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Chapter 6

Metabolism of Plasma Membrane Lipids in Mycobacteria and Corynebacteria

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1. Introduction

Bacteria of the Corynebacterineae, a suborder of the Actinobacteria, comprise *Mycobacterium*, *Corynebacterium*, *Nocardia*, *Rhodococcus* and other genera. This suborder of high GC gram-positive bacteria includes a number of important human pathogens, such as *Mycobacterium tuberculosis*, *Mycobacterium leprae* and *Corynebacterium diphtheriae*, the causative agents of tuberculosis, leprosy and diphtheria, respectively. *M. tuberculosis* is the most medically significant species, a devastating human pathogen infecting around one-third of the entire human population and responsible for more than 1 million deaths annually. The Corynebacterineae also includes non-pathogenic species such as *Mycobacterium smegmatis*, a saprophytic species, and *Corynebacterium glutamicum*, an industrial workhorse for the production of amino acids and other useful compounds. These relatively fast-growing species serve as useful models to study metabolic processes essential to the growth and survival of the slow-growing pathogens.

All these bacteria share a common feature, a distinctive multilaminate cell wall composed of peptidoglycan, complex polysaccharides, and both covalently linked lipids and free lipids/lipoglycans (Fig. 1). Among them, mycolic acids are the hallmark of these species. These long chain α-branched, β-hydroxylated fatty acids are covalently linked to the arabinogalactan polysaccharide layer. This mycolic acid layer is complemented by a glycolipid layer to form an outer "mycomembrane" analogous to the outer membrane of Gram-negative bacteria. [1, 2]. The outer leaflet of the mycomembrane is composed of a variety of lipids including trehalose dimycolates (TDMs), glycopeptidolipids (GPLs), phthiocerol dimycocerosates (PDIMs), sulfolipids, phenolic glycolipids (PGLs), and lipooligosaccharides. Some of these lipids are widely distributed while others are restricted to particular species. For example, TDMs and their structural equivalents are found in both mycobacteria and corynebacteria, while PDIMs and PGLs are restricted to a subset of

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mycobacteria. The structure and hydrophobic properties of the cell wall make it a potent permeability barrier that is responsible for intrinsic resistance of mycobacteria to an array of host microbiocidal processes, many antibiotics and sterilization conditions [3, 4]. Many of the cell wall components of pathogenic mycobacterial species are essential for pathogenesis and *in vitro* growth, hampering efforts to characterize the function of individual proteins in their assembly. In contrast, some non-pathogenic species such as *C. glutamicum* can tolerate the loss of major cell wall components, making them useful model systems for delineating processes involved in the assembly of core cell wall structures.

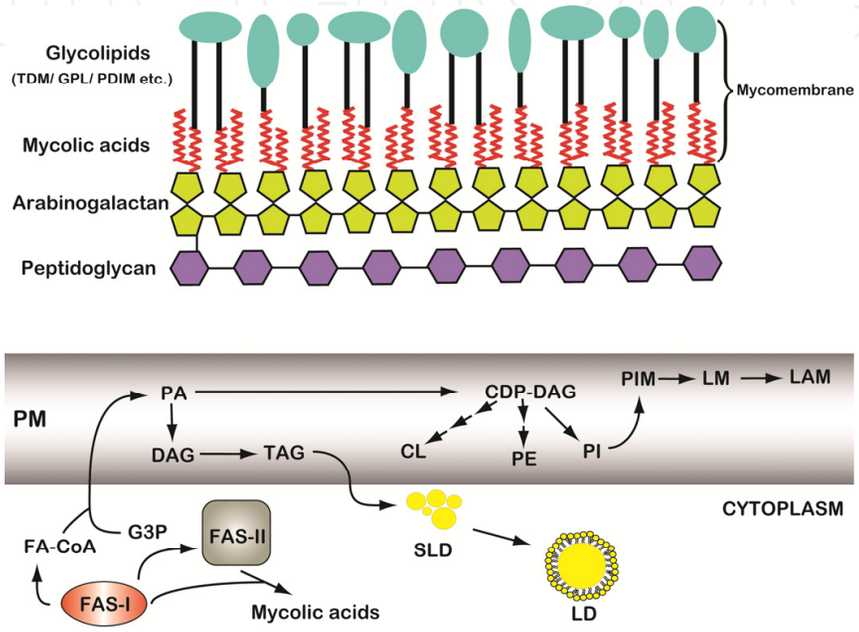


Figure 1. Mycobacterial plasma membrane and cell wall with flow of key metabolic pathways. Some of the metabolites are exported to the mycomembrane. SLD, small lipid droplet; LD, lipid droplet; FA-CoA, fatty acyl-CoA. See text for other abbreviations used in the figure.

Studies on mycobacteria and corynebacteria provide a unique opportunity to illustrate the complexity and diversity of lipid metabolic pathways in bacteria. They have a significantly higher lipid content than other bacteria with cell wall lipids comprising ~40% of the dry cell mass. *M. tuberculosis* produces a diversity of lipids unparalleled in bacteria, from simple fatty acids to highly complex long chain structures such as mycolic acids. It has devoted a significant proportion of its coding capacity to lipid metabolism and produces about 250 enzymes dedicated to fatty acid metabolism, which is around five times the number produced by *Escherichia coli* [5]. Lipid biosynthesis places a significant metabolic burden on the organism but is ultimately advantageous, allowing *M. tuberculosis* to survive and replicate in the inhospitable environment of host macrophages. While capable of *de novo* synthesis, these bacteria also scavenge and degrade host cell membrane lipids to acetyl-CoA, via broad families of β-oxidation and other catabolic enzymes, for incorporation into their own metabolic pathways and to fuel cellular processes.

The plasma membrane provides the platform for lipid metabolism. While some lipid metabolic reactions take place in the cytoplasm or cell wall, the plasma membrane is the

pivotal site for the metabolism of lipids. At the same time, this membrane must perform many other functions associated with energy production, nutrient uptake, protein export, and various sensing/signaling reactions. Studies on how these metabolic and cellular processes might be organized within bacterial plasma membranes are in their infancy. Understanding the homeostasis of the plasma membrane is particularly important in Corynebacterineae organisms because this structure must support the high biosynthetic demands of sustaining such a lipid-rich cell wall. In this chapter, we focus our discussion on processes of lipid metabolism that are critical for the biogenesis and maintenance of the plasma membrane, and illustrate the recent progress on our understanding of plasma membrane biogenesis in mycobacteria and corynebacteria.

2. Functions of plasma membrane lipids in mycobacteria and corynebacteria

In this section we will describe the functions of plasma membrane lipids. First, we will describe the functions of major structural phospholipids. We will then describe quantitatively minor lipids, which have important metabolic/physiological functions. Lastly, we will discuss the functions of neutral lipids because their biosynthesis is closely linked to phospholipid metabolism and neutral lipid storage is a critical part of plasma membrane homeostasis.

