Chapter 13
Cell Walls and Membranes of Actinobacteria

Kathryn C. Rahlwes, Ian L. Sparks and Yasu S. Morita

Abstract Actinobacteria is a group of diverse bacteria. Most species in this class of bacteria are filamentous aerobes found in soil, including the genus *Streptomyces* perhaps best known for their fascinating capabilities of producing antibiotics. These bacteria typically have a Gram-positive cell envelope, comprised of a plasma membrane and a thick peptidoglycan layer. However, there is a notable exception of the Corynebacteriales order, which has evolved a unique type of outer membrane likely as a consequence of convergent evolution. In this chapter, we will focus on the unique cell envelope of this order. This cell envelope features the peptidoglycan layer that is covalently modified by an additional layer of arabinogalactan. Furthermore, the arabinogalactan layer provides the platform for the covalent attachment of mycolic acids, some of the longest natural fatty acids that can contain ~100 carbon atoms per molecule. Mycolic acids are thought to be the main component of the outer membrane, which is composed of many additional lipids including trehalose dimycolate, also known as the cord factor. Importantly, a subset of bacteria in the Corynebacteriales order are pathogens of human and domestic animals, including *Mycobacterium tuberculosis*. The surface coat of these pathogens are the first point of contact with the host immune system, and we now know a number of host receptors specific to molecular patterns exposed on the pathogen's surface, highlighting the importance of understanding how the cell envelope of Actinobacteria is structured and constructed. This chapter describes the main structural and biosynthetic features of major components found in the actinobacterial cell envelopes and highlights the key differences between them.

Keywords Actinobacteria · Arabinogalactan · Cell envelope · Corynebacteria · Glycolipid · Membrane · Mycobacteria · Mycolic acid · Peptidoglycan · Phospholipid · Streptomyces

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Introduction

Actinobacteria is a vast and variable class of bacteria. One unifying feature of this class is the high GC content, generally ranging between 55 and 75%. A morphological feature traditionally used to classify Actinobacteria was filamentous growth, but a phylogenetic analysis using 16S rRNA gene has revealed that this group is much more morphologically diverse than it was previously thought. Their lifestyle is also immensely diverse. Many are environmental species that live in soil and aquatic environments where nutrient availability fluctuates. *Streptomyces* species, for example, have the robust ability to grow using a wide variety of nutrients, carrying numerous genes for metabolic regulation, polysaccharide degradation, and carbohydrate transport (Hodgson 2000; Bertram et al. 2004; Bentley et al. 2002). In contrast, there are symbionts, such as the plant symbiont *Frankia* or the human pathogen *Mycobacterium tuberculosis*, which have a limited number of membrane transporters, implying more restricted strategies to acquire nutrients from the host (Niederweis 2008; Normand et al. 2007). Another important feature of Actinobacteria is the Gram-positive cell wall. Most bacteria in the Actinobacteria class carry typical monoderm cell envelope with a relatively thick peptidoglycan layer. However, some members, such as the well-known *Mycobacterium* species, have evolved a diderm cell envelope (Fig. 13.1). Their unusual cell envelope structure makes Gram staining unreliable and is more readily distinguished from the other envelope types by acid-fast staining. This chapter discusses the cell envelope of Actinobacteria, one of six classes within the Actinobacteria phylum (Gao and Gupta 2012). We will primarily focus on the diderm cell envelope of the Corynebacteriales order and compare with those from other orders within the Actinobacteria class.

Of the Corynebacteriales order, *Mycobacterium* and *Corynebacterium* are the best studied genera mainly due to their medical and industrial importance. To name a few, *M. tuberculosis*, *Mycobacterium leprae*, *Mycobacterium bovis*, and *Corynebacterium diphtheriae* are the etiologic agents of tuberculosis (TB), leprosy, bovine TB, and diphtheria, respectively. *Corynebacterium glutamicum* is an industrial source of producing glutamic acid. Other genera such as *Nocardia*, *Rhodococcus*, *Gordonia*, and *Tsukamurella* also include some pathogenic species, which often infects immunocompromised individuals. However, most species in this order are environmental, with some species, such as *Rhodococcus olei,* showing potential industrial use in degrading petroleum oil in contaminated soil (Chaudhary and Kim 2018). Even in the genus of *Mycobacterium* where you find many pathogens, most species are nonpathogenic. For instance, *Mycobacterium smegmatis* is a nonpathogenic saprophyte. This species has become an established model for mycobacteria research because it is a fast grower, in contrast to the slow-growing pathogenic species, and many aspects of cellular physiology, including the cell envelope structures, are comparable to the pathogens.

Among infectious diseases caused by actinobacterial species, TB is the most devastating, currently being one of the top ten causes of death worldwide. In 2017, 10 million people worldwide fell ill with the disease and 1.6 million died (World

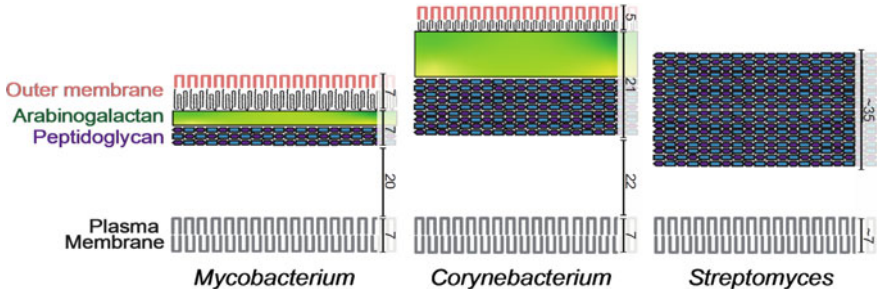

Fig. 13.1 Schematic of the cell envelopes of *Mycobacterium*, *Corynebacterium*, and *Streptomyces*. Bars indicate the height of each layer drawn to scale. In *M. smegmatis*, the plasma membrane, periplasmic space, peptidoglycan-arabinogalactan layer and outer membrane has a thickness of ~7, ~20, ~7 and ~7 nm (Zuber et al. 2008). For *C. glutamicum*, the peptidoglycan-arabinogalactan layer is ~3 times thicker than that of *M. smegmatis*, and outer membrane thinner than that of *M. smegmatis*, presumably due to short mycolic acids (Zuber et al. 2008). The thickness of each layer within the peptidoglycan-arabinogalactan layer is unknown. For *Streptomyces*, there are no precise estimates, but from published images, the peptidoglycan cell wall extends ~35 nm out from the plasma membrane, which appears comparable in thickness to *Mycobacterium* and *Corynebacterium* (Lerat et al. 2012; Yague et al. 2016; Celler et al. 2016)

Health Organization 2018). Additionally, estimated 1.7 billion people are latently infected with *M. tuberculosis* and therefore may develop the disease in their lifetime. Current regimens for the treatment of TB are combinations of the first-line drugs: isoniazid, rifampin, pyrazinamide, and ethambutol, for at least six months. Among them, isoniazid and ethambutol target the biosynthesis of cell envelope components, making cell envelope biosynthesis a proven target of TB chemotherapy. Similar to other microbial infections, the rise in multi-drug resistant *M. tuberculosis* is a global concern. Understanding the diderm cell envelope of *M. tuberculosis* and other Corynebacteriales is important not only for the sake of the unique biology that has evolved in this bacterial lineage, but also from the perspective of identifying novel drug targets to treat the devastating diseases they cause.

The biosynthesis of cell envelope is a tightly regulated process, requiring temporal and spatial controls. In this regard, certain similarities in the actinobacterial cell growth and division are noteworthy. Rapid mechanical separation of daughter cells is found in diverse lineages of Actinobacteria, including *Micrococcus luteus, Brachybacterium faecium, C. glutamicum, M. smegmatis,* and *Streptomyces venezuelae* (Zhou et al. 2016, 2019), suggesting that this mechanism of cell separation is widely conserved in Actinobacteria. From Streptomycetales to Corynebacteriales, polar growth is another well-conserved feature (Daniel and Errington 2003; Thanky et al. 2007; Ramos et al. 2003). Many proteins show specific subcellular localizations, which are likely critical for the function of these proteins and the spatially coordinated cell growth (Puffal et al. 2018). One prominent example is DivIVA, which localizes to the polar ends of *Mycobacterium*, *Corynebacterium*, *Brevibacterium*, and *Streptomyces* cells and helps to coordinate the polar cell envelope biosynthesis

(Ramos et al. 2003; Flärdh 2003; Letek et al. 2008; Hempel et al. 2008; Nguyen et al. 2007; Kang et al. 2008; Meniche et al. 2014; Donovan et al. 2012; Melzer et al. 2018). Spatial coordination is not only dictated by proteins. Plasma membrane has recently been shown to be segregated into functional domains in mycobacteria, and many cell envelope biosynthetic reactions are compartmentalized within the membrane (Hayashi et al. 2016, 2018), further highlighting the intricate spatial controls. More detailed review articles are available on spatial coordination and regulations of actinobacterial cell division and polar envelope growth (Puffal et al. 2018; Donovan and Bramkamp 2014; Logsdon and Aldridge 2018; Flärdh et al. 2012).

In this chapter, we focus primarily on the structure and biosynthesis of the actinobacterial cell envelope (Fig. 13.1), starting with the innermost layer, plasma membrane, followed by the peptidoglycan layer. In Corynebacteriales, the peptidoglycan layer is covalently linked to an arabinogalactan layer, which is covalently linked to a mycolic acid layer. Mycolic acids are long fatty acids and are a core component of the outer membrane. While much less is known, we will also compare and contrast the capsule layer of Actinobacteria.

Plasma Membrane

The plasma membrane is the fundamental innermost layer of the cell envelope. The cryo-electron micrograph of mycobacterial plasma membrane has indicated its thickness to be about 7 nm (Zuber et al. 2008; Hoffmann et al. 2008). The major structural components of the actinobacterial plasma membrane are glycerophospholipids. In Actinobacteria in general, the core plasma membrane is composed of cardiolipin (CL), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylinositol mannosides (PIMs), and other less abundant lipids such as ornithine lipids (OL), and menaquinones (Fig. 14.2a).

CLs and PGs

CL represents one of the most abundant plasma membrane components, constituting roughly 10–50% of the total phospholipids in Actinobacteria (Jackson et al. 2000; Yano et al. 1969; Nampoothiri et al. 2002; Kimura et al. 1967). It is generally considered a plasma membrane phospholipid, but a large amount of cell wall-associated CL has been found in *C. glutamicum* (Bansal-Mutalik and Nikaido 2011). In non-actinobacterial species, CL is typically synthesized by a CL synthase that facilitates transesterification between two PG molecules, producing one CL and one glycerol. In contrast, Actinobacteria use eukaryotic type CL synthase, which produces CL from PG and CDP-diacylglycerol (CDP-DAG), a high-energy molecule that can act as a donor of DAG (Sandoval-Calderon et al. 2009). The use of CDP-DAG makes this reaction energetically favorable, and being consistent with this reaction

kinetics, PG does not significantly accumulate in either *Mycobacterium* or *Streptomyces* (Jackson et al. 2000; Sandoval-Calderon et al. 2009; Hoischen et al. 1997; Mathur et al. 1976; Zuneda et al. 1984; Lechevalier et al. 1977). This biosynthetic approach is energetically more demanding for the cell, likely suggesting evolutionary needs for these lineages of bacteria to have a high CL/PG ratio. While the physiological reasons for this biosynthetic mechanism are unknown, CL is essential for hyphal growth and spore formation in *Streptomyces* (Jyothikumar et al. 2012). Interestingly, *C. glutamicum* does not possess the eukaryotic type CL synthase (Nampoothiri et al. 2002). Being consistent with the lack of the eukaryotic type enzyme, this organism has a low CL/PG ratio (Bansal-Mutalik and Nikaido 2011).

