalose yielding a diacylated trehalose molecule structurally similar to *Mtb* DAT. Consistently, the genetic disruption of *papA3* abolished the synthesis of PAT in *Mtb* (Hatzios *et al*., 2009). Remarkably, the PAT biosynthetic gene cluster also encompasses genes potentially encoding for a lipid transporter

of the RND superfamily (*mmpL10*), a fatty-acyl AMP ligase (*fadD21*) and an acyltransferase (*Rv1184c; chp2*) [Fig. 9]. In order to investigate the function of the products of these genes, *Mtb* knock-out mutants were generated in our laboratory [unpublished results]. Preliminary studies indicate that the *fadD21* null mutant fails to synthesize both DAT and PAT, suggestive of a role for the acyl-AMP ligase FadD21 in the loading of activated fatty acid starter units onto Pks3/4 for the synthesis of mycosanoic and mycolipenic acids. The *mmpL10* mutant, in contrast, is unable to synthesize PAT and accumulates DAT intracellularly, pointing to the involvement of this membrane transporter in the translocation of DAT to the cell surface. The *chp*2 mutant is unable to synthesize PAT and builds up large amounts of DAT, part of which are found at the cell surface. Interestingly, topological studies on Chp2 indicated that it is a membrane-anchored enzyme with a catalytic domain facing the periplasm. Enzyme assays further indicate that Chp2 is capable of synthesizing PAT from DAT. Thus, similar to Chp1 in the biosynthesis of SL-1, Chp2 appears to catalyze three sequential transacylations between DAT molecules yielding the fully elaborated PAT [unpublished results]. Since PapA3 is a cytosolic enzyme, it follows that DAT is synthesized in the cytoplasm prior to being flipped across the plasma membrane to serve as a substrate for Chp2 [Fig. 10]. The fact that PAT are synthesized on the periplasmic side of the membrane and then transported to the OM further suggests that DAT flipping and DAT/PAT translocation between membranes are two separate events. Further investigations are required to determine whether MmpL10 is involved in the first and/or second event(s) and identify the missing components of the translocation machinery. Finally, as reported above, the polyketide synthase Pks8-17 is responsible for the production of the monomethyl-branched unsaturated C_{16} to C_{20} fatty acids found in some forms of SL, DAT and PAT (Dubey *et al*., 2003). The synthesis of DAT and PAT, like that of SL, is under control of the two-component system PhoP-PhoR and the regulatory protein WhiB3 (Gonzalo Asensio *et al*., 2006; Walters *et al*., 2006; Chesne-Seck *et al*., 2008, Singh *et al*., 2009; Goyal *et al*., 2011; Cimino *et al*., 2012).

Several studies have investigated the physiological roles and biological relevance of DAT and PAT in *Mtb* infection. Phenotypic observations made on a *pks3/4* DAT/PAT-deficient mutant indicated a role for these lipids in the retention of the capsular material at the cell surface of *Mtb* (Dubey *et al*., 2002; Rousseau *et al*., 2003b), possibly accounting for the changes observed in the binding and uptake of the mutant by phagocytic and non-phagocytic cells (Rousseau *et al*., 2003b). *In vitro*, DAT are strong immunomodulatory molecules, inhibiting the proliferation of murine and human $CD4^+$ and $CD8^+$ T-cells, and the expression of Th-1 cytokines in murine cells through the disruption of the MAPK signaling pathway (Saavedra *et al*., 2001; Saavedra *et al*., 2006; Palma-Nicolás *et al*., 2010). In another study, purified DAT (but not SL or PDIM) inhibited in a dose-dependent manner LPS- and *Mtb*-induced IL-12p40 and TNF-α production in human monocytes (Lee *et al*., 2007). Mycolipenic acids, the major acyl substituents found in PAT, TAT and some forms of DAT, have been shown to be potent inhibitors of leukocyte migration *in vitro* (Husseini and Eldberg, 1952). Notwithstanding, a *pks3/4* knock-out mutant of *Mtb* deficient in DAT and PAT synthesis replicated similarly to its wild-type parent in mice (Rousseau *et al*., 2003b). Conversely, two independent high-density transposon mutagenesis-based studies aimed at identifying genes required for the optimal replication and survival of *Mtb* in mice

(Sassetti and Rubin, 2003; Lamichhane *et al*., 2005) identified *mmpL10* as a virulence gene; one of these studies also identified *fadD21* and *chp*2 mutants as attenuated *in vivo*. Clearly, further infection studies using individual knock-out mutants and different animal models are required to provide a definite answer as to the role of DAT and PAT in *Mtb* pathogenesis. As in the case of SL, possible explanations for the discrepancy between the potent biological activities of DAT and PAT *in vitro* and their apparent lack of impact on *Mtb* infection *in vivo* may be found in the failure of animal models of infection to accurately mimic human TB infection and the potential functional redundancy of OM polymethyl-branched fatty acids-containing (glyco)lipids. In support of the latter assumption, a recent study comparing the effects of the individual and combined inactivation of the SL, PDIM and DAT/PAT pathways on *Mtb* infection revealed a functional overlap between these molecules with PDIM having a dominant effect over DAT/PAT and SL (Passemar *et al.*, 2013). As noted in the SL section, one of the biological functions shared by these lipids is apparently to alleviate the propionate-mediated stress undergone by the bacilli during growth on host cholesterol as a major carbon source (Singh *et al*., 2009; Lee *et al*., 2013). In addition, the contribution of these lipids to blocking the phagosome acidification of infected macrophages suggests that their presence at the cell surface may promote the intracellular survival of *Mtb* (Brodin *et al*., 2010; Passemar *et al*., 2013).

Lipooligosaccharides (LOS)—LOS are produced by various fast- and slow-growing *Mycobacterium* species. They are found in *M. canettii* and related *M. tuberculosis* complex strains (Daffé *et al*., 1991) but are otherwise apparently absent from *Mtb sensu stricto*. LOS share a poly-*O*-acylated trehalose core glycosylated by a mono- or, more frequently, a oligosaccharidyl unit which is species-specific [Fig. 8] (Daffé and Lemassu, 2000). The trehalose moiety of LOS is acylated by polymethyl-branched fatty acids that can either be saturated (*e. g.* in *M. canettii*) or unsaturated (*e. g.* in *M. smegmatis*).

Although LOS were discovered more **than** 30 years ago (Hunter *et al*., 1983; Saadat & Ballou, 1983), nothing was known of their biosynthesis until recently. While most studies on LOS biosynthesis have focused on *M. smegmatis* and *M. marinum*, the conservation of the core genes involved in *M. canettii* suggests that the biosynthesis of LOS in this species follows a similar pattern. The identification of a LOS biosynthetic gene cluster in *M. marinum* has shed light into the set of steps leading to the synthesis of these glycolipids (Burguière *et al*., 2005; Ren *et al*., 2007). The composition of this gene cluster resembles those described previously for SL and DAT/PAT biosynthesis with genes encoding two polyketide synthases (Pks5 and Pks5.1), a fatty-acyl AMP ligase (FadD25), a lipid transport membrane protein of the RND superfamily (MmpL12) and a polyketide-associated acyltransferase (PapA4) [Fig. 9]. Additionally, the cluster encompasses several genes encoding glycosyltransferases, methyltransferases and other glycosyl modifying enzymes likely to be involved in the synthesis and modification of the species-specific oligosaccharidyl unit (Ren *et al.*, 2007; Alibaud *et al*., 2014). A *M. marinum* mutant carrying a transposon insertion in *papA4* fails to synthesize LOS, confirming the involvement of this acyltransferase in the pathway (Rombouts *et al*., 2011). Interestingly, while a homologous gene cluster is present in the genome of *Mtb* H37Rv, *pks5.1* is missing from this cluster and the H37Rv ortholog of *papA4* is predicted to encode a truncated protein

of only 165 amino acids instead of the 465 residue protein encoded for instance by *papA4* from *M. marinum*. The *papA4* gene of the LOS-producing strain *M. canettii*, in contrast, encodes a full-size protein and this strain is also endowed with an ortholog of *pks5.1* (Rombouts *et al*., 2011). Therefore, the lack of functional Pks5.1 and PapA4 most likely accounts for the inability of *Mtb* H37Rv to produce LOS. Pks5 was shown to synthesize the methyl-branched 2,4-dimethyl-2-eicosenoic acid found in LOS and the disruption of *pks5* in *M. smegmatis* abolished LOS synthesis in this species (Etienne *et al*., 2009).

LOS are highly antigenic molecules (Daffé *et al*., 1991). Recent observations suggest that they play an important role in retaining proteins at the cell surface of some *Mycobacterium* species such as *M. marinum* (van der Woude *et al*., 2012). Their precise role in colony morphology is still a matter of debate and may depend on the species (Belisle & Brennan, 1989; Lemassu *et al*., 1992; Burguière *et al*., 2005). In *M. marinum* for instance, LOS have clearly been associated with colony morphology, sliding motility, biofilm formation and the ability of this *Mycobacterium* to enter macrophages (Ren *et al*., 2007). The *M. marinum* LOS are also endowed with immunomodulatory activities (Rombouts *et al*., 2009) and modulate virulence in the zebrafish embryo model of infection (van der Woude *et al*., 2012).