2.1. Structural lipids

Major structural components of the mycobacterial plasma membrane are phospholipids such as cardiolipin (CL), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and glycosylated PIs (*i.e.* phosphatidylinositol mannosides (PIM), lipomannans (LM) and lipoarabinomannans (LAM), see below). The ratio of these phospholipids may vary depending on the species and growth conditions [6-8]. For example, one study indicated that CL, PE, and PI/PIMs represent about 37, 32, and 28%, respectively, of the total phospholipids in the plasma membrane in *M. smegmatis* [9], while another reported the ratio in *Mycobacterium phlei* to be about 50, 10, and 40% [10]. Phosphatidylglycerol (PG), which is abundant in other bacteria, is a relatively minor species in mycobacteria. Deletion of the PI biosynthetic gene has been shown to be lethal in *M. smegmatis* [9], indicating that PI or glycosylated PIs are essential for mycobacterial viability. In *M. tuberculosis*, putative PI synthetase (*Rv2612c*) and PGP synthetase (*Rv2746c*, involved in CL synthesis) genes are predicted to be essential [11], while the PS synthetase gene (*Rv0436c*, involved in PE synthesis) is not [12]. In corynebacteria, major species of phospholipids are PI, PG, CL, and acylphosphatidylglycerol (APG) [13], and PE appears to be absent.

CL is widely found in both prokaryotes and eukaryotes. It forms aggregates within the membrane bilayer. Nonyl acridine orange (NAO) is a fluorescent dye which is proposed to bind the hydrophobic surface created by the CL cluster [14], allowing microscopic visualization of CL domains. Indeed, using NAO, CLs were found to be enriched in septa and poles of actively dividing *M. tuberculosis* and *M. smegmatis* cells [15, 16]. CL has a non-

bilayer structure [17, 18], and carries a small partially immobilized head group that is more exposed to the aqueous environment than those of other glycerophospholipids [19]. Although the physiological function of CL is unclear, its physical properties may indicate that it provides a platform for membrane-protein interactions. Indeed, some mycobacterial enzymes require CL for activity [20-22], although the molecular basis for these observations has not been clarified. Recent fractionation studies in *C. glutamicum* revealed that CL (as well as other phospholipids) is enriched in the plasma membrane [23, 24]. However, a large proportion of CL is also found to be associated with the outer membrane [24], suggesting that some of these phospholipids are exported to the outer membrane in corynebacteria. Similarly, CL is released from *M. bovis* bacillus Calmette-Guerin residing in host phagosomes, and converted to lyso-CL by a host phospholipase A_2 [25]. It has been suggested that lyso-CL may influence host immune responses during infection.

PE is another major class of glycerophospholipids in mycobacteria. Although PE is generally found in all organisms, it is particularly abundant in bacterial plasma membranes [26]. Mycobacteria are no exception [20], but corynebacteria apparently lack the capacity to synthesize PE [27]. Indeed, PE biosynthetic enzymes, such as PS synthetase and PS decarboxylase, appear to be absent in corynebacterial genomes. *Corynebacterium aquaticum* has been reported to possess PE [28], but this species was later reclassified as *Leifsonia aquatica* [29], which belongs to the suborder Micrococcineae of the order Actinomycetales. The functions of PE remain elusive at the molecular level, but it appears to play important roles as a component of the plasma membrane. For example, TBsmr, a small multidrug resistance family protein from *M. tuberculosis*, shows enhanced catalytic activities when PE is supplemented in a reconstituted liposome [30].

PIs are an important class of phospholipids, and are known to be further modified by extensive glycosylation. The resultant lipoglycans, termed PIMs, LM, and LAM, are essential structural components of mycobacterial and corynebacterial cell walls. Furthermore, in pathogenic species, they have been suggested to perform additional roles in the modulation of host immune responses in favor of the pathogen through myriad effects on macrophages including cytokine production, inhibition of phagosome maturation and apoptosis [31-34]. PIMs are oligo-mannosylated PIs carrying up to 6 mannose residues while LM/LAM carry much longer mannose polymers with arabinan modifications. It remains controversial if these glycolipids are embedded in the plasma membrane or exported to the outer membrane. A recent study suggests that LM/LAM appear to be anchored to both the plasma membrane and outer membrane [35]. In *C. glutamicum*, the outer membrane and plasma membrane were fractionated on sucrose gradients upon cell lysis, and the analysis of these membrane sub-fractions demonstrated that PIMs, LM and LAM are all enriched in the plasma membrane fraction [23]. Another recent study also suggested that PI/PIMs are major components of the plasma membrane of *C. glutamicum* [24]. In the latter study, however, substantial amounts of PI/PIMs were detected in the outer membrane as well. The functional significance of these subcellular localizations, as well as the physiological roles of LM/LAM in each of these locations, remain important questions. The structural importance of PIMs remains unclear as well. For example, a *pimE*-deletion mutant that cannot produce mature

PIM6 species (see below) is viable, but shows severe plasma membrane abnormalities [36], suggesting that higher order PIMs may be involved in the maintenance of plasma membrane integrity.

It is notable that some unusual phospholipids have been identified in corynebacteria. APG is an acylated form of PG which is widespread in corynebacteria [37-40], and is a major phospholipid species in *Corynebacterium amycolatum*. Another interesting phospholipid from *C. amycolatum* is acyl-phosphatidylinositol (API), which was identified by electrospray ionization mass spectroscopy [41]. *C. amycolatum* lacks a mycolic acid-based outer membrane, and does not appear to have a fracture plane other than the plasma membrane [42]. Therefore, APG and API are likely to be components of the plasma membrane, and are suggested to play structural roles. Very little is known about their biosynthesis, and acyltransferases responsible for their synthesis remain to be identified for both lipid species.

2.2. Functional lipids

There are some examples of lipids that appear to play no structural roles in the plasma membrane. They often exist in low quantities but play important functional roles. Among these, polyprenol-phospho-sugars function as sugar donors. Two well-studied examples are polyprenol phosphomannose (PPM) and decaprenol phosphoarabinose (DPA). These molecules are the donors of mannose and arabinose, respectively, and their biosynthesis will be discussed in a later section.

PI 3-phosphate, recently identified in both *M. smegmatis* and *C. glutamicum* [43], may prove to be another interesting example of a functional lipid. It accumulates only transiently upon stimulation by high concentrations of salt, and behaves as if it is involved in a signaling cascade. However, whether PI 3-phosphate represents a mediator of stress responses remains to be addressed. More recently, lysinylated PG was identified as a minor phospholipid species in *M. tuberculosis* [44]. The synthesis of lysinylated PG is mediated by LysX and a *lysX* deletion mutant showed altered phospholipid metabolism and membrane integrity [16, 44], suggesting a regulatory role of lysinylated PG in plasma membrane homeostasis.

Carotenoids are photo-protective pigments and serve to scavenge free radicals or harvest light [45]. Several mycobacterial species are known to produce carotenoids with the notable exception of *M. tuberculosis*, despite the presence of a carotenoid oxidase in the human pathogen [46]. These hydrophobic pigments are thought to be present in the plasma membrane but whether they play structural roles in addition to a photo-protective role remains to be elucidated.