Aminolipids

Aminolipids are widely found as a structural component of the plasma membranes in Actinobacteria, but their abundance can vary. For example, nitrogen-containing lipids are not found in the protoplast of *M. luteus* (Gilby et al. 1958). PE is often a dominant phospholipid in Actinobacteria, but corynebacteria cannot synthesize it (Brennan and Lehane 1971). Two sequential enzymatic reactions synthesize PE: phosphatidylserine synthase produces phosphatidylserine from serine and CDP-DAG, and then phosphatidylserine decarboxylase removes carbon dioxide from phosphatidylserine, producing PE. These two enzymatic activities are differentially enriched in specific membrane domains of *M. smegmatis* (Morita et al. 2005), although the significance of this spatial segregation is unknown (see below for more details on membrane compartmentalization).

Another class of aminolipids includes ornithine- and lysine-amide lipids, in which a long-chain β-hydroxy-fatty acid acylates the α-amino group of ornithine or lysine, and the β-hydroxy group of the long-chain fatty acid is further modified by another fatty acid. These phosphorus-free lipids have been detected in several species of *Mycobacterium* and *Streptomyces* (Kimura et al. 1967; Kawanami et al. 1968; Batrakov and Bergelson 1978; Laneelle et al. 1990). The precise function of these lipids remains unknown, but they are widespread in Gram-negative bacteria and Actinobacteria (Vences-Guzman et al. 2012). In *Streptomyces coelicolor*, ornithine-amide lipids accumulate during phosphorus starvation or sporulation stages (Sandoval-Calderon et al. 2015). Under these conditions where ornithine-amide lipids are abundant, the level of PE becomes negligible. It has been suggested that ornithine-amide lipids are present in both the inner and the outer membranes in Gram-negative bacteria. Therefore, it is possible that these lipids constitute the outer membrane of mycobacteria as well.

Aminolipids can also be produced by amino acid modifications of glycerolipids. *M. tuberculosis* produces lysinylated PG, and the deletion of the biosynthetic enzyme LysX results in defective cell envelope integrity and less effective establishment of infection in mice lungs (Maloney et al. 2009, 2011). A similar modification can occur in *Mycobacterium phlei*, where DAG rather than PG is lysinylated

(Lerouge et al. 1988). Similarly, *C. glutamicum* produces alanylated PG as well as alanylated DAG, and their synthesis is tightly regulated with the synthesis and trafficking of trehalose corynomycolates (Klatt et al. 2018).

Phosphatidylinositols and Mannolipids

PI is a rather unusual phospholipid species to be found in the *Bacteria* domain but constitutes a significant fraction of the total phospholipids in Actinobacteria (Morita et al. 2011). A fascinating feature of PI in eukaryotes is the versatile modifications of the functional head group, inositol. For example, proteins and glycans are anchored to the eukaryotic cell surface through glycosylphosphatidylinositols, playing numerous critical roles on the cell surface. Furthermore, intracellular signaling and membrane trafficking are mediated through phosphorylated PIs. Mycobacterial PI is also modified in similar ways, and the discovery of actinobacterial PIMs predates that of eukaryotic glycosylphosphatidylinositol anchors (Kimura et al. 1967; Lee and Ballou 1964; Ballou et al. 1963; Minnikin et al. 1977). The biosynthetic pathway starts with the production of inositol 3-phosphate from glucose (Glc) 6-phosphate, mediated by the inositol 3-phosphate synthase Ino1 (Haites et al. 2005; Movahedzadeh et al. 2004). Being the first key enzyme, the expression of *ino1* is tightly controlled by the transcription factor IpsA, which senses the decreased cellular levels of inositol and activates the *ino1* gene transcription (Baumgart et al. 2013). PI is produced from inositol and CDP-diacylglycerol, and this energetically favorable reaction is mediated by the PI synthase PgsA, which is an essential enzyme in *M. smegmatis* (Jackson et al. 2000). In mycobacteria, the major PIM species are PIM2 and PIM6, containing 2 and 6 mannose (Man) residues, respectively (Fig. 13.2a). These PIM species can be modified by up to four fatty acids: one at the 6-OH of the Man residue attached to the 6-OH of inositol ring, and another at the 3-OH of the inositol ring, in addition to the DAG moiety of PI. AcPIM2 and AcPIM6 are the most abundant PIM products in *M. smegmatis* grown under standard laboratory conditions, and they have an additional fatty acid attached to the Man residue. AcPIM2 is synthesized by sequential additions of Man residues mediated by PimA and PimB' (Guerin et al. 2009; Lea-Smith et al. 2008; Kordulakova et al. 2002), followed by the acyl addition to the Man residue mediated by PatA (Albesa-Jove et al. 2016; Kordulakova et al. 2003). There has been no report of AcPIM2 or AcPIM6 having the acyl modification on the inositol ring instead of the Man acyl chain, indicating that the inositol acylation occurs only after Man residue is acylated.

PimA and PimB' are GDP-Man-dependent enzymes, suggesting that the reactions take place on the cytoplasmic side of the plasma membrane. In contrast, the later mannosylation steps to produce AcPIM6, lipomannan (LM) and lipoarabinomannan (LAM) are dependent on a lipidic Man donor, which is known as polyprenol-phosphate-Man (PPM). PPM is produced from GDP-Man and polyprenol-phosphate by a membrane-bound PPM synthase, Ppm1, in *M. tuberculosis*. Interestingly, in other species of *Mycobacterium* such as *M. smegmatis*, *Mycobacterium avium*, and

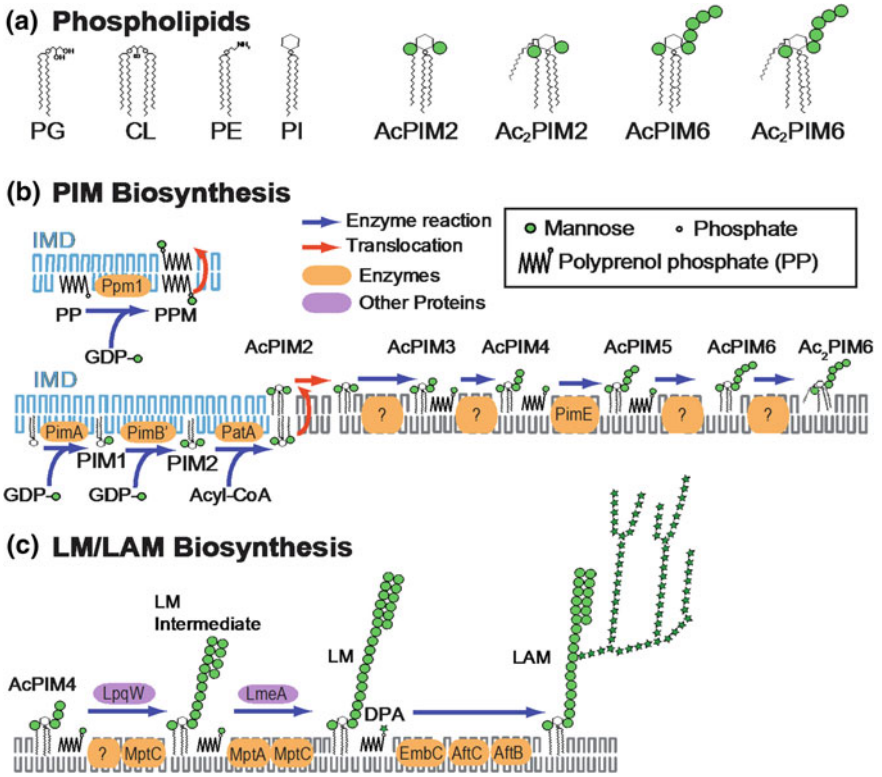

Fig. 13.2 Phospholipids of Actinobacteria. **a** Structures of major phospholipids. The acyl chain compositions can vary. PG, phosphatidylglycerol; CL, cardiolipin; PE, phosphatidylethanolamine; PI, phosphatidylinositol. AcPIM6 and Ac_2PIM6 are found in *Mycobacterium* and not in *Corynebacterium* (see text for details). **b** Biosynthesis of polyprenol phosphate Man (PPM) and PIMs. The question marks indicate currently undetermined or unconfirmed enzymes. The biosyntheses of PPM and the early steps of PIM are proposed to take place in the IMD (Hayashi et al. 2016; Morita et al. 2005). **c** Biosynthesis of lipomannan (LM) and lipoarabinomannan (LAM). Polyprenol phosphate Man (PPM) and decaprenol phosphate β-D-Ara*f* (DPA) are the Man and Ara donors for LM and LAM biosynthesis, respectively

M. leprae as well as in several *Corynebacterium* species, two genes encode separate domains of PPM synthase (Gurcha et al. 2002). Ppm synthase is essential in mycobacteria (Rana et al. 2012), and its deletion in *C. glutamicum* results in a reduced growth rate (Gibson et al. 2003), indicating the critical importance of surface mannosylation. GDP-Man and PPM biosynthetic pathways are also conserved in the genus *Streptomyces* and are important for protein *O*-mannosylation (Wehmeier et al. 2009; Howlett et al. 2018). The disruption of these pathways in *Streptomyces* results in increased antibiotic sensitivities, suggesting the roles of mannosylated proteins in cell envelope integrity.

The mannosyltransferase(s) that extends AcPIM2 to a more polar AcPIM4 is currently unknown in mycobacteria. In corynebacteria, AcPIM2 is produced by a similar pathway and is further elongated by MptB, a mannosyltransferase that is presumably a processive enzyme to create an α1,6 mannan chain (Mishra et al. 2008a). Corynebacteria do not produce PIM6 species but do produce LM and LAM (Crellin et al. 2013), and MptB is involved in the production of these more extensively mannosylated species (see below). The gene encoding the ortholog of corynebacterial MptB was deleted in *M. smegmatis*, and the gene deletion showed no defects in PIMs/LM/LAM synthesis (Mishra et al. 2008a), making it unclear if MptB is redundant with another enzyme in *M. smegmatis*, or if there is another enzyme that mediates the reaction in *M. smegmatis*.

A key mannosyltransferase that drives the synthesis of PIM6 species is PimE, which adds the fifth Man to PIM4 using PPM as a Man donor (Fig. 13.2b). As mentioned above, corynebacteria also produce PIM species, but lack PIM6. Being consistent with this observation, there is no apparent ortholog of PimE in corynebacteria. LpqW, a lipoprotein, is suggested to be involved in regulating the AcPIM4 biosynthetic branch point in *M. smegmatis* (Kovacevic et al. 2006). When the *lpqW* gene was deleted, the *M. smegmatis* mutant became defective in producing LM/LAM and growth was retarded. Suppressor mutants that restored rapid growth were isolated from Δ*lpqW*, and all carried mutations in the *pimE* gene (Crellin et al. 2008). The *pimE* mutation blocked the synthesis of AcPIM6 but allowed the increased production of LM/LAM in Δ*lpqW*. These observations suggested that LpqW is a key regulatory protein controlling the two alternative pathways, AcPIM6 or LM/LAM. This proposed role of LpqW in *M. smegmatis* somewhat contradicts the fact that LpqW is present in corynebacteria yet AcPIM6 is not produced in these bacteria. Instead, LpqW is proposed as a regulatory protein essential for the activity of MptB mannosyltransferase in *C. glutamicum* (Rainczuk et al. 2012).