Acyltrehaloses in serodiagnosis, drug discovery and vaccine development— The essentiality of TMM and TDM for mycobacterial growth makes the enzymes and transporters involved in their biogenesis targets of choice for drug development. Many inhibitors of this pathway in fact already exist if one considers all the compounds either used clinically (e.g., isoniazid, ethionamide) or under development that target the biosynthesis of mycolic acids. A comprehensive review of past and ongoing efforts to target this pathway was recently published (North *et al.*, 2013). Among the targets sought, the mycoloyltransferases of the antigen 85 complex have received some attention lately. High-throughput screening assays were reported for these enzymes (Elamin *et al*., 2009; Boucau *et al.*, 2009; Sanki *et al*., 2009; Favrot *et al*., 2013) and used to identify inhibitors. The molecular mechanism of inhibition of antigens 85 by one of them, known as ebselen (MIC of 20 μg/ml against *Mtb* in culture), was elucidated and found to be particularly interesting in the sense that it is unlikely to promote the emergence of drug-resistant isolates (Favrot *et al*., 2013). Small molecule binders of antigen 85C identified by magnetic resonance spectroscopy also showed activity against *Mtb in vitro* and inside macrophages in the 100 μM range (Warrier *et al*., 2012). Importantly, the return of interest in whole cell-based screening that the TB field has witnessed in recent years also has led to the identification of a variety of chemotypes active against the TMM transporter, MmpL3 (Grzegorzewicz *et al*., 2012; Stanley *et al*., 2012; La Rosa *et al*., 2012; Remuinan *et al.*, 2013; Ioerger *et al.*, 2013; Poce *et al*., 2013; Konddredi *et al*., 2013; Onajole *et al*., 2013; Rao *et al*., 2013) including SQ109, a drug candidate currently undergoing phase II clinical trials (Sacksteder *et al*., 2012; Tahlan *et al*., 2012). The reason why so many different chemical scaffolds apparently inhibit the MmpL3-mediated translocation of TMM is at present unclear. Another approach to targeting acyltrehalose biosynthesis would to screen for inhibitors of OtsB2 since this enzyme was shown to be essential for *Mtb* growth (Murphy *et al*., 2005). To the best of our knowledge, no OtsB2 inhibitor has yet been reported. Finally, a regulatory system of interest in the context of drug development is the two-component transcriptional regulator PhoP-

PhoR (Rv0757-Rv0758) which regulates the biosynthesis of multiple virulence factors including SL, DAT and PAT (Gonzalo-Asencio *et al*., 2006; Walters *et al*., 2006; Frigui *et al*., 2008; Gonzalo-Asencio *et al*., 2008; Ryndak *et al*., 2008; Chesne-Seck *et al*., 2008; Lee *et al*., 2008). No inhibitors of PhoP-PhoR have yet been described.

Beyond drug discovery, acyltrehaloses, either as purified antigens or in the context of attenuated live strains, are also being studied from the perspective of their vaccine potential. Ongoing studies include the testing of CD1-restricted sulfolipid antigens (G. Puzo, pers. comm.) and that of attenuated *phoP* knock-out mutants of *Mtb* (Nambiar *et al*., 2012).

The acyltrehaloses of *Mtb* (TDM, SL, DAT, TAT) are potent inducers of the humoral immune response (Lemassu *et al*., 1991; Muñoz *et al*., 1997). Accordingly, their potential as serodiagnostic tools for the detection of TB has been explored (Simonney *et al*., 1995; Simonney *et al*., 1996; Julian *et al*., 2002; Julian *et al*., 2004). While TB patients seem to exhibit heterogeneous IgG and IgA antibody responses against these glycolipid antigens, the results of these studies were still encouraging in that ELISA tests based on these antigens tended to be more sensitive than protein-based ELISAs in the detection of smear-negative TB patients and in that of patients co-infected with HIV (Simonney *et al*., 1995; Simonney *et al*., 1996; Julian *et al*., 2002; Julian *et al*., 2004).

para-hydroxybenzoic acids and phenolic glycolipids

Structures and distribution of para-hydroxybenzoic acid derivatives and phenolic glycolipids in Mtb—The structures of the phenolic glycolipids (PGLs) and *para*-hydroxybenzoic acid derivatives (*p*HBADs) of *Mtb* are shown on Fig. 11. *p*HBADs and PGLs share the same glycosylated aromatic nucleus. PGLs are found in the capsule and OM of *Mtb* (Ortalo-Magné *et al*., 1996); *p*HBADs, in contrast, are released in culture filtrates and tend not to remain associated with the cell envelope (Constant *et al*., 2002). While all *Mtb* isolates analyzed to date have retained the ability to produce and secrete *p*HBADs, most *Mtb* strains 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). In fact, PGL production appears to be restricted to *M. canettii* and some *Mtb* isolates of the East Asian/Beijing lineage (Daffé *et al*., 1987; Constant *et al*., 2002; Reed *et al*., 2004; Huet *et al*., 2009). The PGLs of *Mtb* are glycosylated phenolic derivatives of phthiocerol dimycocerosates (PDIM), themselves abundant components of the OM of *Mtb* contributing to its impermeability (Camacho *et al*., 2001) [Fig. 11].

Roles in the physiology and virulence of Mtb—The roles of *p*HBADs, PDIM and PGL in the permeability barrier, intracellular survival, modulation of the host immune response and pathogenicity of *Mtb* have been the object of several recent reviews (Jackson *et al*., 2007; Guilhot *et al*., 2008; Daffé *et al*., 2014) and will therefore not be detailed here. Interestingly, in an attempt to correlate the lipid content with the virulence of *Mtb* isolates, Goren *et al.* characterized in 1974 a methoxylated phenolphthiocerol (a non-glycosylated variant of PGL), the so-called “attenuation indicator lipid” (Goren *et al*., 1974b). Recent studies have identified this lipid and its unmethylated form in East Asian/Beijing isolates

and found that they accumulate in all Indo-Oceanic *Mtb* strains examined (Krishnan *et al*., 2011). Similarly, Beijing strains were reported to accumulate variants of PDIM and eventually PGL, known as phthiotriol and glycosylated phenolphthiotriol dimycocerosates (Huet *et al*., 2009). The correlation between the occurrence of these lipids and variations in virulence remains, however, unclear (Huet *et al*., 2009; Krishnan *et al*., 2011). It was proposed that the different lineages of *Mtb* (Gagneux and Small, 2007) may have evolved regionally to tailor their OM lipid composition to the genetic background of their human host (Neyrolles and Guilhot, 2011).

Biogenesis of PGL and pHBADs—Coupled genetic and biochemical strategies have allowed much of the biosynthetic pathways of PDIM, PGL and *p*HBADs to be elucidated (for recent reviews, Guilhot *et al*., 2008; Malaga *et al*., 2008; Daffé *et al*., 2014). The nature of the enzymes involved suggests that most if not all of the elongation and assembly of these compounds takes place on the cytoplasmic side of the plasma membrane. The biosynthesis of the mycocerosic acid and (phenol)phthiocerol moieties of PDIM and PGL follows a similar pattern as the polymethyl-branched fatty acids found in SL, DAT and PAT (see previous section) and involves dedicated type I polyketide synthases, associated FadD-type fatty acyl-AMP ligases for the activation of long-chain (C_{16}-C_{24}) starter fatty acids as acyl-adenylates, and a PapA enzyme (PapA5) to transfer the newly synthesized mycocerosates to their phthiocerol or phenolphthiocerol acceptors. Consistent with their conserved structures [Fig. 11], the biosynthesis of the glycosyl moiety of PGL and *p*HBADs involves the same set of enzymes including three glycosyltransferases (Rv2962c, Rv2957, Rv2958c) and four *O*-methyltransferases (Rv2954c, Rv2955c, Rv2956 and Rv2959c). Upon synthesis, PDIM, PGL and *p*HBADs are exported by a dedicated translocation machinery. All of the work on this topic thus far has focused on PDIM but it is reasonable to assume that the same transporters are involved in the export of PGLs. Both the RND transporter MmpL7 and the ABC-transporter DrrABC are required for the translocation of PDIM to the OM. In addition, the lipoprotein LppX has been found to be required for PDIM to reach the cell surface (Sulzenbacher *et al*., 2006). LppX shares a similar fold with the periplasmic chaperone LolA and the outer membrane lipoprotein LolB which, in Gram negative bacteria, are involved in the localization of lipoproteins to the OM. It is thought that LppX acts downstream from MmpL7 and DrrABC, carrying PDIM across the periplasm. Using a yeast two-hybrid system, Jain and Cox (2005) showed that MmpL7 interacts with the polyketide synthase PpsE involved in the synthesis of the (phenol)phthiocerol moieties of PDIM and PGL. Based on this finding, a model was proposed wherein the synthesis and transport of PDIM are coupled (Jain and Cox, 2005) [Fig. 10]. In many ways, the biogenesis of PDIM and PGL thus resembles that of polymethyl-branched fatty acid-containing acyltrehaloses, involving similar sets of enzymes and transporters interacting with one another. Most of the genes involved in the biosynthetic pathways of PDIM, PGL and *p*HBADs are clustered on a 73-kb fragment of the *Mtb* chromosome (for a recent review, Daffé *et al*., 2014).