2.3. Lipid storage for energy and carbon

Neutral lipids are an important reservoir of stored energy and carbon, and their metabolism is closely linked to plasma membrane phospholipid metabolism. Unlike many other bacteria which use polyhydroxyalkanoates as a lipid storage material [47], Actinobacteria use

triacylglycerides (TAGs) as a major form of lipid storage, and the presence of TAGs has been reported in *Mycobacterium*, *Streptomyces*, *Rhodococcus* and *Nocardia* [48-52]. Interestingly, corynebacteria seem to lack the capacity to synthesize TAG, indicating that some lineages of Actinobacteria have eliminated this capacity at some point in their evolution. Recent evidence suggests that *M. tuberculosis* accumulates TAG-based lipid droplets while residing in macrophages using fatty acids released from host TAGs, and this process is critical for acquiring a dormancy phenotype [53]. Nevertheless, a mutant defective in accumulating TAG remained viable under *in vitro* dormancy-inducing conditions [54]. These somewhat contradictory observations suggest that our understanding of TAG metabolism in mycobacteria is far from complete. As we illustrate later, there appear to be several redundant genes involved in the final step of TAG synthesis, suggesting that it is an important regulatory step of lipid metabolism in these bacteria.

Cholesterol has recently been suggested to be an alternative form of lipid storage in mycobacteria. Neither mycobacteria nor corynebacteria have the capacity to synthesize cholesterol. However, cholesterol is taken up by *M. tuberculosis* cells residing in the host, and components of the *mce4* operon have been shown to be involved in cholesterol import [55]. Cholesterol catabolism is critical in the chronic phase of animal infection, and a fully functional catabolic pathway is encoded by the *M. tuberculosis* genome [56]. Furthermore, cholesterol appears to accumulate in the mycobacterial cell envelope, and this might represent a potential form of lipid storage for *M. tuberculosis* during animal infection [57, 58]. Although the authors of this study suggested that cholesterol accumulates in the outer membrane, it remains possible that the plasma membrane is the true site of accumulation. Therefore, in addition to acting as a lipid storage molecule, cholesterol may play roles in plasma membrane structure and function, and these possibilities await further exploration.

Catabolism of cholesterol, amino acids and odd-chain-length/methyl branched fatty acids produces propionyl-coenzyme A (CoA). Propionate accumulation has been shown to be toxic in various organisms [59-61], and *M. tuberculosis* has multiple pathways to metabolize propionyl-CoA [62]. Metabolized propionyl-CoA is in part incorporated into TAG [63], and it has been suggested that TAG functions as a sink for reducing equivalents in addition to being a source of carbon and energy.

3. Structure and metabolism of plasma membrane lipids in mycobacteria and corynebacteria

In this section, we will describe the structure and metabolism of various lipids found in the plasma membrane of mycobacteria and corynebacteria in more detail. Lipids are categorized into the following four classes based on their key structural features.

3.1. Fatty acids

M. tuberculosis devotes a large proportion of its coding capacity to genes involved in fatty acid metabolism [5], highlighting the importance of lipids to the organism. Fatty acid

metabolism is essential for intracellular survival of the pathogen since it forms the precursors of key membrane components such as plasma membrane phospholipids and outer membrane glycolipids. In particular, mycolic acids, which are very long chain α-alkyl β-hydroxy fatty acids, form the hydrophobic, protective mycomembrane described earlier. *M. tuberculosis* encodes two distinct enzyme systems for biosynthesis of fatty acids, designated FAS (fatty acid synthase) I and II (Fig. 2). Studies on fatty acid synthesis date back to the 1970s when *M. smegmatis* was shown to contain both type I fatty acid synthetase (FAS-I), involving a large multifunctional polypeptide, and type II fatty acid synthetase (FAS-II), consisting of a series of distinct enzymes [64]. The key elongation unit is malonyl-CoA, which is produced by acetyl-CoA carboxylase (ACCase) and the *M. tuberculosis* genome encodes several such enzymes (AccA1-3 and AccD1-6). The resultant malonyl-CoA is incorporated into fatty acids by the two FAS systems.

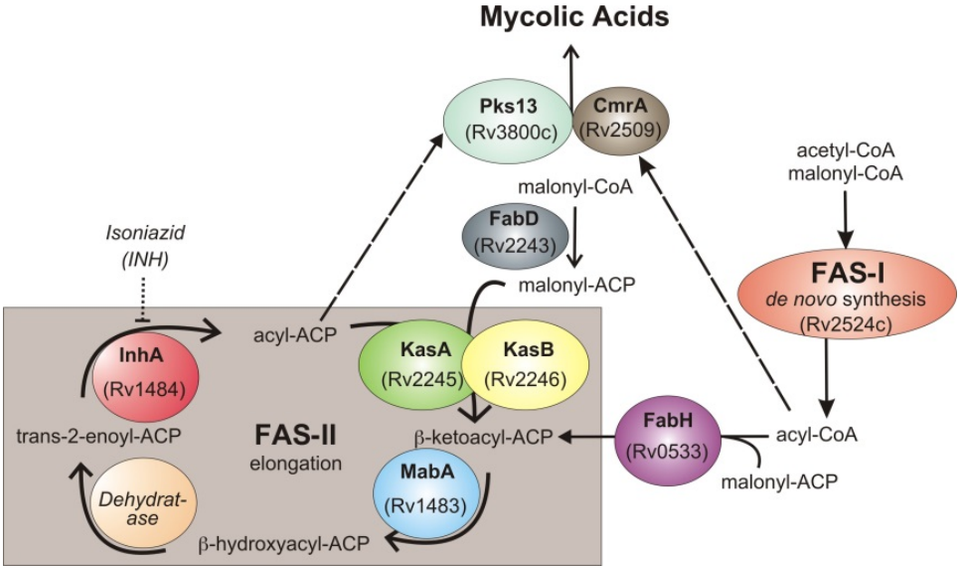


Figure 2. Fatty acid biosynthesis pathways in mycobacteria. Point of inhibition by the front-line tuberculosis drug isoniazid is indicated. Product profile of FAS-I is bimodal, and C_{16}-C_{18}-CoA and C_{24}-C_{26}-CoA are produced. Dashed lines indicate that some of the fatty acid products are further utilized for mycolic acid production.

3.1.1. De novo synthesis by FAS-I

Surprisingly, members of the Corynebacterineae use a eukaryote-like FAS-I system for *de novo* fatty acid synthesis. The single, essential [11], 9.2kb *fas* gene encodes a 326 kDa protein containing all seven domains necessary to perform the iterative series of reactions: acyl transferase, enoyl reductase, β-hydroxyacyl dehydratase, malonyl transferase, acyl carrier protein, β-ketoacyl reductase, and β-ketoacyl synthase [65, 66]. This very large protein elongates acetyl groups by 2-carbon (acetate) units using acetyl-CoA and malonyl-CoA. Early rounds of elongation yield C_{16} to C_{18}-CoA products that are used for synthesis of membrane phospholipids or to feed into the FAS-II system. More extensive elongation yields C_{24}-C_{26} products that ultimately form the α-branch of mycolic acids. Unlike *M. tuberculosis*, *C. glutamicum* encodes two *fas* genes (*fasA* and *fasB*) with FasA taking the

dominant role [67]. The presence of two Fas proteins may compensate for the lack of a FAS-II system in this organism.