Once the mannan chain of LM is extended to an intermediate length of 5–20 residues, another processive mannosyltransferase, MptA, elongates the α1,6 mannan chain to a mature size of 21–34 residues (Kaur et al. 2007; Mishra et al. 2007). The α1,6 mannan backbone is decorated by α1,2 mono-Man branches, and this reaction is mediated by the α1,2 mannosyltransferase MptC (Kaur et al. 2006, 2008; Sena et al. 2010). Two proteins are involved in regulating mannan elongation: one membrane protein encoded by the *C. glutamicum NCgl2760* gene is proposed to play a role in mannan elongation at, or immediately prior to the MptA-dependent elongation step (Cashmore et al. 2017). The deletion of the *M. smegmatis* ortholog, *MSMEG_0317*, was not possible, suggesting that it is an essential gene. This is somewhat surprising as *mptA* is not an essential gene in *M. smegmatis* (Kaur et al. 2007; Fukuda et al. 2013). Another protein, termed LM elongation factor A (LmeA), is an *M. smegmatis* periplasmic protein necessary for the α1,6 mannan elongation mediated by MptA (Rahlwes et al. 2017). LmeA is not essential for in vitro growth, and genetic studies suggested that MptA is epistatic to LmeA, but the precise function of this protein remains unknown.

A single arabinan made of ~70 arabinofuranose (Ara*f*) residues is attached to the mannan backbone of LAM (Kaur et al. 2014). It is composed of a linear α1,5 chain

with α1,3 branches and terminated with a linear tetra-Ara*f* or branched hexa-Ara*f* motif. Both of these terminal motifs end with the non-reducing β1,2 Ara*f* residue. The first arabinosyltransferase that primes the mannan chain is unknown. EmbC is the processive α1,5 arabinosyltransferase that elongates the primed arabinose (Ara) (Zhang et al. 2003; Shi et al. 2006) and is an essential enzyme in *M. tuberculosis* (Goude et al. 2008) (Fig. 13.2c). AftC is an α1,3 arabinosyltransferase that creates branching in LAM biosynthesis (Birch et al. 2008, 2010). AftB is the β1,2 arabinosyltransferase, which forms the terminal motif (Jankute et al. 2017). Both AftC and AftB are also involved in arabinogalactan biosynthesis (see below).

The presence of PI and mannolipid species beyond mycobacteria and corynebacteria becomes less well described. PIM1 and PIM2 are present in *Streptomyces* and can comprise 2–21% of the main polar lipids in the plasma membrane (Kimura et al. 1967; Sandoval-Calderon et al. 2015; Nguyen and Kim 2015). While LAM is suggested to be present in the outer membrane in mycobacteria, LAM can be produced without the outer membrane: *Corynebacterium otitidis* (formerly *Turicella otitidis)*, which lack mycolic acids and therefore the mycolate-based outer membrane, has the ability to synthesize LAM (Gilleron et al. 2005). Furthermore, *Amycolatopsis sulphurea* and *Lechevalieria aerocolonigenes*, which belong to the order Pseudonocardiales (Gibson et al. 2005), do not possess the outer membrane but are known to synthesize LAM. These observations suggest that PIMs/LM/LAM are plasma membrane mannolipids, predating the evolution of the outer membrane.

What are the potential reasons for having these glycolipids in actinobacterial plasma membrane? The method of reverse micellar solution extraction indicated that PIM species are found in the plasma membrane of mycobacteria and corynebacteria (Bansal-Mutalik and Nikaido 2011, 2014). Our previous study in *M. smegmatis* is consistent with this notion because the deletion of *pimE*, the fifth mannosyltransferase of AcPIM6 biosynthesis, resulted in abnormal mesosome-like membrane accumulation in the cytoplasm (Morita et al. 2006). Simultaneously, the *pimE* deletion mutant becomes hypersensitive to various antibiotics as well as a low concentration of copper, which is often included in standard mycobacterial growth media (Eagen et al. 2018). These observations suggest the importance of PimE in the structural integrity of the plasma membrane. It remains unknown if the accumulation of AcPIM4 or the lack of AcPIM6 in the *pimE* deletion mutant is toxic to the cell. One possibility is that AcPIM6 plays a structural role in anchoring the plasma membrane to the peptidoglycan layer, and the lack of anchoring results in destabilization of the plasma membrane, leading to the invagination and mesosome formation. LAM is also suggested to be a plasma membrane lipid in mycobacteria (Hunter et al. 1986), and visualization of surface-exposed LAM using atomic force microscopy indicates that LAM is not exposed on the cell surface unless outer membrane integrity is perturbed by antibiotics (Alsteens et al. 2008). These observations are consistent with the idea that LAM is anchored to the plasma membrane and glycan moiety intercalates the cell wall peptidoglycan, similar to the functions of (lipo)teichoic acids in Gram-positive bacteria (Weidenmaier and Peschel 2008) (see below for the discussion on outer membrane LM/LAM).

Although not PI-anchored, notable DAG-anchored mannolipids are also found in *Corynebacterium* and *Micrococcus*. *C. glutamicum* produces two LM species, termed Cg-LM-A and Cg-LM-B, and DAG-anchored Cg-LM-B is more predominant than the PI-anchored Cg-LM-A (Lea-Smith et al. 2008). Cg-LM-B carries 8–22 mannosyl residues and is anchored to the plasma membrane by α-D-glucopyranosyluronic acid DAG (Lea-Smith et al. 2008; Mishra et al. 2008a, b; Tatituri et al. 2007). The biosynthetic pathway is distinct from that of PI-anchored Cg-LM-A, involving the first priming mannosyltransferase, MgtA, which transfers Man onto the α-D-glucopyranosyluronic acid residue of the lipid precursor (Tatituri et al. 2007). Once MgtA adds the first Man, the same PPM-dependent MptB and MptA that produce Cg-LM-A extend the mannan chain (Mishra et al. 2008a). *Micrococcus* also produces DAG-anchored mannosides: D-mannosyl- α1,3-DAG (Man1-DAG), D-mannosyl- α1,3- D-mannosyl- α1,3-DAG (Man2-DAG) as well as much larger DAG-anchored LM carrying ~50 Man residues (Scher and Lennarz 1969; Lennarz and Talamo 1966; Pakkiri et al. 2004; Powell et al. 1975). *Micrococcus* species lack lipoteichoic acids, and LM is suggested to play a structural role in the cell wall. The biosynthetic pathway of these *Micrococcus* LM is not fully understood, but the first two mannoses are added using GDP-Man in the cytoplasmic side, and the Man2-DAG is proposed to flip to the periplasmic side of the plasma membrane to serve as the lipid anchor for further Man extension using PPM as the Man donor (Pakkiri et al. 2004; Pakkiri and Waechter 2005).

Plasma Membrane Compartmentalization

In *M. smegmatis*, a lipid domain termed the intracellular membrane domain (IMD) has been recently reported (Hayashi et al. 2016) and is suggested to form areas within the plasma membrane that are spatially distinct from the conventional plasma membrane. The IMD is enriched in metabolic enzymes, and many of them are involved in cell envelope biosynthesis. Furthermore, the IMD localizes to the polar region where the active elongation of the cell envelope takes place, suggesting that it is a strategic positioning of membrane-associated enzymes to the locations where the biosynthetic products are needed. Notably, the IMD is a dynamic entity which responds to environmental stresses and repositions its subcellular localization from polar enrichment during active growth to more sidewall localizations under stress exposure (Hayashi et al. 2018). There are many remaining questions in this research area: (1) what are the molecular mechanisms of protein localization to specific plasma membrane regions? (2) how are lipid intermediates able to translocate from one membrane domain to another? (3) what is the molecular mechanism of lipid domain formation? (4) what is the signaling mechanism for the spatial repositioning and how does the IMD relocate its subcellular location? We have provided a more detailed overview of the spatial control of the mycobacterial cell envelope in a recent review (Puffal et al. 2018).

Peptidoglycan

Peptidoglycan is a mesh of carbohydrate polymers crosslinked by short peptide side chains. It acts as an exoskeleton of bacteria, giving cells their shape and strength. This is illustrated by the demonstration that the digestion of the peptidoglycan layer results in the formation of spheroplasts in many rod-shaped bacteria, including Actinobacteria such as corynebacteria and mycobacteria (Melzer et al. 2018; Verma et al. 1989; Udou et al. 1983). The peptidoglycan layer is composed of repeating units of β1,4-linked *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) and tetrapeptides extending from MurNAc residues. Since the original proposal of peptidoglycan types for taxonomic classification (Schleifer and Kandler 1972), the amino acid composition of peptide stems has been widely used as a critical phenotypic feature for species identification in Actinobacteria. In general, the actinobacterial peptide stem comprises L-alanine-D-isoglutamine-L-diamino acid-D-alanine, where the third position L-diamino acid varies in different lineages. For example, the third position is *meso*-2,6-diaminopimelic acid (DAP) in mycobacteria and *C. diphtheriae* (Petit et al. 1969; Kato et al. 1968), while other diamino acids, such as L-2,4-diaminobutyrate, L, L-diaminopimelic acid, L-lysine and L-ornithine, or monoamino acids, such as L-homoserine, are found in various species of *Corynebacterium*, *Streptomyces*, and *Bifidobacterium* (Perkins and Cummins 1964; Perkins 1971; Koch et al. 1970; Veerkamp 1971; Leyh-Bouille et al. 1970).

In contrast to the highly variable third position, the first position L-alanine is almost invariable. Nonetheless, L-serine is found in this first position in some species of the order Micrococcales (von Wintzingerode et al. 2001; Hamada et al. 2009), and glycine substitutes the first position L-alanine in *M. leprae* (Draper et al. 1987). Beyond this core structure, the glycan units and peptide stems are subject to additional modifications, creating further variations in the peptidoglycan structure.

In addition to compositional differences, different types of peptide cross-linking are found not only among various lineages of Actinobacteria but also within a single species. The most common linkage is between the ω-amino group of the position 3 diamino acid and the position 4 D-alanine, mediated by two conventional penicillin-binding proteins (PBP1 and PBP2, encoded by *ponA1* and *ponA2* genes), which catalyze the D,D-transpeptidase reaction. In addition to this 3-4 cross-linking, mycobacteria and some species of *Streptomyces* and *Corynebacterium* insert the 3-3 cross-linking between two residues of diamino acid, catalyzed by L,D-transpeptidases (Leyh-Bouille et al. 1970; Wietzerbin et al. 1974; Lavollay et al. 2009, 2011; Kumar et al. 2012). In fact, this unusual 3-3 cross-linking is the predominant linkage found in *M. tuberculosis* (Kumar et al. 2012; Lavollay et al. 2008). Consistent with the abundance of the 3-3 cross-linking, the genomes of *M. tuberculosis* and *M. smegmatis* respectively carry five and six homologs of L,D-transpeptidases that mediate this reaction. Several of these play critical non-redundant roles in maintaining cell wall integrity, antibiotic resistance, and the establishment of *M. tuberculosis* infection in animal models (Gupta et al. 2010; Schoonmaker et al. 2014; Brammer Basta et al. 2015; Kieser et al. 2015; Sanders et al. 2014). In particular, genome-wide TnSeq

analyses demonstrated that one of the L,D-transpeptidases, LdtB, and two penicillin-binding proteins genetically interacts with distinct sets of genes, suggesting non-redundant functions of these two peptide bridges (Kieser et al. 2015). In addition to the genetic studies, recent cell biological analyses further revealed that cross-linking by L,D-transpeptidases are particularly necessary for creating 3-3 crosslinking in the aging cell wall along the sidewall while D,D-transpeptidases such as PBP1 and PBP2 are critical for polar elongation and repair of damaged sidewall peptidoglycan (Baranowski et al. 2018; Garcia-Heredia et al. 2018). These recent studies reinforce the concept that these enzymes play distinct roles. For more details of the peptidoglycan structure, comprehensive reviews are available (Schleifer and Kandler 1972; Vollmer et al. 2008; Mainardi et al. 2008).