PGL biosynthesis in the context of diagnosis and drug discovery—Although not essential for growth, PDIM and biosynthetically-related PGL contribute to a significant extent to the ability of *Mtb* to replicate and survive *in vivo*. These lipids also play important roles in the permeability barrier of the cell envelope (Camacho *et al*., 2001) suggesting that

compounds inhibiting their synthesis could synergize with or potentiate existing anti-TB drugs. A compound inhibiting the production of PGL in whole *Mtb* cells has been reported (Ferreras *et al*., 2008). As noted earlier in this review, promising compounds targeting the Ser/Thr kinases of *Mtb* that regulate the synthesis of PDIM and PGL (among other physiological processes) (Molle and Kremer, 2010) are also under development. In addition, several studies have explored the potential of PGL as serodiagnostic tools for TB detection (Simonney *et al*., 1995; Simonney *et al*., 1996; Constant *et al*., 2002). As most clinical isolates of *Mtb* do not produce PGL (Daffé *et al*., 1987; Daffé *et al*., 1988; Constant *et al*., 2002), it is likely that the antibodies detected in patients were in fact directed against *p*HBADs (Constant *et al*., 2002).

Mannosyl-β-1-phosphomycoketides

Mannosyl-β-1-phosphomycoketides (MPM) are glycoconjugates found in minute quantities (in the range of 1 nM concentration) inside the cells and released in the culture medium of pathogenic slow-growing *Mycobacterium spp.* including *Mtb*, *M. bovis* BCG, *M. africanum*, *M. canetti*, *M. avium*, *M. avium paratuberculosis*, *M. marinum* and *M. ulcerans* (Matsunaga and Sugita, 2012). They are apparently not found in rapidly growing mycobacteria (Matsunaga *et al*., 2004). MPM consist of a mannosyl-β-1-phosphate moiety reminiscent of polyprenol phosphomannose (PPM) and an alkyl chain of varying length (C30-C34) made of a fully saturated 4, 8, 12, 16, 20-pentamethylpentacosyl unit (the mycoketide) [Fig. 12]. The alkyl chain of MPM is elongated by the polyketide synthase Pks12 (Rv2048c) (Matsunaga *et al*., 2004; Chopra *et al*., 2008), the largest predicted protein of *Mtb* (430 KDa). Pks12 consists of two complete sets of fatty acid synthase (FAS)-like catalytic domains capable together of using alternating C2 (malonyl-CoA) and C3 (methylmalonyl-CoA) units to elongate the alkyl backbone of mycoketides. After 5 cycles of C3 and C2 chain elongation, the alkyl chain is thought to be released from Pks12 upon hydrolysis yielding mycoketidic acid which is further reduced to the corresponding long-chain alcohol, mycoketide, and finally phosphorylated and mannosylated to generate MPM (Matsunaga and Sugita, 2012). The identity of the enzymes catalyzing the hydrolysis, reduction, phosphorylation and mannosylation steps is not known.

In line with the restricted distribution of MPM to pathogenic slow-growing *Mycobacterium spp.*, studies comparing the virulence of MPM-deficient mutants of *Mtb*, *M. avium* and *M. marinum* to that of their wild-type parent in animal models of infection have provided support for their involvement in pathogenicity (Matsunaga and Sugita, 2012). MPM have the ability to activate human CD1c-restricted T-cells (Moody *et al*., 2000; Ly *et al*., 2013). In addition, they have been proposed to contribute to the suppression of phagosomal acidification and to act as signaling molecules regulating cell division and virulence (Matsunaga and Sugita, 2012).

Glycoproteins

Several proteins of *Mtb* complex species have been identified as glycoproteins on the basis of lectin binding (Espitia *et al*., 1989; Garbe *et al*., 1993; Gonzalez-Zamorano et al., 2009; Sartain and Belisle, 2009) or by using liquid chromatography-mass spectrometry approaches and bioinformatic analyses (Smith *et al*., 2013), but the detailed structure of the glycosyl

appendages of only two of them have been characterized so far. The 45-47 kDa (Apa) antigen of *Mtb* was shown to be modified at threonine residues with one to three linear α–(1,2)-linked oligomannosides, whereas the MBP83 antigen of *M. bovis* is modified at threonine residues with one to three linear α–(1,3)-linked oligomannosides (Dobos *et al*., 1996; Michell *et al*., 2003). *Mtb* proteins may also be *O*-glycosylated on serine residues as shown in the case of the superoxide dismutase SodC (Sartain and Belisle, 2009). The glycosylation pattern of Apa and MBP83 is reminiscent of eukaryotic short-chain mannoproteins (Lengeler *et al*., 2007). Consistently, the yeast-like protein-*O*-mannosyltransferase Rv1002c was identified in *Mtb* as the enzyme responsible for the first mannosylation step of Apa (VanderVen *et al*., 2005). Analogous to eukaryotic systems, Sec-translocation is required for the mannosylation of extracytoplasmic proteins to occur in *Mtb* (VanderVen *et al*., 2005). Rv1002c belongs to the GT-C superfamily of glycosyltransferases as would be expected for a protein transferring a mannosyl residue from the mannose donor, Dec-P-Man, on the periplasmic side of the plasma membrane. The glycosyltransferases responsible for the further elongation of the α-(1,2) or α-(1,3)-linked oligomannoside motifs have not yet been identified in *Mtb*. The presence of identical linear α–(1,2)-linked oligomannosides in polar forms of PIMs (PIM_6) or capping the non-reducing termini of the arabinosyl side chains of ManLAM led us to investigate the putative involvement of the α-(1,2) mannosyltransferases Rv2181 (Kaur *et al*., 2006; Kaur *et al*., 2008) and PimE (Rv1159) (Morita *et al*., 2006) in this process. Interestingly, while the disruption of *pimE* or *Rv2181* in *Mtb* failed to reveal any effect on the glycosylation pattern of Apa in the mutant strains (G. Larrouy-Maumus, M. Jackson, D. Kaur, K. Dobos *et al*., unpublished results), disruption of the *pimE* gene in *M. smegmatis* yielded a mutant devoid of triglycosylated forms of FasC (a major mannosylated secreted protein in this species) and producing almost exclusively the monoglycosylated forms of the protein with only trace amounts of the diglycosylated forms (Liu *et al*., 2013). Importantly, wild-type mannosylation was restored in the mutant complemented with the *pimE* gene of *Mtb*. Disruption of *MSMEG_4247* (the ortholog of *Rv2181* in *M. smegmatis*), in contrast, had no effect on the glycosylation pattern of FasC (Liu *et al*., 2013). Collectively, these data point to the involvement of PimE in the deposition of the second and perhaps third Man*p* residue of the glycosyl appendages of mannoproteins, even though compensatory enzymatic activities may exist in *Mtb* accounting for the absence of protein mannosylation phenotype in the *Mtb* knock-out mutant.

In contrast to eukaryotic protein-*O*-mannosyltransferase, Rv1002c is not an essential protein of *Mtb* and *M. smegmatis* indicating that protein *O*-mannosylation is not essential for viability in mycobacteria (Liu *et al*., 2013). Disruption of *Rv1002c* in *Mtb*, however, dramatically alters the growth properties of the bacterium in certain liquid and solid media and negatively impacts its virulence in cellular and animal models of infection (Liu *et al*., 2013). Protein *O*-mannosylation thus plays critical roles in the physiology and pathogenesis of the tubercle bacillus. One of its proposed functions is to regulate the proteolytic processing and subcellular localization of exported proteins (Herrmann *et al*., 1996; Sartain and Belisle, 2009; Wilkinson *et al*., 2009). As in other bacterial pathogens, the glycosylation of mycobacterial proteins also influences their interactions with the host (Torrelles and Schlesinger, 2010). The mannosyl appendages of Apa for instance have been implicated in 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).