3.1.2. Elongation by FAS-II

The FAS-II system is commonly found in bacteria and plants and, unlike FAS-I, is composed of a series of separate enzymes, each performing one step in the pathway. FAS-II elongates medium chain fatty acids derived from FAS-I using malonyl-CoA, producing C_{18}-C_{30} fatty acids [68]. FAS-II has been extensively studied in *E. coli* [69] and orthologs of the *fab* genes have been identified in mycobacteria. AcpM is a mycobacterial acyl carrier protein (ACP) and plays a key role in transferring acyl groups between the various enzyme components [70]. The seven genes are located in two clusters on the *M. tuberculosis* chromosome [5], comprising *mtfabD-acpM-kasA-kasB-accD6* and *mabA-inhA*. Initially, the malonate group is transferred from malonyl-CoA to AcpM by the MtFabD protein. Then MtFabH performs a Claisen condensation of malonyl-ACP with acyl-CoA to form β–ketoacyl-ACP. A four-step cycle is then initiated [64] in which:

1. β–ketoacyl-ACP reductase MabA reduces the β–keto group with concomitant oxidation of NADPH
2. β-hydroxyacyl-ACP dehydratase dehydrates the β-hydroxyl to enoyl-ACP
3. enoyl-ACP reductase InhA, a target of the first-line anti-tuberculosis drug isoniazid (INH) [71], reduces enoyl-ACP to acyl-ACP with concomitant oxidation of NADPH
4. β-ketoacyl-ACP synthase KasA/B elongates acyl-ACP by 2 carbon units, forming β-ketoacyl-ACP, which can feed back into step 1.

In this way, the hydrocarbon chain increases by 2 carbons each cycle. Further elongation and processing of the products of FAS-II produces the precursors of the long meromycolate chains that are condensed with the α-branches derived from FAS-I by the large polyketide synthase Pks13 [72]. Reduction of the β–keto group by CmrA forms the mature C_{60}-C_{90} mycolic acid [73].

3.2. Glycerolipids

Glycerolipids include both nonpolar lipids and polar phospholipids. Their biosynthesis is overlapping and 1,2-diacyl-*sn*-glycerol 3-phosphate, commonly known as phosphatidic acid (PA), is an important intermediate at the branch point (Fig. 3) [74]. In this section, we focus our discussion on the biosynthesis of PA and its conversion to non-polar lipids. Non-polar lipids are generally divided into three different classes depending on the number of fatty acids attached to glycerol: monoacylglycerol (MAG), diacylglycerol (DAG) and TAG. TAG is a glycerol carrying three fatty acyl chains, and its biosynthesis diverges from phospholipid synthesis after the synthesis of PA. TAG is a major component of lipid droplets, which accumulate in the cytoplasm. How TAG is made in the plasma membrane and incorporated into lipid droplets remains largely unclear. Here, we provide an overview of the TAG metabolic pathway.

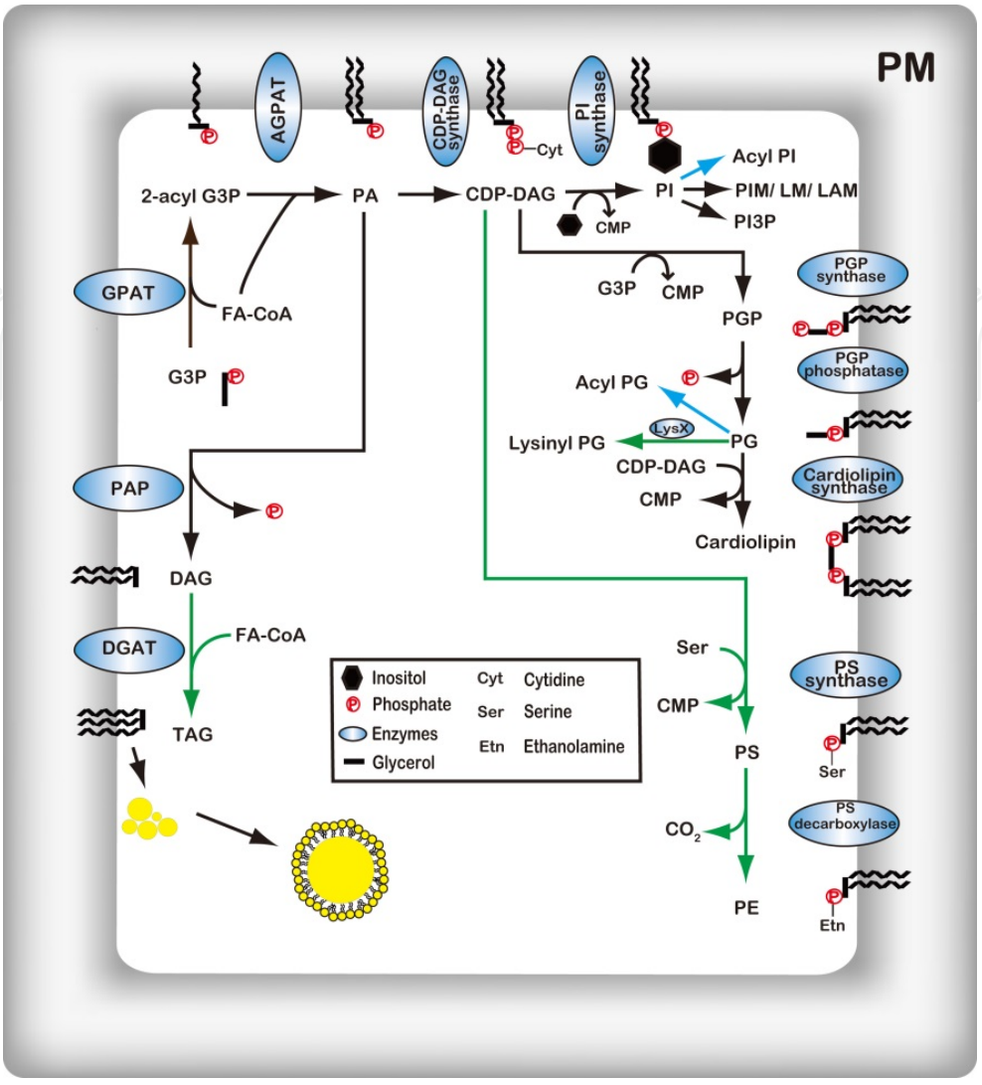


Figure 3. Glycerolipid/phospholipid biosynthesis pathways. Some pathways such as TAG and PE biosynthesis (shown as green arrows) do not occur in corynebacteria while some others (shown as blue arrows) are known to occur only in corynebacteria. PG is abundant in corynebacteria, but is a minor species in mycobacteria.