The biosynthesis of peptidoglycan in Actinobacteria is generally similar to the evolutionarily conserved pathway found in other bacteria. It is separated into cytoplasmic and membrane steps: cytoplasmic enzymes make pentapeptidyl MurNAc, and membrane-bound enzymes produce the polyprenol-linked peptidoglycan precursor lipid II on the cytoplasmic side of the plasma membrane (Fig. 13.3a). MurF is the last enzyme of the cytoplasmic steps, mediating the UDP-MurNAc-tripeptide-D-alanyl-D-alanine ligase. One of the MurF substrates, D-alanyl-D-alanine, is synthesized by a D-alanine-D-alanine ligase. In *Mycobacterium* and *Streptomyces* species, this ligase belongs to the DdlA group (Noda et al. 2004). In contrast, *Amycolatopsis orientalis*, an actinobacterial species in the order Pseudonocardiales, uses VanA group D-alanine-D-lactate ligase, producing a lipid II capped with D-lactate instead of D-alanine (Marshall and Wright 1998). This bacterium is the producer of the antibiotic vancomycin, which binds the terminal D-alanine-D-alanine residue of lipid II to prevent its utilization for peptidoglycan biosynthesis. This VanA-mediated modification is a clever way for this bacterium to prevent its antibiotic product from inhibiting its lipid II. The first membrane step is mediated by MraY, the polyprenyl transferase, which conjugates pentapeptidyl MurNAc to a polyprenol phosphate. The resulting intermediate, termed lipid I, is then modified by GlcNAc to become a complete precursor, lipid II, mediated by the GlcNAc transferase, MurG. MurJ is the proposed flippase, which translocates lipid II from the cytoplasmic side to the periplasmic side of the plasma membrane. PBP1 and PBP2 function as the trans-glycosylases that transfer the de novo synthesized peptidoglycan subunit to the elongating chain, and also as the D,D-transpeptidases to introduce the classical 3-4 peptide bridges (Fig. 13.3a).

Given its structural importance, it is not surprising that many regulatory mechanisms control the peptidoglycan biosynthesis. De novo synthesis of peptidoglycan precursor starts in the cytoplasm, with the committing step mediated by MurA. This enzyme, UDP-*N*-GlcNAc enolpyruvyl transferase, is regulated in response to nutrient availability through physical interactions with a cytoplasmic regulator CwlM. The serine/threonine kinase PknB phosphorylates CwlM, and this phosphorylated form activates MurA (Boutte et al. 2016). Since PknB carries the extracellular peptidoglycan-binding domain known as the PASTA domain, it seems that intracytoplasmic MurA is regulated by sensing the periplasmic peptidoglycan biosynthetic activities. CwlM orthologs are widely found in Actinobacteria, suggesting

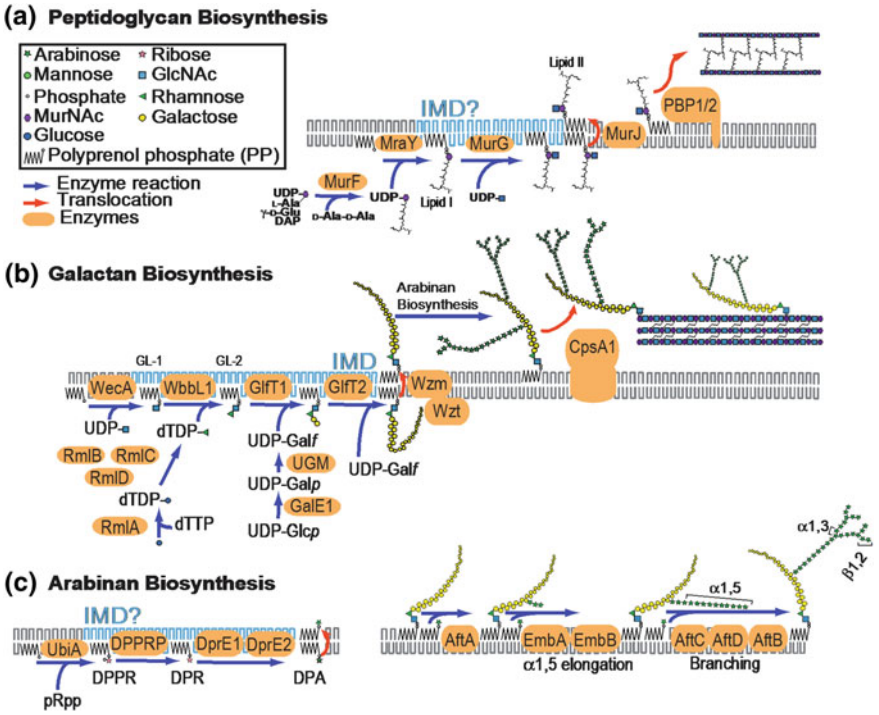

Fig. 13.3 Biosynthesis of peptidoglycan-arabinogalactan layer. **a** De novo synthesis of peptidoglycan. The pentapeptide compositions vary (see text for details). Remodeling of peptidoglycan layer by hydrolases and L,D-transpeptidases is not shown (see text for details). The IMD association of MurG is suggested from the proteomic analyses (Hayashi et al. 2016). **b** Biosynthesis of galactan. The biosyntheses of galactan and arabinan (see panel **c**) are found in species of the Corynebacteriales order. The representative pathway in *Mycobacterium* is shown. UGM, UDP-Gal*p* mutase. **c** Biosynthesis of decaprenol-phosphate-β-D-Ara*f* (DPA) and arabinan. pRpp, 5-phosphoribosyl-1-pyrophosphate; DPPR, decaprenol-phosphate-5-β-D-phosphoribofuranose; DPR, decaprenol-phosphate-β-D-ribofuranose. In *M. tuberculosis*, Rv3789 is a proposed DPA flippase (not shown). In *M. smegmatis*, a DPPR phosphatase (DPPRP) candidate (MSMEG_6402), DprE1 and DprE2 are suggested to associate with the IMD based on the proteomic analyses (Hayashi et al. 2016). The biosynthesis of arabinan follows that of galactan (see panel **b**)

that this is a highly conserved regulatory mechanism (Boutte et al. 2016). Furthermore, there are additional examples of potential regulations of peptidoglycan biosynthesis by phosphorylation. MurC, which mediates the addition of L-alanine onto UDP-MurNAc, is phosphorylated by the protein kinase PknA in *C. glutamicum*, and the phosphorylation results in decreased enzymatic activity (Fiuza et al. 2008). In *M. tuberculosis*, PknA phosphorylates the next enzyme in the pathway, MurD, which mediates the addition of D-glutamate onto UDP-*N*-acetylmuramoyl-L-alanine (Thakur and Chakraborti 2008). In addition to CwlM, PknB also phosphorylates PBP1 in *M. tuberculosis*, and the phosphorylation plays a critical role in polar cell

envelope elongation (Kieser et al. 2015). These observations collectively suggest that phosphorylation by serine/threonine kinases is a critical mechanism of regulating peptidoglycan biosynthesis at multiple different steps. *S. coelicolor* possesses 34 putative serine/threonine kinases, and some of them carry the extracellular PASTA peptidoglycan-binding domain (Petrickova 2003), suggesting that similar regulation of peptidoglycan biosynthesis by phosphorylation of key enzymes may occur in *Streptomyces* species.

Mycobacteria grow from the polar ends, and polar peptidoglycan synthesis supports cell elongation. When dividing, peptidoglycan synthesis is needed for the septum formation. Furthermore, sidewall peptidoglycan synthesis occurs in response to cell wall damage (Garcia-Heredia et al. 2018). These observations indicated that the synthesis of peptidoglycan is a controlled process likely requiring the coordination of biosynthetic enzymes as well as hydrolyzing enzymes. RipA is a peptidoglycan endopeptidase, which forms a complex with the peptidoglycan hydrolases RpfB and RpfE (Hett et al. 2007, 2008), or with the biosynthetic enzyme PBP1 (Hett et al. 2010). When fluorescent protein-tagged *M. tuberculosis* RipA and RpfB are heterologously expressed in *M. smegmatis*, they localized to the septum (Hett et al. 2007), and these proteins are suggested in coordinating septum resolution and cell separation (Chao et al. 2013). Being consistent with a known interaction with PBP1, an *M. smegmatis* mutant lacking all four Rpf homologs showed a reduced level of 4-3 crosslinking (Ealand et al. 2018). Other peptidoglycan modifying enzymes also play important roles. The peptidoglycan hydrolase, ChiZ (Rv2719c), is a protein in the mycobacterial divisomal complex (Chauhan et al. 2006; Vadrevu et al. 2011). Four peptidoglycan degrading amidases (Ami1-4) are present in the genomes of *M. tuberculosis* and *M. smegmatis* (Machowski et al. 2014). One of them is CwlM (Ami2), the above mentioned cytoplasmic regulator for which the amidase activity is not essential (Boutte et al. 2016). Of the remaining three, Ami1 (MSMEG_6281) is required for cell division (Senzani et al. 2017). Finally, DacB2 is a mycobacterial enzyme that shows D,D-carboxypeptidase and D,D-endopeptidase activities, and is proposed to play a role in converting the peptide bridges from 4-3 to 3-3 crosslinking (Baranowski et al. 2018; Bansal et al. 2015). The recent discoveries of many peptidoglycan hydrolyzing enzymes indicate the presence of complex mechanisms to maintain the peptidoglycan integrity.

Do Actinobacteria produce teichoic acids, which are important structural components of Gram-positive peptidoglycan layer? It is well established that teichoic, teichuronic and teichulosonic acids are widespread in bacteria such as *Streptomyces*, *Micrococcus*, *Propionibacterium*, *Kribbella, Catellatospora*, and *Actinoplanes* (Tul'skaya et al. 2011; Naumova et al. 1980), which do not have mycolic acid-based outer membrane (see below). Furthermore, lipoteichoic acids have been reported in *Streptomyces*, *Agromyces*, and *Thermobifida* species (Rahman et al. 2009; Cot et al. 2011) (and references therein). These molecules appear to be absent in bacteria that produce mycolic acid-based outer membrane. These mycolic acid-producing bacteria are sometimes called "mycolata," and are found in all known families within the Corynebacteriales order: *Corynebacteriaceae*, *Dietziaceae*, *Gordoniaceae*, *Mycobacteriaceae*, *Nocardiaceae*, *Segniliparaceae*, *Tsukamurellaceae*,

and *Williamsia*. With a recent demonstration of the Gram-negative outer membrane having a load-bearing function (Rojas et al. 2018), the evolution of the unique outer membrane in mycolata bacteria (see below) might have provided an alternative load-bearing function to the cell envelope and might have made teichoic acids and related molecules unnecessary.