Capsular polysaccharides

The capsular material of Mtb—The outermost compartment of the cell envelope of *Mtb* consists of a loosely bound structure referred to as 'capsule' (Daffé and Draper, 1998) primarily made of proteins and polysaccharides (~ 97% of the total material) with only small amounts of lipids (Lemassu and Daffé, 1994; Ortalo-Magné *et al*., 1995). While the nature of this material has essentially been studied in axenically grown bacilli, the major capsular polysaccharides of *Mtb* have also been found coating the bacilli during infection (Schwebach *et al*., 2001; Schwebach *et al*., 2002). The amount of capsular polysaccharides produced by *Mtb* varies between isolates and this diversity is thought to impact the way that *Mtb* interacts with host cells (Cywes *et al*., 1997; Ehlers and Daffé, 1998; Daffé and Etienne, 1999; Torrelles and Schlesinger, 2010). The three major capsular polysaccharides of *Mtb* are an α-D-glucan, a D-mannan and a D-arabino-D-mannan representing, respectively, approximately 70%, 15% and 13% of the total polysaccharide content of the capsule (Lemassu and Daffé, 1994; Ortalo-Magné *et al*., 1995). In addition, traces (2-5%) of xylose were also detected (Ortalo-Magné *et al*., 1995). All are devoid of acyl substituents and are not covalently linked to the rest of the cell envelope.

α-D-glucan—α-D-glucan is a linear polymer of α–(1,4)-Glc*p* substituted at some 6-positions by oligoglucoside chains comprised of 1 to 9 Glc residues (Dinadayala *et al*., 2008) [Fig. 13]. It is structurally very similar to the intracellular glycogen of *Mtb* and *M. bovis* BCG although its 3D-structure appears to be more compact and its molecular mass, as determined by analytical ultracentrifugation, slightly higher (13×10^6 versus 7.5×10^6 Da) (Dinadayala *et al*., 2004; Dinadayala *et al*., 2008; Sambou *et al*., 2008). Consistently, the biosynthetic pathways of both glucopolymers share common enzymes that resemble the glycogen biosynthetic enzymes of *E. coli*. Enzymatic studies and phenotypic analyses of knock-out mutants of *Mtb* identified the α-(1,4)-glucosyltransferases Rv3032 and GlgA (Rv1212c), the ADP-glucose pyrophosphorylase GlgC (Rv1213) and the branching enzyme GlgB (Rv1326c) as components of their biosynthetic machinery (Sambou *et al*., 2008; Garg *et al*., 2007) [Fig. 14]. In addition, UDP-glucose which also serves as a Glc donor in the biosynthesis of α-(1,4)-linked glucans (Stadthagen *et al*., 2007) may be formed from glucose-1-phosphate and UTP by the UDP-glucose pyrophosphorylase GalU (Rv0993) (Lai *et al*., 2008). Disruption of *glgA* reduced the capsular α-D-glucan content of *Mtb* by half while that of *glgC* reduced by half both the α-D-glucan and glycogen contents of the cells (Sambou *et al*., 2008). Attempts to create a double *glgA-Rv3032* mutant were unsuccessful indicating that a functional copy of at least one of the two α-(1,4)-glucosyltransferases is required for *Mtb* growth. The targeted inactivation of the only *Rv3032* gene yielded a mutant with dramatically reduced glycogen and methylglucose lipopolysaccharide (MGLP) contents (Stadthagen *et al*., 2007). MGLPs are intracellular 6-*O*-methylglucose lipopolysaccharides of intermediate size which share with α-D-glucan and glycogen an α-(1,4)-linked glucan backbone. The precise physiological function of these cytosolic lipopolysaccharides is currently not known although a regulatory role in fatty acid

biosynthesis has been proposed (Jackson and Brennan, 2009). The disruption of *glgB* was not achievable (Sambou *et al*., 2008) most likely due to the toxic accumulation of maltose-1-phosphate that follows the inactivation of this gene (Kalscheuer *et al*., 2010). Nevertheless, altering the level of expression of this gene in *Mtb* or replacing the *Mtb glgB* gene by that of *E. coli* resulted in profound quantitative and qualitative effects on the α-D-glucan and glycogen of the cells, thereby implicating GlgB in the branching of both polysaccharides in *Mtb* (Sambou *et al*., 2008). In contrast to the reactions leading to polymerization and branching, nothing is known of the initiation reactions of α-D-glucan and glycogen. In particular, it is not known whether they involve an endogenous acceptor such polyprenyl-phosphate, diacylglycerophosphate or D-3-phosphoglycerate (Whitfield, 2006; Kaur *et al*., 2009), or if they directly occur on the α-(1,4)-glucosyltransferases as reported for the glycogen synthase of *Agrobacterium tumefaciens* (Ugalde *et al*., 2003). In addition to the classical Glg pathway, a second pathway for the biogenesis of α-(1,4)-glucans exists in *Mtb*. This four-step pathway generates α-(1,4)-glucans from trehalose and involves the trehalose synthase TreS, the maltokinase Pep2, the maltose-1-phosphate maltosyltransferase GlgE, and GlgB [Fig. 14] (Pan *et al*., 2004; Pan *et al*., 2008; Kalscheuer *et al*., 2010, Elbein *et al*., 2010; Mendes *et al*., 2010; Miah *et al*., 2013). Disruption of *glgE*, like that of *glgB*, results in the accumulation of maltose-1-phosphate which is toxic to the cells. The crystal structures of TreS from both *Mtb* and *M. smegmatis* were recently reported (Roy *et al*., 2013; Caner *et al*., 2013) and the mechanism of association of the C-ter domain of this enzyme with oligosaccharides and possibly glycogen investigated (Caner *et al*., 2013). Importantly, it was found that TreS forms a hetero-octameric complex with Pep2. The formation of this complex boosts the maltokinase activity of Pep2 suggesting that it may serve to regulate α-(1,4)-glucan synthesis (Roy *et al*., 2013). Another point of control of this pathway appears to be at the level of GlgE which was recently reported to be negatively phosphoregulated by the Ser/Thr kinase PknB (Leiba *et al*., 2013).

Consistent with the intracellular localization of glycogen and MGLPs, GlgA and Rv3032 are nucleotide sugar-utilizing glycosyltransferases predicted to catalyze the elongation of polysaccharides on the cytosolic face of the plasma membrane, and GlgB, GlgC, GlgE, Pep2 and TreS are also predicted to be cytosolic proteins. Consistently, α-D-glucan is likely to be elongated and branched on the cytosolic side of the plasma membrane before or in the process of being translocated across the plasma membrane. Given the major challenge represented by the translocation of such a high-molecular-weight hydrophilic molecule across the different layers of the mycobacterial cell envelope, the existence of molecular scaffolds providing continuity between the cytoplasm and the surface of *Mtb* functionally similar to the transport systems of Gram-negative group 2 and 3 capsules (Whitfield, 2006), seems likely. To this date, however, none of the components of the dedicated translocation machinery have been identified.

Recent studies using cellular models of infection have highlighted the roles of capsular polysaccharides in host-pathogen interactions. α-D-glucan and D-mannan mediate the nonopsonic binding of *Mtb* to CR3 (Cywes *et al*., 1997). CR3 is one of the main phagocytic receptors on monocytes and neutrophils and bacilli uptake through this receptor is thought to promote intracellular survival by suppressing the production of IL-12 and limiting

respiratory burst (Ehlers *et al*., 1998; Fenton *et al*., 2005). Further studies have highlighted the antiphagocytic properties of the *Mtb* capsule and suggested that this structure may serve to limit and control the interactions of the bacilli with macrophages (Stokes *et al*., 2004). More recently, Sani *et al*. (2010) showed that the presence of capsular material at the surface of *M. bovis* BCG enhanced the binding of the bacterium to human monocyte-derived macrophages and modulated the pro-inflammatory cytokine response of these cells. Along the same lines, Gagliardi *et al*. (2007) showed that *Mtb* capsular α-D-glucan blocked CD1 expression and suppressed IL-12 production in monocyte-derived DCs. The ability of α-D-glucan to bind the C-type lectin DC-SIGN may account at least in part for its biological activities (Geurtsen *et al*., 2009). Other studies have shown that antibodies to *Mtb* capsular polysaccharides can modify the course of infection to the benefit of the host (Glatman-Freedman and Casadevall, 1998; Teitelbaum *et al*., 1998). Owing to its glycogen-like structure, α-D-glucan was also proposed to be involved in *Mtb's* evasion of the immune system by molecular mimicry (Lemassu and Daffé, 1994). Finally, studies aimed at elucidating the basis of the immunotherapeutic properties of *M. bovis* BCG against bladder cancer have highlighted the anti-tumor activity of α-D-glucan (Wang *et al*., 1995; Zlotta *et al*., 2000). Since most of these studies have focused on the interactions of non-isogenic strains of *Mtb* or purified α-D-glucan with cellular models, it is important to keep in mind that they may not accurately reflect the relevance and individual contribution of capsular polysaccharides in mycobacterial infections. Further studies are warranted to determine the contribution of α-D-glucan to the pathogenicity of *Mtb* when carried by whole bacilli. Such studies will require isogenic mutants of *Mtb* specifically deficient in the production of capsular α-D-glucan (and producing wild-type levels of glycogen and MGLPs) that are presently not available.