3.2.1. Biosynthesis of PA

The first step of PA biosynthesis is mediated by glycerol phosphate acyltransferase (GPAT) transferring an acyl chain from acyl-CoA to glycerol-3-phosphate, forming acyl-glycerol 3-phosphate. In general, this reaction produces 1-acyl-*sn*-glycerol 3-phosphate. However, mycobacteria are unusual in that 2-acyl-*sn*-glycerol 3-phosphate is used as the main intermediate for the production of PA [75]. Another unusual feature is that oleic acid, an unsaturated fatty acid often found at the *sn*-2 positions of glycerolipids, is found at the *sn*-1 position in mycobacteria. Instead, palmitic acid, a saturated fatty acid, is the preferred fatty acid attached to the *sn*-2 position in mycobacteria [75, 76]. In the second step, acylglycerol phosphate acyltransferase (AGPAT) further transfers a fatty acid from acyl-CoA to 2-acyl-*sn*-glycerol 3-phosphate, producing PA. PA can be diverted to TAG synthesis, or activated

to form cytidine diphosphate-diacylglycerol (CDP-DAG), which is the precursor for the synthesis of phospholipids. Therefore, PA represents an important branch point for the synthesis of TAG and phospholipids [74]. An alternative pathway for PA synthesis is phosphorylation of DAG by DAG kinase, and Rv2252 has been suggested to be involved in this reaction [77]. Disruption of this enzyme results in altered PIM biosynthesis, but precise functions of this metabolic pathway remain unclear.

3.2.2. TAG Biosynthesis

TAG is *de novo* synthesized by two steps. First, PA is dephosphorylated to become DAG, and this reaction is mediated by phosphatidic acid phosphatase (PAP). PAP was discovered from animal tissues in 1957 by the group of Eugene Kennedy [78], and the gene encoding this activity was recently identified in *Saccharomyces cerevisiae* [79]. Nothing is known about this enzyme in mycobacteria or corynebacteria. In the second step, diacylglycerol acyltransferase (DGAT) catalyzes the addition of a fatty acyl-CoA to DAG to form TAG. Until recently, little was known about the genes involved in this final step of TAG synthesis in mycobacteria. Analysis of this final step is complicated because there are multiple genes encoding TAG synthetase in mycobacteria and corynebacteria. For example, the *M. tuberculosis* genome encodes 15 putative TAG synthetase genes [48, 80]. Despite the redundancies, recent studies reported that some of these *tgs* genes are critical for TAG synthesis in *M. tuberculosis* [48, 54]. Specifically, TAG synthetases encoded by *Rv3130c* (*tgs1*), *Rv3734c* (*tgs2*), *Rv3234c* (*tgs3*), and *Rv3088* (*tgs4*) have been shown to have TAG synthetase activities [53]. Furthermore, Tgs1 has been demonstrated to be the main contributor to TAG synthesis and lipid droplet formation in *M. tuberculosis* [53]. More recently, Ag85A, which is known as a mycolyltransferase involved in TDM biosynthesis, was shown to possess DGAT activity [81]. Ag85A is not homologous to other *tgs* genes, and may represent a novel class of TAG biosynthetic enzymes. TAG not only forms a lipid droplet in the cytoplasm, but also accumulates in the cell wall of mycobacteria [82]. Therefore, Ag85A located in the cell wall might be involved in the production of surface-exposed TAGs.

3.2.3. Utilization of TAG

Under starvation conditions where stored TAG needs to be mobilized for energy production, TAG is catabolized by lipases. In 1977, TAG lipase was purified from stationary phase *M. phlei* and predicted to have a molecular weight of about 40 kDa [83]. More recently, LipY, encoded by the *M. tuberculosis Rv3097c* gene, was identified as a TAG lipase [84]. LipY appears to play a critical role in TAG catabolism because a *M. tuberculosis lipY* deletion mutant cannot utilize accumulated TAG under starvation conditions. Another recent study demonstrated that LipY has a dual localization pattern [85]: while a fraction of LipY was found in the cytoplasm, consistent with its role in the catabolism of intracellular TAG, a significant fraction of LipY was also localized to the outer membrane of the cell wall, indicating that it may be involved in the breakdown of exogenously available TAGs. Indeed, it has been long known that *M. tuberculosis* depends on fatty

acids as a preferred energy source during infection [86], and LipY may well be a critical enzyme for the utilization of host lipids during an *M. tuberculosis* infection. Another lipase encoded by *Rv0183* shows preference for MAG over DAG and TAG, and is localized to the cell wall [87], suggesting its involvement in subsequent reactions of TAG breakdown. However, whether it is involved in degradation of host-derived TAG or intracellular TAG remains to be determined.

3.2.4. Lipid droplet formation

In eukaryotes, lipid droplets form in between the two leaflets of the endoplasmic reticulum membrane [88]. In bacteria, a distinct mechanism of lipid body formation has been proposed. For example, in rhodococci, TAG is formed in the cytoplasmic surface of the plasma membrane. Small lipid droplets are then fused to each other, coated by a monolayer of phospholipids, and released from the surface of the plasma membrane into the cytoplasm as mature lipid droplets [89]. Although no endogenous proteins have been found to associate with lipid droplets in rhodococci or mycobacteria, heterologous expression of known lipid droplet-associated proteins resulted in correct targeting of these proteins to lipid droplets in both *R. opacus* and *M. smegmatis* [90, 91], allowing visualization of lipid droplets in these organisms.

3.3. Phospholipids

3.3.1. CDP-DAG

In both eukaryotic and prokaryotic cells, PA is activated by CTP to form CDP-DAG, and this reaction is mediated by CDP-DAG synthase [92]. The synthesis of CDP-DAG commits the pathway to phospholipid biosynthesis, and CDP-DAG is a common precursor for the biosynthesis of all glycerophospholipids in mycobacteria and corynebacteria. The activity of CDP-DAG synthetase is associated with plasma membrane in *M. smegmatis*, and is possibly encoded by the *cdsA* (*Rv2881c*) gene in *M. tuberculosis* H37Rv [93].

3.3.2. CL

CL is composed of four acyl chains, three glycerols and two phosphates, and is structured in a 1,3-diphosphatidylglycerol configuration [94]. It is a common phospholipid in bacteria, and is one of the abundant phospholipids in mycobacteria and corynebacteria. To initiate CL synthesis, PG phosphate synthase first produces PG phosphate (PGP) using CDP-DAG and glycerol 3-phosphate as substrates. An *M. smegmatis* strain engineered to overexpress *M. tuberculosis* PgsA3 (encoded by *Rv2746c*) was shown to overproduce PG, suggesting that PgsA3 is the PGP synthase [9]. PGP is then converted into PG via PGP phosphatase. Three phosphatases, PgpA, PgpB, and PgpC, have been identified as PGP phosphatases in *E. coli* [95-97]. Furthermore, Gep4 and PTPMT1 have been identified as PGP phosphatases in yeast and mammals, respectively [98, 99]. Some homologs exist in the genomes of mycobacteria and corynebacteria, but experimental verification of these genes remains to be performed.