Arabinogalactan

The arabinogalactan layer is composed primarily of a galactose (Gal) polymer of repeating β1,5- and β1,6-linked D-galactofuranose (Gal*f*) units, covalently modified by stretches of α1,5-linked Ara*f* residues which are branched by α1,3 branching sites (Daffe et al. 1990; Jankute et al. 2015; Angala et al. 2014). The linear galactan chain of the arabinogalactan is attached to the MurNAc residue of peptidoglycan through the α-L-Rha*p*-α1,3-D-GlcNAc-1-phosphate linker (McNeil et al. 1990). This layer is found in mycolata and is not known to be present in conventional Gram-positive Actinobacteria such as *Streptomyces* or *Micrococcus* species. It is best-studied in *Mycobacterium* and *Corynebacterium* species. In particular, *C. glutamicum* has been a useful model for delineating the structure and biosynthesis of this layer because arabinan biosynthesis is dispensable under laboratory growth conditions (Alderwick et al. 2005). The complete absence of arabinan biosynthesis in *Corynebacterium* results in a slower growing but viable mutant (Jankute et al. 2018). Even though the general structures are similar among mycolata, there are some notable differences. The galactan chain consists of ~30 residues in mycobacteria while it is much shorter in corynebacteria (Daffe et al. 1990; Alderwick et al. 2005). More drastic differences are found in *Nocardia* and *Rhodococcus*, in which galactan is primarily composed of linear β1,5-D-Gal*f* without the alternating β1,6 linkages (Daffe et al. 1993). Furthermore, the Gal*f* residues are partially modified by β1,6 mono-Glc side chains in *Nocardia* or by β1,2- and β1,3-linked Gal*f* residues in *Rhodococcus*. The arabinan portion of arabinogalactan is also differentially modified: galactosamine and succinate in *M. tuberculosis* and some slow-growing mycobacteria (Bhamidi et al. 2008; Draper et al. 1997; Lee et al. 2006; Peng et al. 2012) or rhamnose (Rha) in *C. glutamicum* (Alderwick et al. 2005). In some *Corynebacterium* species, arabinogalactan can be additionally modified by other monosaccharides such as Man and Glc (Abou-Zeid et al. 1982). The non-reducing termini of the arabinan moiety appear to be the most complex in mycobacteria, consisting of the branching motif: Ara*f*-β1,2-Ara*f*-α1,5-(Ara*f*-β1,2-Ara*f*-α1,3-)Ara*f*-α1,5-Ara*f*-α1-. Other mycolata bacteria tend to have simpler terminal ends. For example, *Nocardia* species cap the non-reducing ends of the arabinan residue by the linear motif: Ara*f*-β1,2-Ara*f*-α1,5-Ara*f*-α1-.

Galactan biosynthesis starts from building the linker moiety on a decaprenol-phosphate lipid. In mycobacteria, a homolog of WecA is the proposed first enzyme that transfers GlcNAc-phosphate from UDP-GlcNAc to the polyprenol lipid, forming an intermediate termed GL-1 (Jin et al. 2010). The deletion of the encoding gene, MSMEG_4947, in *M. smegmatis* resulting in severe morphological changes is

consistent with its role in the cell wall galactan biosynthesis. The rhamnosyltransferase WbbL adds L-rhamnopyranose (Rha*p*) from dTDP-Rha*p* to the GlcNAc residue of GL-1, forming GL-2 (Mills et al. 2004). The Rha*p* donor, dTDP-Rha*p*, is synthesized by sequential actions of four enzymes, RmlA-D, starting from dTTP and D-Glc-α1-phosphate substrates (Ma et al. 2001; Li et al. 2006; Ma et al. 1997, 2002; Qu et al. 2007; Stern et al. 1999) (Fig. 13.3b).

The next step in the pathway is to prime the GL-2 with two Gal*f* residues. The donor of Gal*f* is UDP-Gal*f*, which is produced by the sequential actions of two enzymes. The first enzyme, GalE1, is the UDP-Glc 4-epimerase, which epimerizes UDP-glucopyranose (UDP-Glc*p*) to UDP-galactopyranose (UDP-Gal*p*) (Weston et al. 1997; Pardeshi et al. 2017). UDP-Gal*p* is then converted to UDP-Gal*f* by an essential enzyme, UDP-Gal*p* mutase (Weston et al. 1997; Pan et al. 2001), for which the atomic resolution structure was recently revealed (van Straaten et al. 2015). GlfT1 is the galactosyltransferase responsible for adding the first two Gal*f* residues to GL-2 forming Gal*f*-Gal*f*-Rha*p*-GlcNAc-phosphate-decaprenol (GL-4) (Mikusova et al. 2006; Alderwick et al. 2008; Belanova et al. 2008). GlfT2 is the processive galactosyltransferase, which extends the galactan polymer (Szczepina et al. 2009; Wheatley et al. 2012). A comparative study between *Mycobacterium* and *Corynebacterium* showed that GlfT2 can dictate the chain length of galactan (Wesener et al. 2017). We and others have demonstrated that GlfT2 is enriched in the polar region of mycobacterial cells and bound to the IMD (Meniche et al. 2014; Hayashi et al. 2016), where the enzyme perhaps coordinates and facilitates the spatially localized biosynthesis of the galactan layer. Once the decaprenol-linked galactan precursor is synthesized, it is flipped to the periplasmic side of the plasma membrane, and a putative ABC transporter, composed of two proteins, Wzm and Wzt, is implicated in this process (Dianiskova et al. 2011).

Once the decaprenol-linked galactan precursor is translocated to the periplasmic side, the galactan chain is modified by several arabinans. An earlier study suggested that there are three arabinans attached in one galactan polymer (Alderwick et al. 2005), but a more recent study suggests that only two arabinan chains are present per galactan (Bhamidi et al. 2011). The donor of Ara is decaprenol-phosphate-β-D-Ara*f* (DPA) (Alderwick et al. 2005; Lee et al. 1995, 1997; Wolucka and de Hoffmann 1995; Wolucka et al. 1994; Xin et al. 1997). DPA biosynthesis starts with UbiA, a 5-phospho-α-D-ribose-1-pyrophosphate:decaprenol phosphate 5-phosphoribosyltransferase, which produces decaprenol-phosphate-5-β-D-phosphoribofuranose (DPPR) using 5-phosphoribosyl-1-pyrophosphate (pRpp) and decaprenol phosphate as substrates (Scherman et al. 1995; Alderwick et al. 2011). The deletion of *ubiA* gene in *C. glutamicum* results in the complete abrogation of cell wall arabinan, suggesting that this enzyme is the sole enzyme that diverts pRpp into the biosynthesis of arabinan (Alderwick et al. 2005). In *M. tuberculosis*, the genome region spanning Rv3789-Rv3809c are dedicated to arabinan biosynthesis, and a putative phosphorylase in this region, Rv3807c, is proposed to act as the next enzyme, DPPR phosphatase, which forms decaprenol-phosphate-β-D-ribofuranose (DPR). However, the deletion of the ortholog in *M. smegmatis* showed only a mild impact on arabinan content of arabinogalactan (Jiang et al. 2011), suggesting that

there are phosphatases that can surrogate the function of this enzyme. The third and fourth enzymes in the DPA biosynthesis pathway are oxidoreductases: DprE1 oxidizes DPR to decaprenol-phosphate-2-keto-β-D-*erythro*-pentofuranose (DPK), which is then reduced by DprE2 to DPA (Mikusova et al. 2005) (Fig. 13.3c). A homolog of *dprE2* is present in *C. glutamicum*, making it a redundant gene, and this second gene is also present in *M. tuberculosis* (Rv2073c) (Meniche et al. 2008). Inhibition of DprE1 results in the accumulation of DPR and kills *M. tuberculosis*, validating this enzyme as a potential target of TB chemotherapy (Grover et al. 2014; Makarov et al. 2009). Since DprE2 is an NADH-dependent oxidoreductase (Mikusova et al. 2005), it seems likely that biosynthetic enzymes upstream of DprE2 are active on the cytoplasmic side of the plasma membrane. DprE1 does not appear to carry trans-membrane domains while DprE2 does (data not shown). Nonetheless, both have been identified as IMD-associated proteins by proteomic analysis (Hayashi et al. 2016), likely suggesting a peripheral association of DprE1 with the membrane domain. Once DPA is produced, it is flipped to the periplasmic side of the plasma membrane, and Rv3789 has been proposed as the flippase candidate (Larrouy-Maumus et al. 2012).

Using DPA as the Ara donor, coordinated actions of multiple arabinosyltransferases, which belong to the GT-C glycosyltransferase superfamily, drive the biosynthesis of arabinan on the periplasmic side of the plasma membrane. The first enzyme that primes the galactan chain with Ara is AftA (Alderwick et al. 2006). In *C. glutamicum*, the next enzyme that extends the α1,5 Ara chain of the core arabinan is Emb (Alderwick et al. 2005). The homologs in mycobacteria, EmbA and EmbB, are proposed to function redundantly in the core α1,5 arabinan synthesis (Alderwick et al. 2005; Escuyer et al. 2001). Notably, these enzymes, EmbB in particular, are the targets of the frontline drug ethambutol (Takayama and Kilburn 1989; Mikusova et al. 1995; Telenti et al. 1997; Lety et al. 1997; Belanger et al. 1996). The α1,3 branching is mediated by the arabinosyltransferase, AftC, which plays a role in both arabinogalactan and LAM biosynthesis in mycobacteria (Birch et al. 2008, 2010). AftD was initially proposed as α1,3 branching enzyme in mycobacteria (Skovierova et al. 2009), but a more recent study suggests that it is a processive α1,5 arabinosyltransferase, which extends the α1,3 branching Ara primed by AftC (Alderwick et al. 2018). Finally, AftB adds the terminal β1,2 capping (Seidel et al. 2007). When *aftB* is deleted, mycoloylation sites on arabinogalactan are severely reduced (Bou Raad et al. 2010). Nevertheless, the *aftB* deletion mutant can still produce an outer membrane, although the stability of the outer membrane becomes significantly compromised.

Once arabinan is synthesized onto the galactan polymer, the arabinogalactan complex is attached to the 6-OH of MurNAc residues in the peptidoglycan glycan chain (Fig. 13.3c). The enzymes that mediate this reaction are the LytR-CpsA-Psr (LCP) phosphotransferases, which are variably termed CpsA1/CpsA2, LcpA/LcpB, or Lcp1/CpsA by different groups (Wang et al. 2015; Harrison et al. 2016; Baumgart et al. 2016; Grzegorzewicz et al. 2016). CpsA1 is widely conserved and appears to play a primary role in arabinogalactan anchoring. In contrast, CpsA2 is not found in fast-growing species of *Mycobacterium* and is implicated in processes associated with the host-pathogen interaction (Koster et al. 2017).

Outer Membrane

The outer membrane (OM) , also known as the mycomembrane, is a mycolic acid-rich pseudo-bilayer of lipids. It has 7 nm thickness as determined by cryo-electron microscopy in mycobacteria (Zuber et al. 2008; Hoffmann et al. 2008; Sani et al. 2010). In a proposed model, the inner leaflet of the OM is composed primarily of mycolic acids, which are covalently attached by an ester linkage to the non-reducing end of arabinan. The outer leaflet of the OM is comprised of diverse lipid species. Some of these extractable outer membrane lipids are conserved throughout mycolata. For instance, the OM of both *Corynebacterium* and *Mycobacterium* likely consist of trehalose di(coryno)mycolates, free mycolic acids and fatty acids (Bansal-Mutalik and Nikaido 2011, 2014). In contrast, other lipids such as glycopeptidolipids (GPLs), phthiocerol dimycocerosates (PDIMs) and phenolic glycolipids (PGLs) are specific to certain species within mycolata. Some of the major outer membrane lipids are highlighted below.