D-mannan and D-arabino-D-mannan—The structure of D-mannan is identical to that of the mannan domain of LM, and the structure of AM is identical to that of the arabinomannan domain of LAM [Fig. 6]. It is likely that D-mannan and AM are released from LM and LAM upon hydrolysis of their phosphatidyl-*myo*-inositol anchor. As pointed out earlier in this review, D-mannan and AM are expected to share with LM and LAM common properties in their interactions with the host.

Capsular polysaccharides in the context of drug discovery—Two enzymes involved in the formation of the α-(1,4)-glucans of *Mtb*, namely the branching enzyme GlgB and the α-(1,4)-glucan:maltose 1-phosphate maltosyl transferase GlgE, may represent good targets for drug development since their inactivation is expected to result in the lethal accumulation of maltose-1-phosphate (Sambou *et al*., 2008; Kalscheuer *et al*., 2010). To the best of our knowledge, no inhibitors of these enzymes have yet been reported.

Conclusions and Future Prospects

The progress made in elucidating the biosynthetic pathways of the major cell envelope glycoconjugates of *Mtb* since the beginning of the mycobacterial genomic/genetic era in the late 1990s has been substantial and so has our understanding of the roles of these molecules in the physiology and pathogenesis of this paramount bacterial pathogen. Yet, important challenges lay ahead.

Several key biosynthetic enzymes have yet to be identified. In the PIM pathway, these include the α-(1,6)-ManT(s) initiating the elongation of the mannan backbone of LM from PIM_3; the α-(1,2)-ManT responsible for the formation of PIM_6 from PIM_5; and the acyltransferase catalyzing the acylation of position 3 of *myo*-Ins. In the LM and LAM pathways, an activity consistent with the priming AraT that transfers the first Ara*f* residue to the mannan backbone of LM was recently detected in cell-free assays but the identity of the corresponding enzyme is not known. The number and identities of the α-(1,5)-AraTs involved in the elongation of the arabinan domains of AG and LAM and the precise contribution of the Emb proteins in this process also remain to be defined. In light of the renewed interest in PG synthesis and recycling as a target for new and repurposed drugs (Jackson *et al*., 2013), more work is required to fully define the enzymes involved. With regards to glycoproteins, further work will need to determine whether *Mtb* proteins may be modified with other sugars than mannose and the identity the underlying glycosyltransferases, whether shared with other biosynthetic pathways as seems to be the case with the α-(1,2)-ManT PimE in *M. smegmatis* (Liu *et al*., 2013), or otherwise. Another biosynthetic step of considerable interest for its potential to uncover new drug targets is the attachment of AG to PG. The missing enzymes involved in all of these key aspects of the physiology of *Mtb* may be found in the numerous as yet unannotated ORFs of the *Mtb* genome, including some bearing conserved motifs suggestive of sugar-modifying enzymes (Pavelka *et al*., 2014; Berg *et al*., 2007; Slayden *et al*., 2013). Concomitantly, our continuously evolving view of the fine structures of *Mtb* glycoconjugates may reveal the existence of previously unsuspected ‘decorating’ enzymes, such as the ones involved in the biosynthesis of the MTX motif of ManLAM or the succinylation and galactosaminylation of the arabinan chains of AG and ManLAM. Based on the restricted distribution of some of these modifications to pathogenic *Mycobacterium* species and what is known of the biological activities of minor covalent modifications of lipopolysaccharides in other bacterial pathogens (Raetz *et al*., 2007; Kanistanon *et al*., 2008; Hamad *et al*., 2012), one may expect these discrete substituents to play a role in pathogenesis. Validation of this assumption, however, awaits in most cases the availability of *Mtb* mutants specifically deficient in the production of these motifs.

Beyond the identification and characterization of individual enzymes, more substantial challenges to be faced in terms of biogenesis reside in defining the sequential order of the reactions leading to the elongation, assembly and export of *Mtb* glycoconjugates, the processes involved in chain termination, and the nature and spatial organization of the translocation machineries. As illustrated by this review, recent years have seen a number of breakthroughs made on these fronts in the context of PG, PIM, LM/LAM, AG, glycoprotein and acyltrehalose biosynthesis allowing for more accurate models of these pathways [Fig. 4,5,6,10]. A pattern that has begun to emerge from these studies is that, analogous to other prokaryotic and eukaryotic systems, much of the biosynthesis of glycoconjugates in mycobacteria seems to rely on multiprotein complexes (e. g., acyltrehaloses, AG, PG), and involves tightly coordinated polymerization, modification and translocation events on both sides of the plasma membrane (e. g., AG, LM/LAM, glycoproteins). In some cases, biosynthesis and export may be coupled (e. g., acyltrehaloses, glycoproteins). Unlike other systems, however, the polymerization of building blocks in the assembly of complex

glycoconjugates has not yet been reported in *Mtb*, and evidence to date instead points to the sequential addition of mannosyl, arabinosyl and galactosyl residues in the biosynthesis of AG, LM and LAM. Moreover, mycobacteria seem to have evolved somewhat unusual translocation mechanisms to export their (lipo)polysaccharides and glycolipids possibly reflecting the unique structure and composition of their cell envelope (e. g, MmpL proteins and periplasmic LppX-like lipoproteins in the transport of acyltrehaloses, lipids, siderophores and PIMs). Certainly, the missing components of the translocation machineries of AG, PIM, LM, LAM, MPM, PGL, acyltrehaloses and capsular polysaccharides are to be found among the numerous putative transporters of unknown function encoded by the *Mtb* genome (Slayden *et al*., 2013). Yet, the poor sequence similarity typically shared by prokaryotic transporters and general lack of identifiable motifs in their primary sequence complicates their identification. Moreover, our preliminary evidence indicates that the relaxed substrate specificity and thus redundancy of many of these transporters (e. g., the Dec-P-Ara flippase Rv3789) represents a major obstacle to their functional characterization as it limits the usefulness of genetic strategies based on the phenotypic analysis of knock-out or knock-down mutants.

Clearly, much remains to be done on the topic of mycobacterial glycoconjugates, particularly in establishing the composition of the multiprotein complexes involved in their biogenesis and elucidating the pivotal processes responsible for the translocation of biosynthetic intermediates and end products of these pathways across the different layers of the *Mtb* cell envelope. Pursuing this fascinating avenue of research is required for a complete understanding of the physiology and pathogenesis of *Mtb* as much as for the development of new drugs, vaccines, diagnostics and biomarkers.

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Abbreviations

AG	arabinogalactan
AM	D-arabino-D-mannan
Ara*f*	arabino-furanose
AraT	arabinosyltransferase
Dec-P	decaprenyl-monophosphate
Dec-PP	decaprenyl diphosphate
Dec-P-Man	decaprenyl-monophospho-mannose
DAT	diacyltrehaloses

Dec-P-Ara	decaprenyl-monophospho-arabinose
EMB	ethambutol
Gal*p*	galactopyranose
Gal*f*	galacto-furanose
LM	lipomannan
LAM	lipoarabinomannan
LPS	lipopolysaccharide
Man*p*	mannopyranose
ManT	mannosyltransferase
***myo*-Ins**	*myo*-Inositol
PAT	polyacyltrehaloses
PDIM	phthiocerol dimycocerosates
PG	peptidoglycan
PI	phosphatidyl-*myo*-inositol
PIMs	phosphatidyl-*myo*-inositol mannosides
SL	sulfolipids

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PIM is used to describe the global family of PIM that carries one to four fatty acids and 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 hexamannoside PIM_6 carrying two acyl groups attached to the glycerol (the diacylglycerol substituent) and one acyl group esterified to the Man*p* residue.

The *Mtb* gene nomenclature used is that of the *Mtb* strain H37Rv. The *M. smegmatis* gene nomenclature used in this review is that currently in use for the *M. smegmatis* strain mc^2155.