Typically, the final step of CL synthesis in prokaryotes is mediated by a reaction that utilizes two PG molecules, producing one molecule of CL and one molecule of glycerol. However, in mycobacteria, the eukaryote-like reaction, which utilizes PG and CDP-DAG to produce CL, has been shown to occur [100], and Jackson and colleagues have suggested that PgsA2 might be the enzyme responsible for this reaction [9].

3.3.3. PE

The precise structure of PE was recently reported as 1-*O*-tuberculostearoyl-2-*O*-palmitoyl-*sn*-glycero-3-phosphoethanolamine in *M. tuberculosis* using tandem mass spectrometry [101]. In the initial step of PE synthesis, PS synthetase transfers serine to CDP-DAG and produces PS. In the second step, PE is produced by decarboxylation of PS mediated by PS decarboxylase. Genes encoding putative PS synthetase (*pssA*, *Rv0436c*) and PS decarboxylase (*psd*, *Rv0437c*) are found in tandem in the *M. tuberculosis* genome [9]. However, there is no experimental evidence demonstrating the identities of the genes. In *M. smegmatis*, PS synthetase and PS decarboxylase activities are enriched in different membrane fractions, which can be distinguished by sucrose gradient sedimentation [102]. However, the significance of differential membrane localization remains to be clarified.

3.3.4. PI

PI is a major phospholipid in both mycobacteria and corynebacteria and forms the anchor for the PIMs, which are substrates for heavy mannosylation to form LMs and additional arabinosylation to produce LAMs. PI is formed by the PI synthase PgsA (Rv2612c) from CDP-DAG and *myo*-inositol [9, 103]. Inositol is not a common metabolite in bacteria. It is therefore surprising that mycobacteria produce copious amounts of inositol through pathways shared with eukaryotes for incorporation into a range of metabolic pathways (recently reviewed in [104]). The enzyme D-*myo*-inositol 3-phosphate synthase (Ino1) converts glucose-6-phosphate to D-*myo*-inositol 3-phosphate, which is dephosphorylated by one of several inositol monophosphatases to produce *myo*-inositol [105].

3.3.5. PIMs

All Corynebacterineae synthesize PIMs that are important components of the cell envelope. Polar PIM species can also serve as membrane anchors for LM and LAM. Many of the steps of PIM/LM/LAM biosynthesis have now been elucidated [106]. Extensive genetic and biochemical studies have demonstrated that the synthesis of PIMs occurs linearly in mycobacteria with PI as the starting substrate (reviewed in [107]) (Fig. 4A). Early steps of the pathway occur on the cytoplasmic face of the plasma membrane. A PIM biosynthetic membrane, enriched in the early steps, has been purified by sucrose gradient fractionation as a membrane subdomain termed PMf, which is distinct from the bulk plasma membrane [102]. Mannosyltransferases performing the early steps utilize the water-soluble mannose donor, GDP-Man, which can be produced from exogenously acquired mannose or via *de novo* synthesis from the glycolytic pathway when fructose-6-phosphate is transformed by

the enzymes ManA (Rv3255c), ManB (Rv3257c) and ManC (Rv3264c) [108-111], with a degree of redundancy reported at the ManC (NCgl0710) step in *C. glutamicum* [112]. The first step of PIM synthesis involves mannosylation of the C2-position of the inositol ring of PI by the enzyme PimA (Rv2610c) to form PIM1 [113, 114]. PimA is an essential enzyme in mycobacteria [11, 113] and absent in humans, making it a current target for drug development by several groups. The crystal structure of PimA from *M. smegmatis* has been solved in complex with GDP and GDP-Man at resolutions of 2.4Å and 2.6Å, respectively [115, 116]. PimA has a typical GT-B fold of glycosyltransferases consisting of two Rossmann-fold domains with a deep fissure at the interface, which contains the active site. Close to the GDP-Man binding site, the *N*-terminal domain displays a deep pocket containing highly conserved hydrophobic residues. This pocket is proposed to bind the acyl moieties of the acceptor substrate PI [116].

Next, *O*-6 mannosylation of the *myo*-inositol ring is performed by the cytoplasmic α-mannosyltransferase PimB' (Rv2188c) [117, 118] resulting in the formation of PIM2. While the mycobacterial enzyme Rv0557 was originally assigned this function [119], later studies showed that Rv2188c was the true PimB (designated PimB') [118], with Rv0557 being renamed MgtA and included in the LM-B pathway [120, 121] (see below). PimB' is an essential enzyme in mycobacteria [11, 117] and the crystal structure of the equivalent corynebacterial enzyme has been solved at high resolution complexed with nucleotide [122]. In corynebacteria, PIM2 is detected mainly in its mono-acylated form, but in mycobacteria PIM2 accumulates in the cell envelope as monoacyl (AcPIM2) and diacyl (Ac_2PIM2) forms, the former produced by the acyltransferase Rv2611c [123] which acts optimally on the product of the PimB' reaction [117].

The next enzyme in the pathway, PimC, has been identified in *M. tuberculosis* strain CDC1551 and could produce trimannosylated PIMs [124]. However, the absence of this enzyme in other strains indicates redundancy at this step and the enzymes involved remain to be identified, as does the putative "PimD" protein. Flipping of PIM intermediates from the cytoplasmic face of the membrane to the periplasm is thought to occur at this point of the pathway, but the precise intermediate and transporter involved are also undefined.

AcPIM4 species can be further mannosylated to form more polar PIMs in reactions thought to take place on the periplasmic side of the cytoplasmic membrane. These reactions are performed by glycosyltransferases that require a lipid sugar donor in the form of PPM, since these reactions are amphomycin-sensitive [36, 125-128]. In mycobacteria, AcPIM4 is proposed to be a branch point for synthesis of polar PIM end products and LM/LAM. PimE (Rv1159) has been shown to elongate AcPIM4 with one or more α1-2 linked mannoses to form AcPIM6 [36]. This polytopic membrane protein has sequence similarities with eukaryotic PIG-M mannosyltransferases and localizes to a cell wall-associated plasma membrane subdomain in *M. smegmatis*, termed PM-CW, to which enzymatic activities of AcPIM4-6 synthesis are enriched [102]. Its catalytic activity was successfully mapped to a conserved aspartate residue in the first outer loop of the protein. Whether PimE also forms AcPIM6 is unknown. Interestingly, no PimE orthologue is present in *C. glutamicum* and

Ac/Ac2PIM6 do not accumulate in this species, so the formation of Ac/Ac2PIM5/6 appears to be a mycobacterium-specific side-branch of the pathway.

Surprisingly, studies in *M. smegmatis* have revealed a role for a lipoprotein in PIM/LAM synthesis, since *lpqW* mutants produce reduced levels of LM/LAM [129]. As the only non-enzymatic component of the pathway identified to date, LpqW has been proposed to have a regulatory role at the bifurcation point of the pathways, as well as a functional connection with PimE, since mutations in the *pimE* gene can bypass the requirement for LpqW [130]. Very recent studies have implicated the corynebacterial ortholog of LpqW in the LM-B pathway as well (Rainczuk *et al*, submitted). Structural studies on the *M. smegmatis* LpqW revealed a scaffold similar to substrate binding proteins associated with ABC transporters, which has evolved to fulfill a new role in the regulation of PIM/LAM biosynthesis [131].