Mycolic Acids

Mycolic acids are long α-alkyl β-hydroxy fatty acids with the meromycolic acid carbon backbone ranging from C18 to C76 and the alkyl side chain ranging from C24 to C26. Comprehensive surveys of the mycolata from the 1980s demonstrated that the length of the mycolic acids varies significantly among species (Collins et al. 1982; Goodfellow et al. 1982). Additional modifications such as cyclopropane rings, double bonds, and methylations further add diversity to the structures of mycolic acids (Marrakchi et al. 2014; Minnikin et al. 2015; Quemard 2016). Because of the structural variety, mycolic acid structures have been used for taxonomic purposes. For instance, *Corynebacterium* produces some of the shortest mycolic acids, ranging from C22 to C36 (Collins et al. 1982; Welby-Gieusse et al. 1970). In contrast, recent studies revealed that *Segniliparus rotundus* produces the longest mycolic acid (C100), which is perhaps the longest fatty acyl chain currently known (Hong et al. 2012; Laneelle et al. 2013). *Mycobacterium* produces relatively long mycolic acids, typically ranging from C60 to C90 (Barry et al. 1998). However, *Hoyosella altamirensis* and *Hoyosella subflava*, two recently discovered environmental cocci that belong to the *Mycobacteriaceae* family and are closely related to the *Mycobacterium* genus, produce relatively short mycolic acids, ranging from C30 to C36 (Laneelle et al. 2012).

In mycobacteria, mycolic acid biosynthesis is initiated by type I and type II fatty acid synthases (FAS-I and FAS-II) (Fig. 13.4). FAS-I is a large multifunctional enzyme that utilizes acetyl-CoA and malonyl-CoA to create short chain fatty acyl-CoAs (C16–C18 and C24–C26) (Brindley et al. 1969). The longer of the two distinct pools of the products are then carboxylated, and the carboxyacyl-CoA serves as the donor of the alkyl side chain for the mycolic acid synthesis.

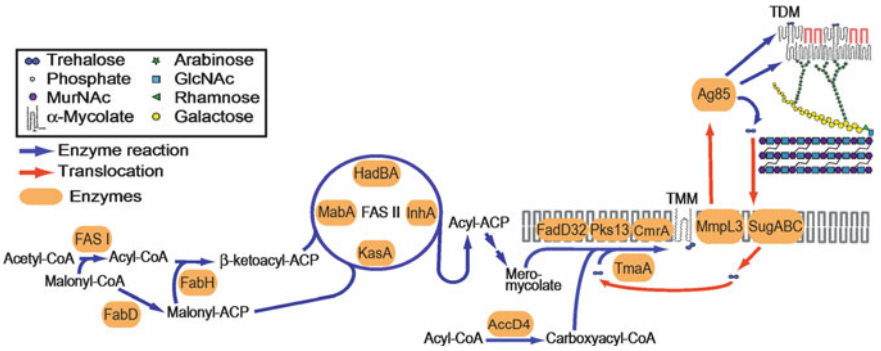

Fig. 13.4 Biosynthesis of mycolic acids. The chain length of mycolic acids can vary dramatically among mycolata bacteria. Further modifications of mycolic acids can also vary among species. For example, cyclopropane, methoxy, keto, and hydroxy modifications are found in *M. tuberculosis*, while epoxy and methyl modifications are found in *M. smegmatis*. Representative structures are shown. Biosynthesis of trehalose monomycolate (TMM) and trehalose dimycolate (TDM) is coordinated in conjunction with the biosynthesis of mycolic acid and its attachments to the arabinan layer

Next, β-ketoacyl-acyl carrier protein (ACP) synthase III (FabH) condenses malonyl-ACP and acyl-CoA produced by the FAS-I enzyme, producing β-ketoacyl-ACP. The FAS-II elongates the fatty acyl chain of β-ketoacyl-ACP to produce fully elongated acyl-ACPs (Odriozola et al. 1977; Bloch 1977; Slayden and Barry 2002). As mentioned above, *Corynebacterium* produces the shortest mycolic acids, and being consistent with this observation, *C. glutamicum* lacks the FAS-II elongation system. Instead, its genome encodes two FAS-I genes (*fasA* and *fasB*) with FasA playing the dominant role (Radmacher et al. 2005). In contrast to FAS-I being a single polypeptide carrying multiple catalytic domains, the FAS-II system is composed of several separate enzymes. First, MabA, the β-ketoacyl-ACP reductase, reduces the β-keto moiety of the β-ketoacyl-ACP. Second, dimeric β-hydroxyacyl-ACP dehydratases, HadBA/HadBC, dehydrate the product of MabA reaction, β-hydroxyacyl-ACP. Third, the resultant enoyl-ACP is reduced by *trans*-2-enoyl-ACP reductase, InhA. Finally, the fully saturated acyl-ACP is elongated by the β-ketoacyl-ACP synthases, KasA or KasB, using malonyl-ACP (Marrakchi et al. 2014; Duan et al. 2014).

Why are there two different heterodimers of β-hydroxyacyl-ACP dehydratases? HadB is proposed as the catalytic component (Biswas et al. 2015), implying that HadA and HadC play non-catalytic roles within the heterodimers HadBA and HadBC. Interestingly, HadC is mutated in an avirulent strain of *M. tuberculosis* (Lee et al. 2008; Zheng et al. 2008), and targeted gene disruptions in *M. tuberculosis* and *M. smegmatis* demonstrated changes in mycolic acid profile, and attenuation of virulence in the case of *M. tuberculosis* (Slama et al. 2016; Jamet et al. 2015). It is proposed that HadBA is the dehydratase in the early stage of meromycolic acyl chain elongation and HadBC mediates the late elongation steps. Furthermore, another recent study in *M. smegmatis* revealed an additional dehydratase, termed

HadD, which is involved in α- and epoxy-mycolic acid biosynthesis (Lefebvre et al. 2018). HadD is conserved in *Mycobacterium* genus, including *M. leprae*, but is absent in other genera of mycolata, suggesting that it is involved in specific steps found in mycobacteria.

Acyl-ACPs, produced by the FAS II, are modified by cyclopropane synthases and methyltransferases, resulting in meromycolates (Crellin et al. 2013; Marrakchi et al. 2014; Minnikin et al. 2015). Meromycolates are then activated to meromycoloyl-AMP by the fatty acyl-AMP ligase FadD32. Next, meromycoloyl-AMP is loaded onto Pks13, which condenses the meromycoloyl-AMP with carboxyacyl-CoA, and covalently links the resulting α-alkyl β-ketoacyl chain to the C-terminal ACP domain of Pks13 (Leger et al. 2009; Gavalda et al. 2009). Pks13 has an acyltransferase activity, which transfers the α-alkyl β-ketoacyl chain onto a trehalose, releasing the mono-α-alkyl β-ketoacyl trehalose (Gavalda et al. 2014). The released product is then reduced by CmrA to produce trehalose monomycolate (TMM) (Lea-Smith et al. 2007; Bhatt et al. 2008). In *C. glutamicum*, the TMM equivalent, namely trehalose monocorynomycolate (TMCM), is transiently acetylated by the acetyl transferase TmaA, and this acetylation is critical for the translocation of TMCM across the plasma membrane (Yamaryo-Botte et al. 2014). Acetylated TMCM is transported through the plasma membrane by the transporter MmpL3 (Grzegorzewicz et al. 2012; Varela et al. 2012; Xu et al. 2017; Li et al. 2016). TmaA is conserved in mycobacteria, suggesting that TmaA-mediated acetylation is a conserved mechanism of licensing mature TMM/TMCM for transport. Finally, Ag85 transfers mycolic acid from TMM to either another TMM molecule to produce TDM, or to the arabinan layer of the cell wall to create mycoloyl arabinogalactan peptidoglycan cell wall (Belisle et al. 1997; Backus et al. 2014) (Fig. 13.4).

Trehalolipids

Trehalolipids are bio-surfactants, which are important for bacteria to emulsify and utilize hydrophobic molecules. *Rhodococcus* species are prominent trehalolipid producers, and its production is induced when *Rhodococcus* is grown in the presence of hydrophobic molecules such as alkanes (Yakimov et al. 1999; Lang and Philp 1998). The capability of *Rhodococcus* species to produce trehalolipid surfactants attracts considerable interest in industrial applications, especially in oil recovery and oil spill treatment (Pacheco et al. 2010; Liu and Liu 2011). Structurally diverse variants of trehalolipids, which *Rhodococcus* can produce, include: mycoloylated trehaloses, such as TMM, TDM, and trehalose trimycolates (Niescher et al. 2006), and various forms of acylated trehalose, in which the acyl chains are generally shorter straight chain fatty acids (Singer et al. 1990; Philp et al. 2002; Uchida et al. 1989; Tuleva et al. 2008; Tokumoto et al. 2009; White et al. 2013; Espuny et al. 1995). Other mycolata bacteria, such as *Nocardia farcinica* and several species of *Tsukamurella*, are also known to produce tetraacyl or diacyl trehalose, respectively (Christova et al. 2015; Pasciak et al. 2010; Kugler et al. 2014; Vollbrecht et al. 1998). However, tre-

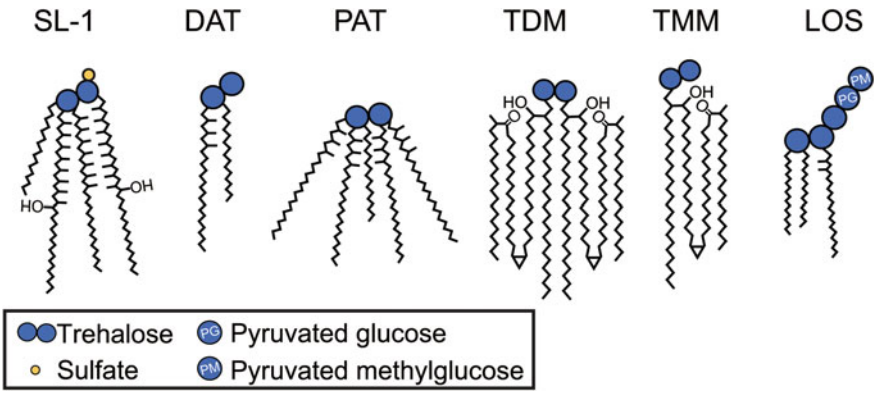

Fig. 13.5 Structures of trehalose-containing lipids. SL-1, a sulfolipid species; DAT, diacyltrehalose; PAT, pentaacyltrehalose; TMM, trehalose monomycolate; TDM, trehalose dimycolate; LOS, lipooligosaccharide. Fatty acid structures vary. Ester linkages and some unsaturated bonds are abbreviated. See text for additional structural variations

halolipids are not restricted to mycolata. *M. luteus* and *Arthrobacter* species produce trehalose tetraester (Tuleva et al. 2009; Passeri et al. 1991). These bacteria belong to the Micrococcales order, and exhibit a more typical Gram-positive cell wall without the outer membrane, suggesting that the presence of the outer membrane is not a prerequisite of producing trehalolipids. Rather, we wonder if the production of trehalolipid surfactants allowed the evolution of the outer membrane in mycolata.