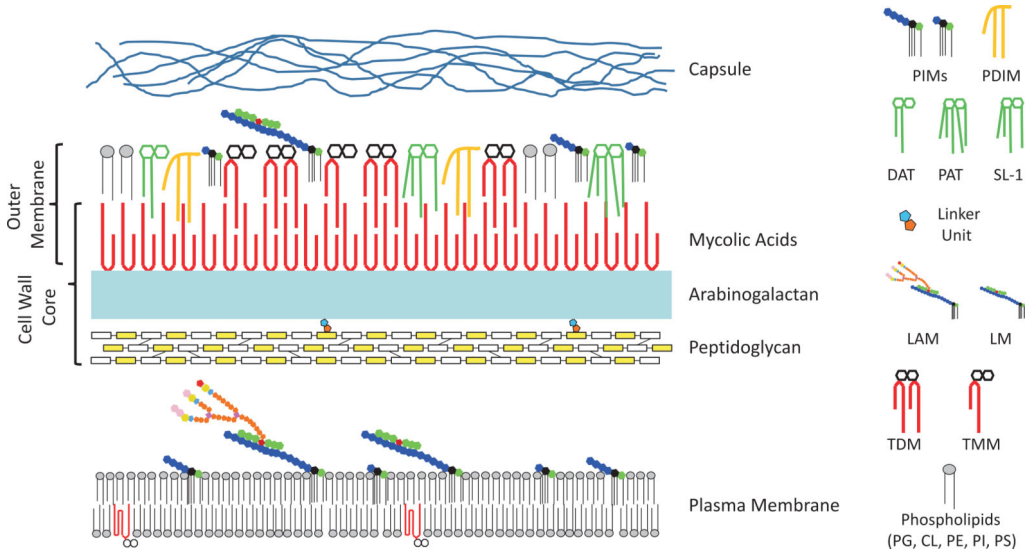

Figure 1. Schematic representation of the *Mtb* cell envelope

Many of the classes of lipids and glycolipids discussed in the review are represented schematically and are shown in probable locations in the cell envelope. The overall schematic and individual structures are not drawn to scale. Proteins and peptides are not shown for the sake of clarity. The color code used in the representation of LM and LAM is the same as in Fig. 6. PE, phosphatidylethanolamine, PI, phosphatidyl-*myo*-inositol; CL, cardiolipin; PS, phosphatidylserine; PG, phosphatidylglycerol.

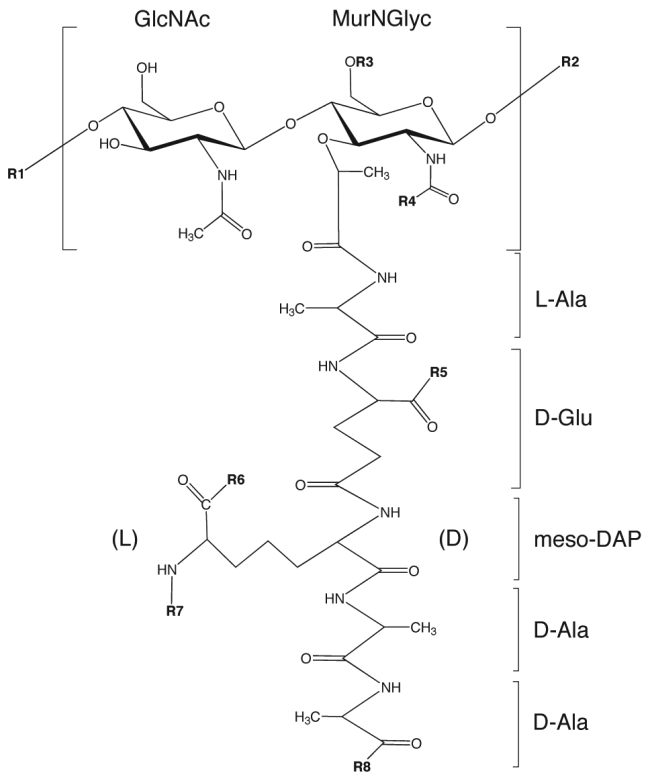

Figure 2. Structures of a representative monomer of mycobacterial PG prior to peptide trimming

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.

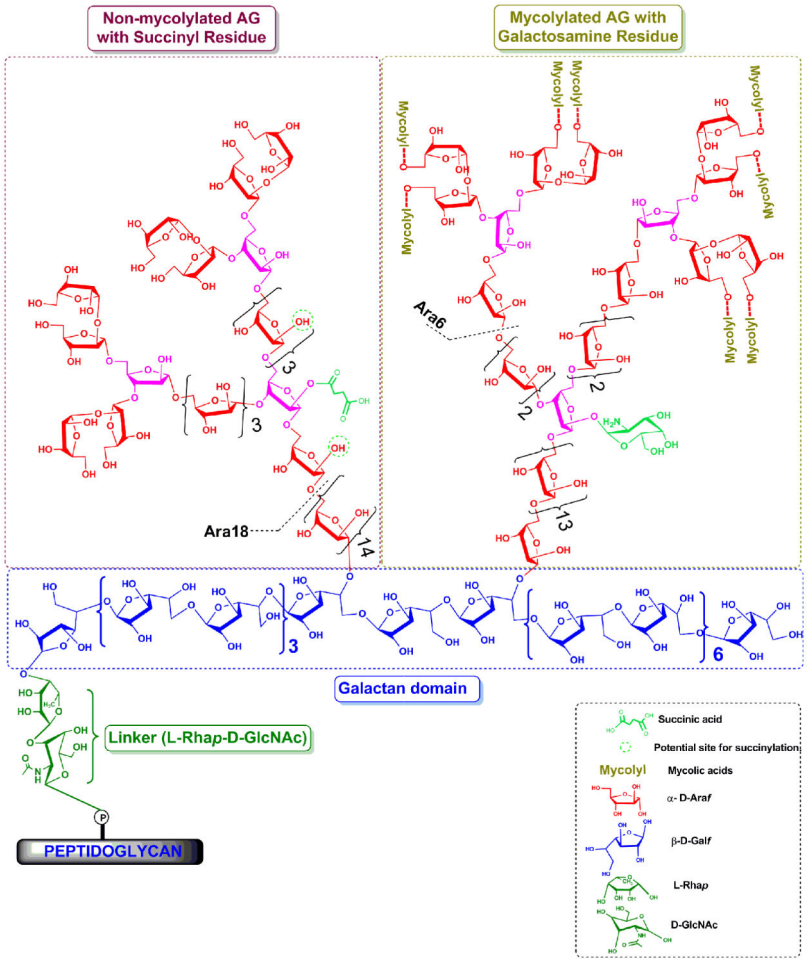

Figure 3. Structure of arabinogalactan

See text for details.

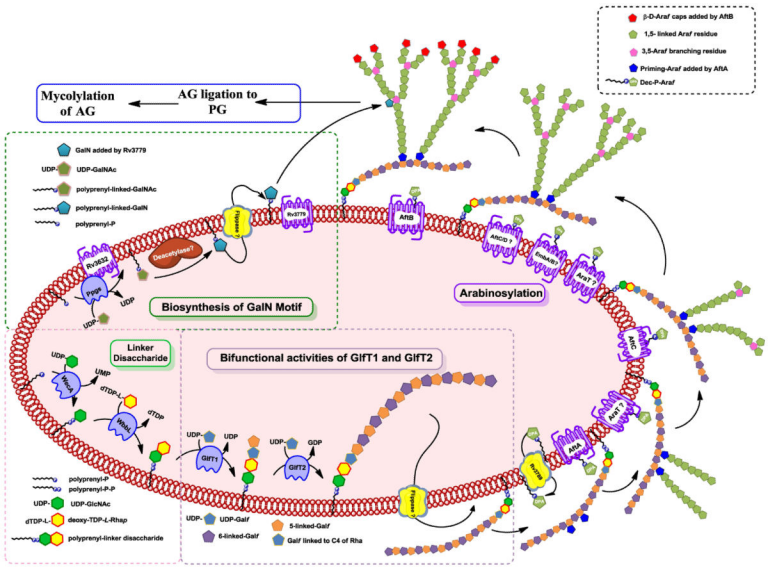

Figure 4. Schematic diagram of arabinogalactan biosynthesis

The synthesis of AG begins with the cytoplasmic formation of the linker unit on a decaprenyl monophosphate carrier lipid followed by the addition of Gal*f* residues still on the cytosolic face of the plasma membrane and that of Ara*f* residues and other decorating motifs (e.g., GalN motif) on the periplasmic side of the membrane. See text for details.

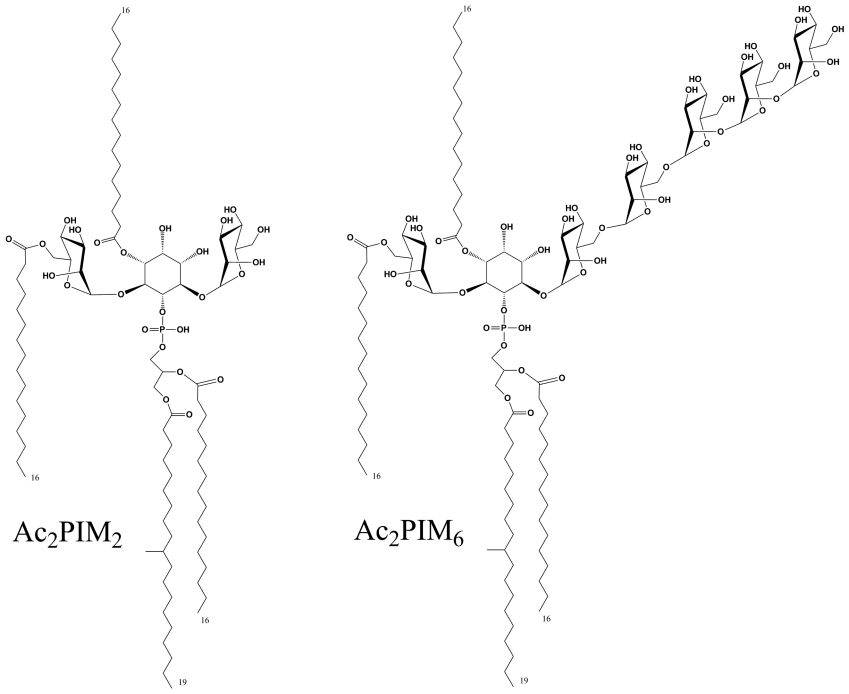

Figure 5. Structures of the two major tetracylated forms of PIM_2 and PIM_6

The forms of PIM_2 and PIM_6 represented here both harbor three palmitic and one tuberculostearic acyl chains.