3.3.6. LM/LAM

A subpopulation of PIMs (AcPIM4 in mycobacteria [128, 129, 132] and AcPIM2 in corynebacteria [133]) can be extended with chains of α1-6 linked mannose to form LM that is further modified with a number of single α1-2 mannose side chains [134-136]. MptB is a PPM-dependent mannosyltransferase involved in extending AcPIM2 to form the proximal α1-6 mannan backbone of LM in *C. glutamicum* but is redundant in *M. smegmatis* [133], indicating additional complexity at this step in mycobacteria. Further elongation is performed by MptA (Rv2174) [135, 136], with MptC (Rv2181) required for addition of α1-2 linked Man side chains [106, 134, 137, 138]. Further additions of arabinose units by EmbC (Rv3793), AftC (Rv2673), AftD (Rv0236c) and at present unidentified α1-5 arabinofuranosyltransferases, result in the formation of mature LAM [139-142]. In *M. tuberculosis* and other pathogenic mycobacteria, additional mannose capping is present [143], synthesized by the enzymes MptC [137] and Rv1635c [144]. Alternatively, *M. smegmatis* LAM is capped with inositol phosphate [145].

While the general PI→PIM→LM→LAM pathway is conserved in corynebacteria, a second pathway of lipoglycan biosynthesis exists in which a sub-population of LM lipoglycans is assembled on a glucopyranosyluronic acid diacylglycerol (Gl-A, GlcADAG) glycolipid anchor [118, 121, 133]. In this pathway (Fig. 4B), Gl-A is first mannosylated by MgtA (NCgl0452 in *C. glutamicum* and previously termed PimB, see above) forming mannosyl-glucuronic acid diacylglycerol (Gl-X, ManGlcADAG), which is subjected to further α1-6 (backbone) and α1-2 (side unit) mannosylation resulting in LM [121]. This pathway shares some PPM-dependent enzymes with the PI-based LM pathway including MptB, since an *mptB* (*NCgl1505*) mutant of *C. glutamicum* fails to produce intermediates beyond Gl-X or AcPIM2 [133]. For clarity, the PI-based LM pool has been designated LM-A while this second pathway produces LM-B [121, 146], the major LM pool in *C. glutamicum* [118]. While *C. glutamicum* produces LAMs via LM-A using a similar pathway to mycobacteria, its LAMs are smaller and structurally distinct, with more extensive mannosylation and singular Ara*f* capping [147].

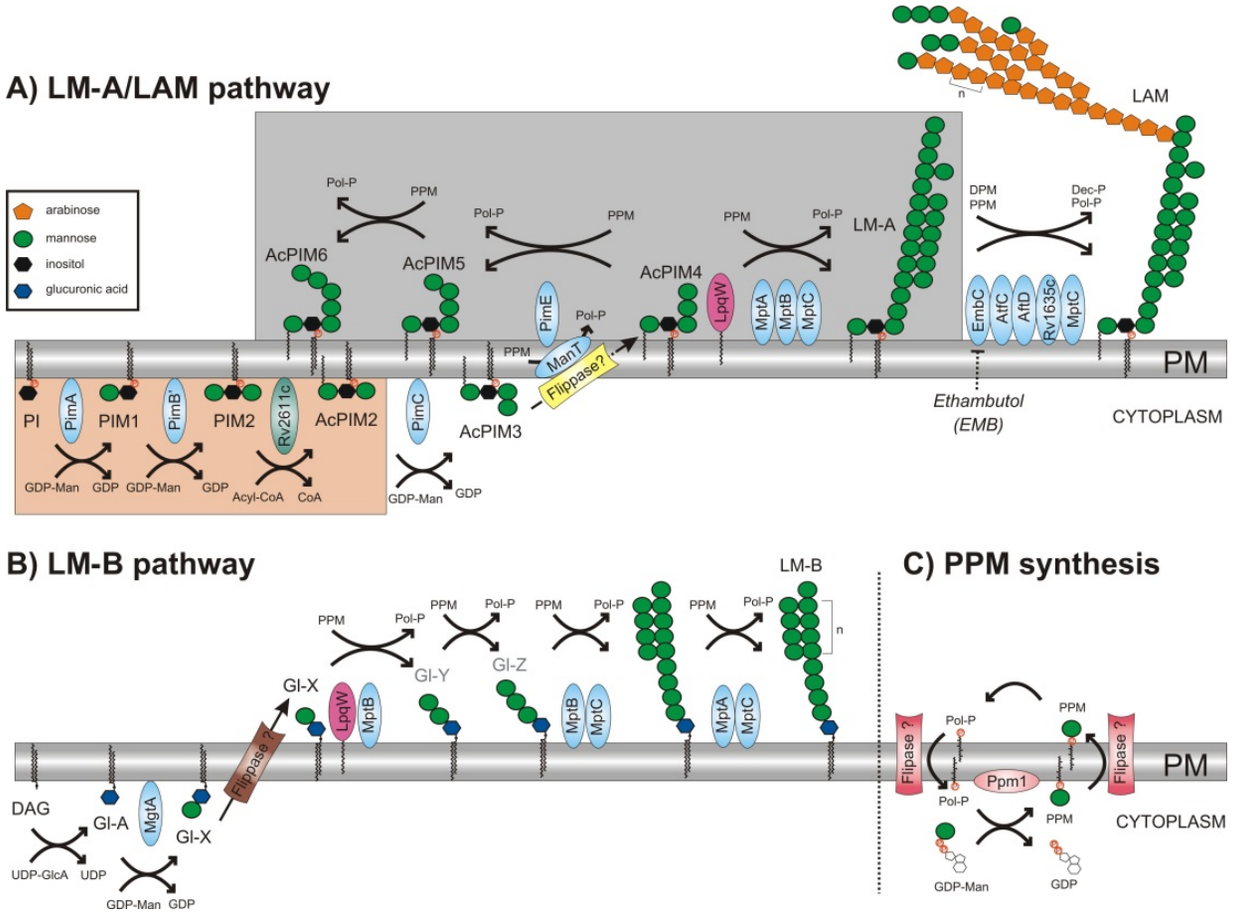


Figure 4. Summary of Lipoglycan Biosynthesis Pathways in Corynebacterineae. A) The mycobacterial pathway and structures for LM-A/LAM are shown, although several steps are inferred from studies in *C. glutamicum*. A progression from PIM1 to AcPIM2 via AcPIM1 can also occur but is sub-optimal in mycobacteria. AcPIM3 is shown as the substrate for the unidentified flippase but AcPIM2 may be the preferred substrate: AcPIM2 appears to be the flipped intermediate in *C. glutamicum*. Reactions associated with the PMf and PM-CW subfractions of *M. smegmatis* are shaded orange and grey, respectively. B) LM-B pathway in *C. glutamicum*. Its presence in mycobacteria remains to be determined. Intermediates representing Gl-Y and Gl-Z have been detected in very recent studies (Rainczuk et al, submitted). C) PPM serves as the lipid-linked donor of mannose for both pathways. The mechanism of flipping is undetermined. PM, plasma membrane. Other abbreviations are as defined in the text.