Biosynthesis of trehalolipids has been studied more extensively in pathogenic *Mycobacterium* species than in other mycolata bacteria. In addition to TMM and TDM, bacteria that belong to the *M. tuberculosis* complex produce diacyl, triacyl and pentaacyl trehalose (DAT, TAT, and PAT) (Fig. 13.5). A unique feature of these mycobacterial trehalolipids is the extensive methyl branching of fatty acyl moieties. Mycolipenic acid is one such fatty acid, which is tri-methylated with one unsaturated bond. Mycolipenoyl modification of trehalose is only found in the *M. tuberculosis* complex such as *M. tuberculosis*, *M. bovis*, and *Mycobacterium africanum*. Other *Mycobacterium* species, such as *Mycobacterium fortuitum*, do produce acyl trehaloses, but the acyl chains are not multi-methylated (Ariza et al. 1994; Lopez-Marin et al. 1994). The polyketide synthase Msl3 synthesizes mycolipenic and mycosanoic acids, and the activation and loading of the fatty acid substrate are mediated by the fatty acid ligase FadD21 (Dubey et al. 2002; Rousseau et al. 2003; Belardinelli et al. 2014). Similarly, another polyketide synthase Msl5 produces a minor monomethyl branched unsaturated C16–C20 fatty acid found in acyl trehaloses (Dubey et al. 2003). Once fatty acids are made, PapA3 mediates the acyltransferase reactions using trehalose as the acceptor (Hatzios et al. 2009). It has been proposed that PapA3 can successively transfer fatty acyl groups to the 2- and 3-positions of trehalose at least in vitro. The acyltransferase Chp2 then mediates the last three acylation events to produce PAT (Belardinelli et al. 2014; Touchette et al. 2014). MmpL10 is the

proposed plasma membrane flippase for the translocation of acyl trehalose species (Belardinelli et al. 2014; Touchette et al. 2014).

Sulfolipids are trehalolipids that are sulfonated at the 2-position of trehalose (Fig. 13.5) (Middlebrook et al. 1959; Goren 1970). The most abundant species is a tetra-acylated species known as SL-1, in which three acyl groups at the 6, 3′ and 6′-positions are hepta- or octa-methyl phthioceranic acids or hydroxyphthioceranic acids and one acyl group at the 2′-position is either palmitic or stearic acid. The biosynthesis starts with the sulfotransferase Sft0, a widely conserved protein in mycolata, which transfers sulfate from 3′-phosphoadenosine-5′-phosphosulfate to trehalose (Mougous et al. 2004). The second step is mediated by the acyltransferase PapA2, which transfers a straight chain fatty acid, palmitate or stearate, from its CoA donor substrate to the 2′-position of trehalose 2-sulfate, producing the monoacyl intermediate termed SL659 (Kumar et al. 2007). The third step is another acyltransferase reaction, in which PapA1 transfers (hydroxy) phthioceranoyl group from the polyketide synthase Pks2 to the 3′-position of trehalose residue of SL659 forming the diacyl intermediate SL1278 (Kumar et al. 2007; Sirakova et al. 2001; Bhatt et al. 2007). FadD23 is the proposed fatty acyl AMP ligase involved in Pks2-mediated (hydroxy)phthioceranoyl biosynthesis (Gokhale et al. 2007). Similar to the role of Chp2 for PAT synthesis, the acyltransferase Chp1 adds the remaining acyl groups at the 6- and 6′-positions of trehalose (Seeliger et al. 2011). The product, SL-1, is translocated across the plasma membrane, and MmpL8 and Sap are implicated in this process (Seeliger et al. 2011; Domenech et al. 2004; Converse et al. 2003). Precise functions of sulfolipids remain unknown, but these lipids are implicated in host-pathogen interactions and the establishment of infection (Angala et al. 2014; Schelle and Bertozzi 2006; Daffe et al. 2014).

Lipooligosaccharides (LOSs) are another type of trehalolipids found in *Mycobacterium* species, including *M. smegmatis, Mycobacterium kansasii, M. marium,* and *Mycobacterium canettii* (Hunter et al. 1983; Saadat and Ballou 1983; Daffe et al. 1991). LOSs play critical roles in colony morphology, biofilm formation, motility, as well as immune modulation during host infection (Alibaud et al. 2014; Rombouts et al. 2009; Ren et al. 2007; Sarkar et al. 2011; van der Woude et al. 2012). The core structure of LOS is similar to other acyl trehaloses in that trehalose is modified with either straight chain or branched chain fatty acids. The feature of LOS that distinguishes it from other trehalolipids is the additional glycan modification of the acyl trehalose core. The glycan structures vary among different *Mycobacterium* species. For example, *M. smegmatis* LOS consists primarily of Glc*p* (Fig. 13.5), whereas *M. kansasii, Mycobacterium gastri,* and *Mycobacterium marinum* produce several different LOS species containing Rha*p*, xylopyranose and *N*-acyl kanosamine in addition to Glc*p* (Saadat and Ballou 1983; Rombouts et al. 2009, 2010, 2011; Gilleron et al. 1993; Hunter et al. 1984). The biosynthesis of LOSs is not fully understood. Similar to other trehalolipid biosynthesis, the core acyl trehalose synthesis requires specific polyketide synthases such as Pks5 and Pks5.1, fatty acyl-AMP ligases such as FadD25, and acyltransferases such as PapA4 and PapA3 (Rombouts et al. 2011; Etienne et al. 2009). Glycosyltransferases presumably transfer glycans to the acyl

trehalose core, but only a few genes have been experimentally validated (Ren et al. 2007; Sarkar et al. 2011; Burguiere et al. 2005; Chen et al. 2015; Nataraj et al. 2015).

Mannolipids

As discussed above, PIMs, LM, and LAM are at least partially present in the plasma membrane. Nevertheless, substantial evidence also suggests that these molecules are anchored to the outer membrane of mycolata bacteria as well. First, the majority of LM/LAM was accessible to surface biotinylation in *M. bovis* BCG (Pitarque et al. 2008). Second, only residual amounts of LM/LAM remain in the spheroplast of *M. smegmatis* (Dhiman et al. 2011). These data both suggest that the majority of LM/LAM could be in the outer membrane. Furthermore, LAM from pathogenic mycobacteria is capped with α1,2 Man residues, which are an epitope recognized by host lectins (Ishikawa et al. 2017; Kallenius et al. 2016; Turner and Torrelles 2018). Although host cell receptors can function to detect cell fragments rather than intact cells, the diverse repertoire of host immune molecules to identify these molecules may be more consistent with the concept that these molecules are exposed on the surface of mycobacterial cells. Finally, as detailed in the *Capsules and Extracellular Polysaccharides* section, mannan and arabinomannan are components of mycobacterial capsule. While the biosynthetic relationship between LM/LAM and mannan/arabinomannan is not established, there must be a mechanism to transport either lipidated or delipidated glycans across the cell wall and outer membrane. There have been studies suggesting that the lipoprotein LprG plays a role in this process (Drage et al. 2010; Alonso et al. 2017). However, there is also compelling evidence suggesting that LprG is involved in triacylglycerol trafficking, and acts in a more complex way by physically interacting with two other lipoproteins, LppK and LppI, as well as Ag85A (Touchette et al. 2017; Martinot et al. 2016).

Glycopeptidolipids

Glycopeptidolipids (GPLs) are found in the nontuberculous *Mycobacterium* species, such as *M. smegmatis, M. avium, Mycobacterium intracellulare*, and *Mycobacterium abscessus* and are important virulence factors for the pathogenic species (Schorey and Sweet 2008; Gutierrez et al. 2018; Mukherjee and Chatterji 2012). The defects in GPL biosynthesis results in compromised cell envelope integrity and abnormalities in growth, biofilm formation and sliding motility among others, suggesting their critical roles as a component of the outer membrane (Recht et al. 2000; Recht and Kolter 2001; Zanfardino et al. 2016). GPLs have a common core structure consisting of three parts: a mono-unsaturated 3-hydroxy/methoxy C26–C34 acyl chain, a D-phenylalanyl-D-*allo*-threonyl-D-alanyl-L-alaninol tetrapeptide, and two carbohydrate modifications, a 6-deoxy-α-L-talose linked to the D-*allo*-threonine residue

and an α-L-Rha linked to L-alaninol (Fig. 13.6a). GPLs can be further glycosylated, and these glycan residues are additionally methylated to give rise to serotype-specific GPLs (Chatterjee and Khoo 2001). During the biosynthesis of GPLs, monounsaturated 3-hydroxy/methoxy acyl chain is synthesized by the actions of the acyl-CoA dehydrogenase FadE5, the polyketide synthase Pks, and *O*-methyltransferase Fmt (Jeevarajah et al. 2002; Sonden et al. 2005; Jeevarajah et al. 2004). Fmt is proposed to convert 3-hydroxy to 3 methoxy during the acyl chain synthesis. Mps1 and Mps2 are the nonribosomal peptide synthases that produce the tetrapeptide core (Sonden et al. 2005; Billman-Jacobe et al. 1999). PapA3, an acyltransferase involved in acyl trehalose biosynthesis in *M. tuberculosis*, is proposed to transfer the acyl chain bound to Pks to the tetrapeptide in nontuberculous mycobacteria (Ripoll et al. 2007). Gtf1 and Gtf2 are the 6-deoxytalosyltransferases and rhamnosyltransferase involved in the synthesis of the core glycan residues, respectively (Miyamoto et al. 2006). Additional rhamnosyltransferases, fucosyltransferases and glucosyltransferases, as well as glycan *O*-methyltransferases and *O*-acetyltransferase, produce serotype-specific GPLs (Recht and Kolter 2001; Jeevarajah et al. 2004; Miyamoto et al. 2006; Patterson et al. 2000; Miyamoto et al. 2007, 2008, 2010; Maslow et al. 2003; Eckstein et al. 1998; Naka et al. 2011; Fujiwara et al. 2007; Nakata et al. 2008). Once synthesized, GPLs are transported to the outer membrane by the actions of MmpL4a/b, Gap, and MmpS4 (Sonden et al. 2005; Medjahed and Reyrat 2009; Nessar et al. 2011; Bernut et al. 2016) (Fig. 13.6b).

GPLs are specific to certain species of *Mycobacterium*, but structurally different types of GPLs have been reported from other bacteria in the Corynebacteriales order. *Gordonia hydrophobica* produces a mono-glucosylated *N*-acyl tridecapeptide, in which the beta-hydroxy residue of the fatty acid is interlinked to the C-terminus of the peptide chain, forming a cyclic lactone ring (Moormann et al. 1997). Related molecules are also found in *Rhodococcus erythropolis* (Koronelli 1988).