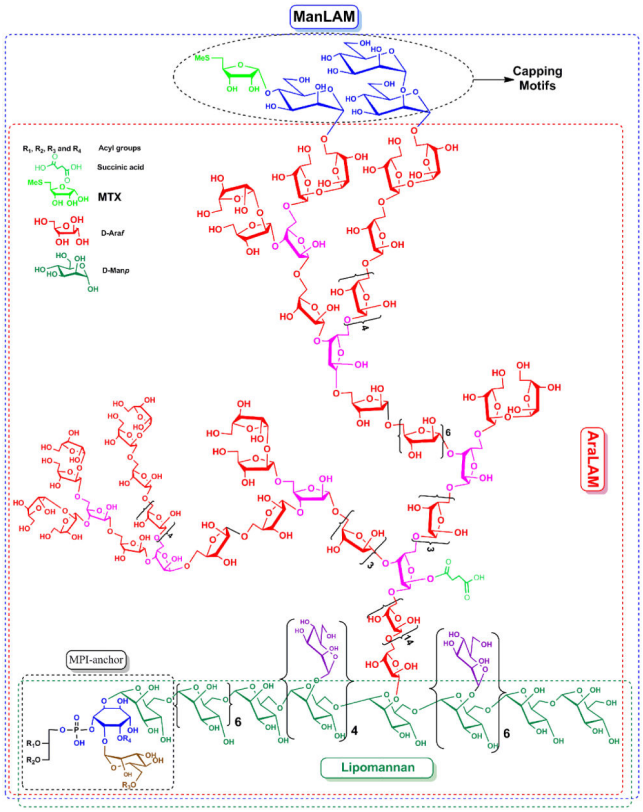

Figure 6. Structures of LM and LAM

See text for details. MPI, mannosylated phosphatidyl-*myo*-inositol anchor.

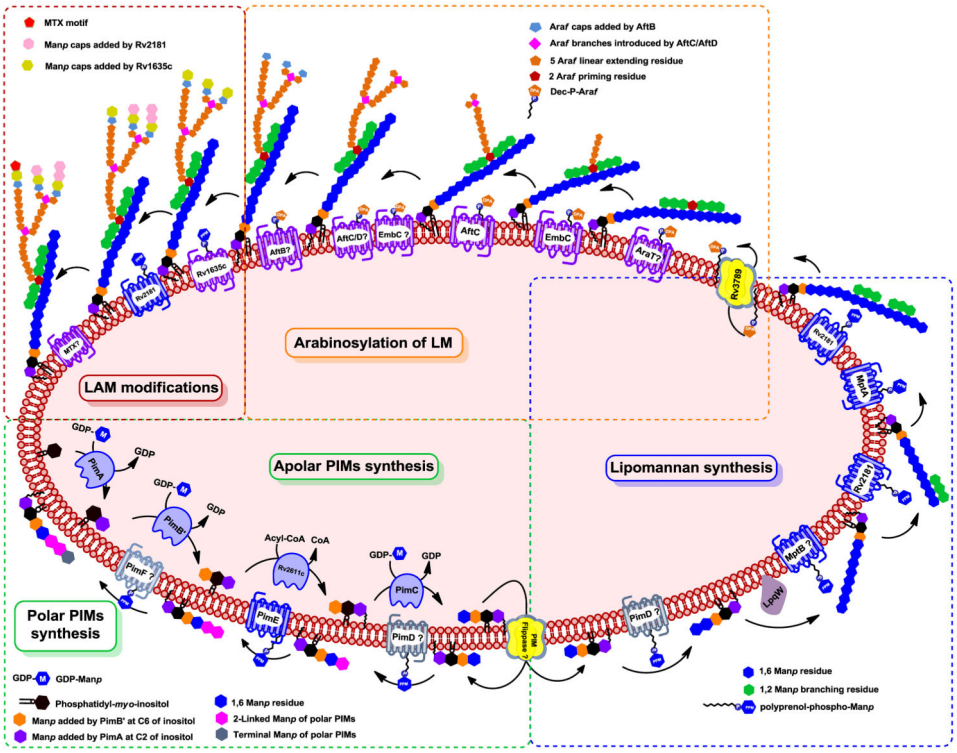

Figure 7. Schematic diagram of PIM, LM and LAM biosynthesis

The biosynthesis of PIM, LM and LAM is initiated on the cytoplasmic side of the plasma membrane by GDP-Man*p*-utilizing ManTs that catalyze attachment of mannosyl residues to the *myo*-inositol ring of PI. Di- or tri-mannosylated forms of PIMs are then flipped to the periplamic face of the membrane where they undergo further elongation catalyzed by integral membrane polyprenyl-monophospho-mannose-dependent ManTs and β-D-arabinofuranosyl-1-monophosphoryl-decaprenol (DPA)-dependent AraTs to generate polar forms of PIMs, LM, and ManLAM. See text for details.

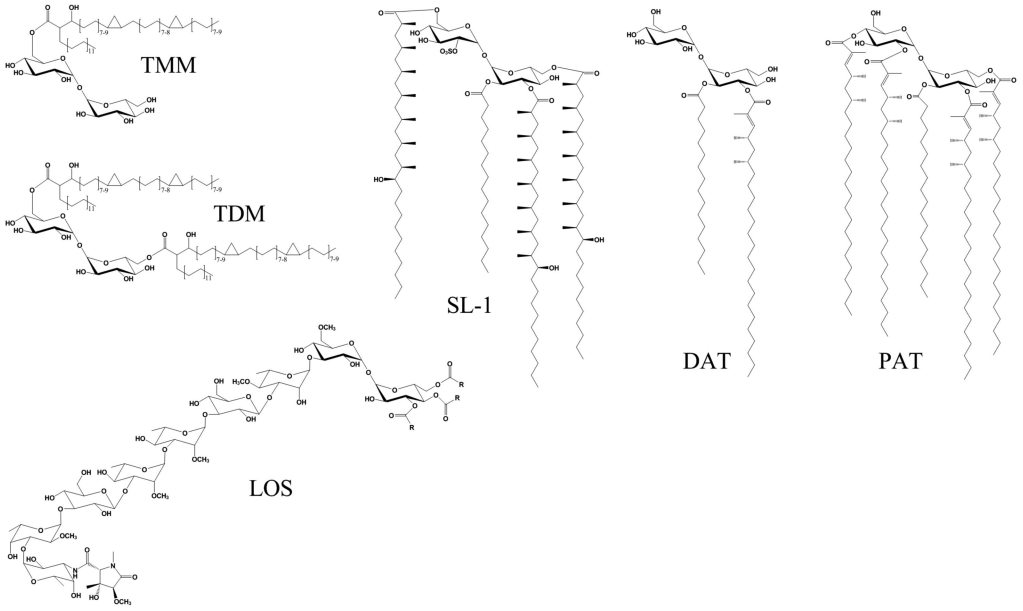

Figure 8. Structures of the acyltrehaloses of *Mtb*

In TMM and TDM, trehalose is here shown esterified with alpha-mycolic acid chains. In SL-I (2,3,6,6'-tetraacyl α–α'-trehalose-2'-sulfate), 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 palmitic acid and the multimethyl-branched mycosanoic acid. In PAT (penta-acyltrehalose), trehalose is esterified with stearic acid and the multimethyl-branched mycolipenic acids. The oligosaccharide of the LOS of *Mtb* Canettii 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-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.

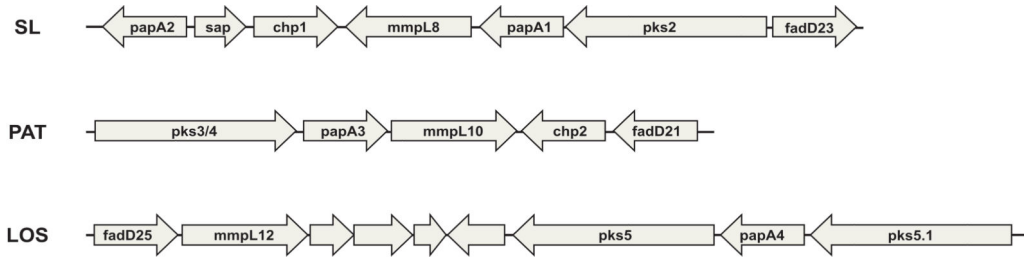

Figure 9.