3.4. Prenol lipids

Polyprenol phosphate (Pol-P) is a key carrier lipid in synthesis of the core structures of the mycobacterial cell wall, including peptidoglycan and arabinogalactan. Unlike most bacteria, mycobacteria contain multiple types of Pol-P. For example, *M. smegmatis* produces decaprenol phosphate (C_{50}, Dec-P, [148]) and heptaprenol phosphate (C_{35}, Hep-P, [149]). Polyprenols are thought to be synthesized via the condensation of two C_5 lipids, isopentenyl diphosphate [150-152] and dimethylallyl diphosphate derived from the mevalonate-independent methylerythritol 4-phosphate (MEP) pathway. These reactions are catalyzed by prenol diphosphate synthases and two *M. tuberculosis* proteins, Rv2361c and Rv1086, acting

sequentially, are thought to fulfill this role [148, 149, 153-155]. Finally, the C_{50} decaprenol diphosphate is dephosphorylated to produce Dec-P by an unknown phosphatase.

3.4.1. Polyprenol-phospho-sugars

3.4.1.1. PPM

PPM, a β-D-mannosyl-1-monophosphoryldecaprenol, is utilized by periplasmic mannosyltransferases for synthesis of polar PIM species and LMs [106]. C_{35}/C_{50}-P-Man*p* is formed by the PPM synthase Ppm1 (Rv2051c) from GDP-Man*p* and Pol-P [126, 127] (Fig. 4C). In *M. tuberculosis* Ppm1 consists of two domains: a C-terminal catalytic domain and a N-terminal membrane anchor with 6 transmembrane helices. However, the equivalent domains of the *M. smegmatis* PPM synthase are two distinct, but interacting, proteins [156]. The importance of PPM in cell wall synthesis has been highlighted by analysis of a *ppm1* mutant in *C. glutamicum* that failed to produce PPM, resulting in severe defects in lipoglycan biosynthesis. While the mutant could synthesize PIM2 species, all downstream products (LM, LAM) were absent, indicating their reliance on the PPM donor [127]. These findings were consistent with others showing that amphomycin, an antibiotic specific for PPM-dependent polymerases, blocked the PIM pathway at the PIM2 or PIM3 stage [125, 128]. The product of the *Rv3779* gene has also been implicated in PPM synthesis but its role remains unclear [157, 158].

3.4.1.2. DPA

DPA is the only known donor of arabinose (Ara) for mycobacterial cell wall synthesis, contributing Ara*f* units to arabinogalactan and LAM [106] with concomitant release of Pol-P. The Ara*f* portion is derived from the pentose-phosphate pathway [159-161]. 5-phosphoribose 1-diphosphate is transferred to Dec-P by the *Rv3806c* gene product [162] and the resultant Dec-P-β-D-5-phosphoribose is dephosphorylated to form Dec-P-β-D-ribose. Oxidation of the 2′ hydroxyl is followed by a reduction reaction to form DPA. This two-step epimerization reaction is catalyzed by the combined activities of DprE1 (Rv3790) and DprE2 (Rv3791) [163]. Since DPA is the sole donor of Ara*f* residues for mycobacterial cell wall synthesis, this pathway is of interest for drug development. Indeed, DprE1 is the target of dinitrobenzamide derivatives (DNBs) [164] and a set of nitro-compounds related to DNBs, the nitro-benzothiazinones (BTZ), a class of compounds with nanomolar anti-*M. tuberculosis* activities but minimal host-cell toxicity [165-167]. The essential nature of DprE1 in species beyond *M. tuberculosis* [168] reinforces it as a "magic" drug target [169]. The crystal structure of *M. tuberculosis* DprE1 complexed with BTZ inhibitors has been reported very recently, revealing the mode of inhibitor binding [170].

3.4.2. Carotenoids

Carotenoids are isoprenoid pigments widely distributed in biology and mostly based on C_{40}-polyene. Synthesis of these pigments has been poorly studied in mycobacteria but they have proven useful for taxonomic and identification purposes. Mycobacterial pigments are

generally yellow or orange and most have been confirmed as carotenoids [171]. While carotenoid genetics has been best studied in plants, the key enzymes of the pathway have been identified in bacteria, including mycobacteria [172-174]. There are two classes of carotenoids in bacteria, carotenes and xanthophylls, the latter of which contain oxygen. Both classes are composed of eight isoprenoid units with a long central chain of double bonded carbons. A consensus pathway for carotenoid biosynthesis in bacteria has been elucidated with orthologues of key enzymes identified in several species of mycobacteria [175]. As described above for prenol lipids, the carotenoid pathway begins with isopentenyl diphosphate and dimethylallyl diphosphate derived from the MEP pathway [176]. Head-to-tail condensation of these terpenes produces geranylgeranyl pyrophosphate (GGPP) due to the activity of GGPP synthase (CrtE). Condensation of two GGPP molecules [177] is followed by desaturation to phytoene by phytoene synthase (CrtB). Phytoene desaturase (CrtI) converts phytoene to lycoprene followed by cyclization to β-carotene by lycoprene cyclase (CrtY) [178].

4. Concluding remarks

Lipid metabolism in mycobacteria and corynebacteria is a highly complex network of catabolic and anabolic reactions. While the metabolic pathways and many of the enzymes involved have been actively elucidated over the past decade, substantial efforts are still needed to draw a comprehensive map of lipid metabolism in these organisms. In particular, our understanding of regulatory mechanisms of lipid metabolism is currently at an early stage. In addition, there are very few studies describing the interactions between multiple metabolic pathways of lipid biosynthesis. One promising approach for the comprehensive understanding of lipid metabolism is lipidomics, which is the study of lipid biosynthetic and catabolic pathways at a global level [179]. In the past, metabolic pathways have generally been examined in isolation without consideration of how different pathways might interact with, and influence, one another. Since the plasma membrane is a shared platform for most lipid biosynthetic pathways, and some donors are shared between different pathways (see above), it seems unlikely that the various pathways are truly independent. Recent advances in mass spectrometry (*e.g.* MALDI-MS, ESI-MS), nuclear magnetic resonance spectroscopy and associated computational methods have fuelled the development of this field [180]. Members of the Corynebacterineae, with their extensive lipid repertoires and complex metabolic pathways, would seem to be ideal targets to assess the true potential of lipidomics technologies. Recently, appropriate databases and methods for detection and identification of all major lipid classes of *M. tuberculosis* from a single crude extract have been developed [181, 182]. Very recently, a lipidomics profiling platform has been reported that uses high-performance liquid chromatography/mass spectrometry to resolve more than 12,000 molecules from *M. tuberculosis* [183]. These exciting advances provide a basis for future studies on the regulation of lipid metabolism and may allow, for the first time, a true appreciation of the interactive lipid networks of mycobacteria and corynebacteria.

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