A schematic representation of the SL, DAT/PAT and LOS biosynthetic gene clusters of *Mtb* H37Rv (SL; DAT/PAT) and *Mtb canettii* (LOS).

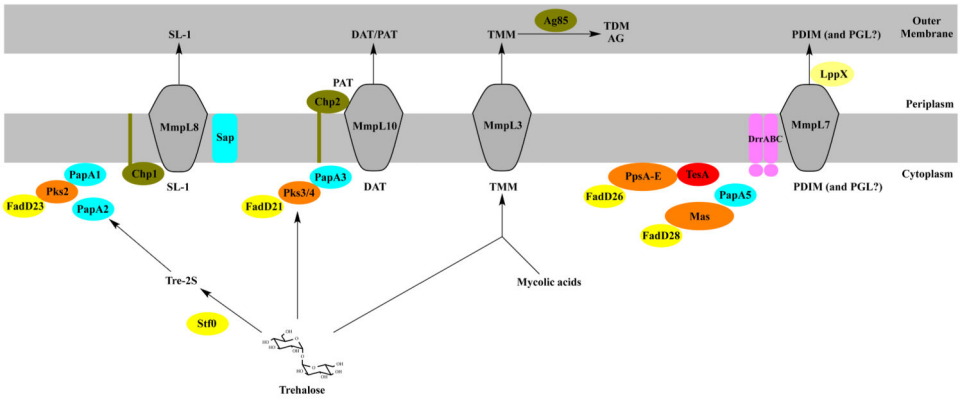

Figure 10. Biogenesis of SL, DAT/PAT and PDIM/PGL in *Mtb*

The enzymes and transporters that have been involved in the elongation, assembly and export of SL, DAT/PAT and PDIM/PGL and their localization in the bacterium are represented.

FadD enzymes are fatty acyl-AMP ligases; PapA and Chp enzymes are acyltransferases; Pks, Mas and PpsA-E are the polyketide synthases responsible for the elongation of the polymethyl-branched fatty acids found in DAT/PAT, SL and PDIM/PGL; TesA is a thioesterase; Stf0 is a sulfotransferase; Antigens 85 (Ag85) are mycolyltransferases; DrrABC is an ABC-transporter; LppX is a periplasmic lipoprotein required for the translocation of PDIM to the outer membrane; MmpL proteins are integral membrane RND superfamily transporters required for the translocation of acyltrehaloses and PDIM to the periplasmic space and outer membrane. The precise extent of (glyco)lipid translocation mediated by MmpL proteins, LppX and DrrABC has has not yet been defined. See text for further details.

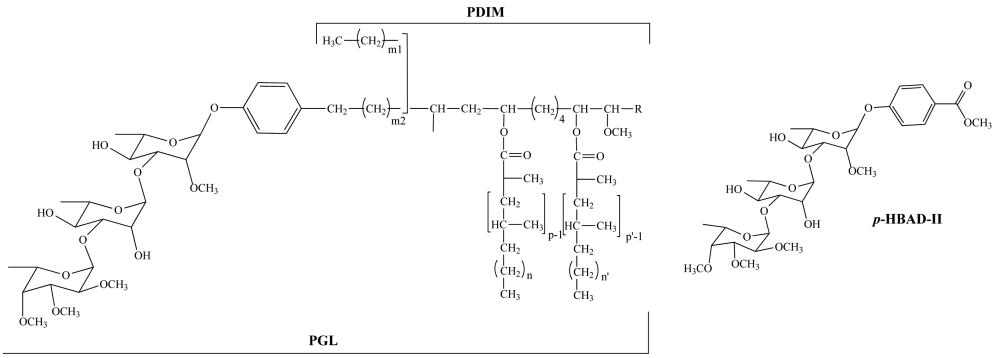

Figure 11. Structures of the phthiocerol dimycocerosates (PDIM), phenolic glycolipids (PGL) and *p*-hydroxybenzoic acid derivatives (*p*-HBADs) of *Mtb*

The lipid core of PGL from *Mtb* is composed of phenolphthiocerol esterified by mycocerosic acids (p, p'=3-5; n, n'=16-18; m2=15-17 ; m1= 20-22; R= CH_2-CH_3 or CH_3). The trisaccharide substituent of PGL and *p*-HBAD-II (i.e., the fully elaborated form of *p*-HBAD produced by *Mtb*) 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*.

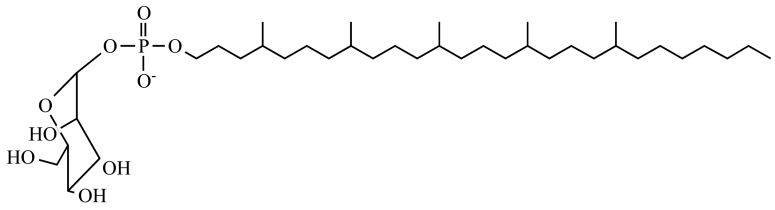

Figure 12.
Structure of the predominant mannosyl-β-1-phosphomycoketide from *Mtb* H37Rv.

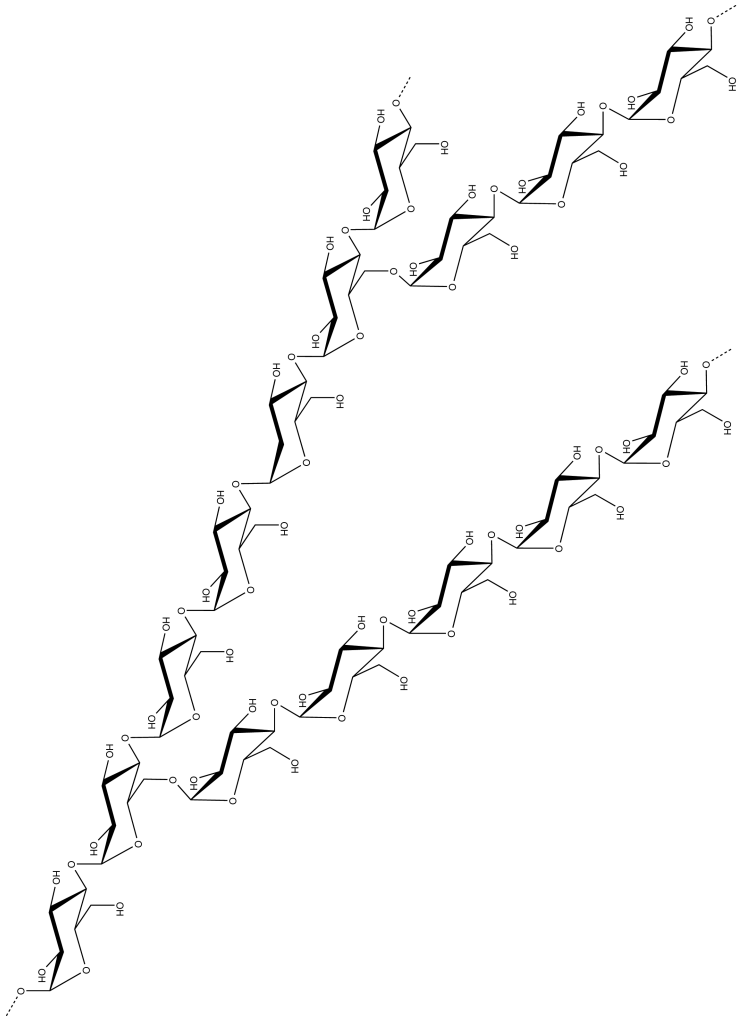

Figure 13.
Structure of the capsular α-D-glucan of *Mtb*.

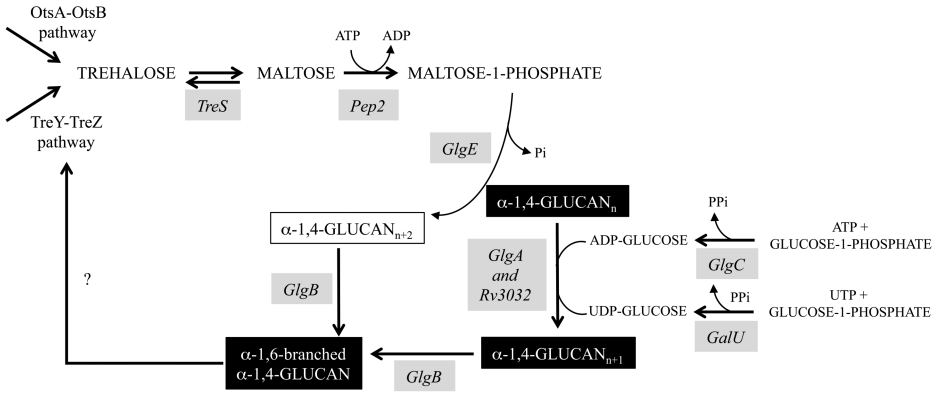

Figure 14. Biosynthesis of α-D-glucans in *Mtb*

See text for details.