Mycocerosyl Lipids

Phthiocerol dimycocerosates (PDIMs) are waxy lipids, in which a phthiocerol (a long (C33-C41) carbon chain β-diol) is esterified with two poly-methylated fatty acids called mycocerosic acids (Fig. 13.6a). PDIMs are found in pathogenic *Mycobacterium*, such as *M. tuberculosis*, *M. bovis*, and *M. leprae* (Daffe et al. 2014; Daffe and Laneelle 1988). Instead of mycocerosic acids, some species such as *Mycobacterium ulcerans* and *M. marinum* utilize phthioceranic acids, in which the chirality of the methyl branches are L configurations instead of D configurations found in mycocerosic acids. PDIMs are essential for *M. tuberculosis* to establish infection in animal models (Goren et al. 1974; Cox et al. 1999; Camacho et al. 1999), and the molecular mechanisms governing the host-pathogen interaction are actively investigated (Arbues et al. 2014). The PDIM synthesis is proposed to take place in four distinct stages (Trivedi et al. 2005) (Fig. 13.6c). First, FadD26, a fatty acyl-AMP ligase, activates long-chain fatty acids to fatty acyl-AMP and transfers the acyl moiety

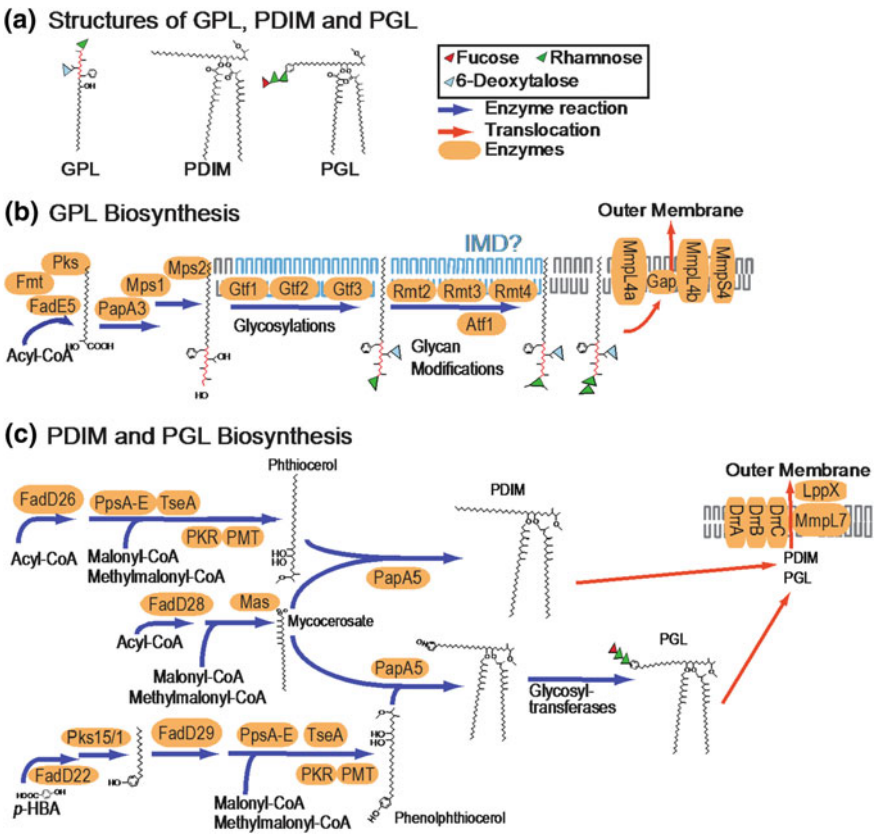

Fig. 13.6 Biosynthesis of glycopeptidolipids and mycocerosyl lipids. **a** Structures of glycopeptidolipids (GPLs) , phthiocerol dimycocerosates (PDIMs) and phenolic glycolipids (PGLs) . The structure of the core (nonspecific) GPL is shown. GPLs are modified by additional glycosylation, methylation and acetylation that result in a variety of serotypes, most well characterized in *M. avium*. The red line indicates the peptide backbone. Significant variations are also found in PDIMs and PGLs, and representative structures are shown. Fucose and rhamnose in PGLs are often methylated (not shown). **b** Biosynthesis of GPL. Gtf, glycosyltransferases; Rmt, *O*-methyltransferases; Atf, *O*-acetyltransferase. The IMD association of Gtf1-3 and Rmt2-4 is suggested from the proteomic analyses (Hayashi et al. 2016). **c** Biosynthesis of PDIMs and PGLs. Acyl-CoA is synthesized from FAS I. PKR, phthiodiolone ketoreductase; PMT, phthiotriol methyltransferase; *p*-HBA, *p*-hydroxybenzoic acid. PGLs are likely exported through the same machinery as that for PDIMs. Some structural details are abbreviated for simplicity

to the polyketide synthase PpsA for the synthesis of phthiocerol moiety (Trivedi et al. 2004, 2005; Camacho et al. 2001; Azad et al. 1997). PpsA and the next enzyme PpsB lack the dehydratase and the enoylreductase domains, allowing the formation of the β-diol structure of phthiocerol. Second, additional polyketide synthases PpsC, PpsD and PpsE continue the chain extension of phthiocerol using malonyl-CoA or methyl malonyl-CoA as the hydrocarbon donor. TesA is a thioesterase, which interacts with PpsE (Rao and Ranganathan 2004), and is proposed to release the phthiodiolone product upon completion of the synthesis (Waddell et al. 2005; Chavadi et al. 2011). Structural variations are introduced by phthiodiolone ketoreductases and phthiotriol methyltransferases (Pérez et al. 2004a; Onwueme et al. 2005; Simeone et al. 2007) to produce mature phthiocerol. Third, FadD28 activates and transfer a fatty acid onto the mycocerosic acid synthase Mas, which elongates the fatty acyl substrate with methyl malonyl-CoA to produce poly-methylated mycoserosic acids (Cox et al. 1999; Trivedi et al. 2005; Simeone et al. 2010; Rainwater and Kolattukudy 1985; Azad et al. 1996; Mathur and Kolattukudy 1992; Rainwater and Kolattukudy 1983; Fitzmaurice and Kolattukudy 1997, 1998). In contrast to modular polyketide synthases PpsA-E, Mas is an iterative polyketide synthase, which can extend the methyl branched carbon chain by multiple rounds of the elongation reaction. Finally, mycocerosic acids are transferred onto the diol of phthiocerol by the acyl transferase PapA5 (Trivedi et al. 2005; Onwueme et al. 2004). PapA5 is proposed to directly transfer the mycoserosic acid attached to Mas to the hydroxyl groups of phthiocerol.

Once PDIMs are synthesized, they are transported to the outer membrane. MmpL7 is a transporter of the RND permease superfamily and DrrABC are homologous to ABC transporters. Both of these putative transporters are proposed to function as PDIM transporters (Cox et al. 1999; Camacho et al. 2001; Waddell et al. 2005; Choudhuri et al. 2002). MmpL7 has been suggested to interact with PpsE, the last enzyme of the modular phthiocerol biosynthesis (Jain and Cox 2005), which may play a role in efficient transport of newly synthesized PDIMs across the plasma membrane. LppX is a β-barrel protein proposed to translocate PDIM across the outer membrane (Sulzenbacher et al. 2006).

PGLs are structurally related to PDIMs. PGLs harbor a phenolphthiocerol instead of a phthiocerol, and the phenolic residue is further modified by carbohydrates (Fig. 13.6a). The synthesis of the phenolphthiocerol moiety is similar to that of phthiocerol. However, before being loaded onto PpsA, a fatty acid must first be modified to *p*-hydroxyphenylalkanoate. The production of *p*-hydroxyphenylalkanoate is achieved by two enzymes. First, the fatty acyl-AMP ligase FadD22, which is specifically involved in PGL biosynthesis, activates *p*-hydroxybenzoic acid to form *p*-hydroxybenzoyl-AMP and transfers the *p*-hydroxybenzoyl moiety onto Pks15/1 (Simeone et al. 2010; Ferreras et al. 2008). Second, Pks15/1 extends the acyl chain by using 8–9 molecules of malonyl-CoA as the carbon donor. The product, *p*-hydroxyphenylalkanoate, is released from Pks15/1 and loaded onto PpsA by another fatty acyl-AMP ligase, FadD29 (Simeone et al. 2010). Once loaded onto PpsA, the biosynthesis of PGLs is the same as that of PDIMs except that the final product of the PapA5 reaction is further glycosylated to become PGLs (Pérez et al. 2004b). PGLs are involved in many aspects of host-pathogen interaction, including macrophage

recruitment in zebrafish infection model of *M. marinum*, the escape of *M. marinum* from macrophages, inhibition of pro-inflammatory cytokines and Th1 response by *M. tuberculosis*, and nerve damage in leprosy patients (Cambier et al. 2014, 2017; Madigan et al. 2017; Reed et al. 2004; Ordway et al. 2007).

Capsules and Exopolysaccharides

Bacterial capsules surround the cell envelope and play a part in a variety of processes including biofilm development, protection from the environment, and pathogenesis. Capsules are typically constructed of secreted polysaccharides that adhere to the cell surface, though other macromolecules may also be present within this matrix. Released polysaccharides that do not form an adherent glycocalyx are called exopolysaccharides. Though they are not capsular under observed conditions, exopolysaccharides may share common functions and biosynthetic pathways to capsules. Actinobacterial capsules and exopolysaccharides are varied in structure and function, though some commonality between taxa is evident.

Mycobacterial capsules have attracted far more research interest than those of other Actinobacteria. The mycobacterial capsule is a loose, non-covalently attached layer with a thickness ranging from negligible to 40 nm in vitro (Sani et al. 2010). In pathogenic mycobacteria, capsules can be seen in vivo by electron microscopy as a 50–100 nm thick electron transparent zone (ETZ) surrounding the phagocytized bacilli between the envelope of the bacteria and the host material (Chapman et al. 1959). This ETZ remains stable in the phagosome during infection, but degrades quickly from phagocytized dead cells, implying mycobacteria actively maintain this structure within macrophages (Frehel et al. 1986). Electron-dense material, assumed to be host-derived, can be seen excluded to the outer boundary of the ETZ within these phagosomes, indicating that the capsule layer prevents the diffusion of host macromolecules (Daffe and Etienne 1999). Furthermore, the capsule layer of *M. bovis* BCG plays roles in binding human monocyte-derived macrophages and dampening cytokine response (Sani et al. 2010). While the function of capsules is evident in pathogenesis, a less extensive but similar structure is also observed by electron microscopy in non-pathogenic species (Daffe and Draper 1998). For example, non-pathogenic *M. smegmatis* has a thinner capsule than the high capsule producing pathogens *M. marinum, M. bovis*, and *M. tuberculosis* (Sani et al. 2010). In addition to thickness, mycobacterial capsules can differ in composition. Though all are composed of glycans and protein, the ratio of glycan/protein comprising the capsule can vary between species. Capsular material derived from the slow-growing *M. gastri* and *M. kansasii* contains up to 95% carbohydrate, while capsules of fast-growing *M. phlei* and *M. smegmatis* are highly proteinaceous. *M. avium* has a more balanced mix of protein and carbohydrate within their capsules (Lemassu et al. 1996).

Mycobacterial capsular glycans consist primarily of three types of neutral polysaccharides: α-glucan, arabinomannan, and mannan. The α-glucan is the most abundant species and consists of α1,4-D-Glc polymer with extensive α1,6-D-Glc branching

(Lemassu and Daffe 1994; Ortalo-Magne et al. 1995). These extracellular α-glucans are >100 kDa in size, which is 1,000 times smaller than structurally related cytoplasmic α-glucans (Lemassu and Daffe 1994). The α-glucans are recognized by host receptors, complement receptor 3 and DC-SIGN, and play a role in survival within the host (Geurtsen et al. 2009; Stokes et al. 2004; Sambou et al. 2008; Cywes et al. 1997). The structure of arabinomannan and mannan are identical or near-identical to that of the carbohydrate moieties of LAM and LM, which are described above (Lemassu and Daffe 1994; Ortalo-Magne et al. 1995; Maes et al. 2007). The arabinomannan has an approximate mass of 13 kDa, while the mannan has an approximate mass of 4 kDa. Due to the structural similarities, arabinomannan and mannan are presumed to be derived from LAM and LM, respectively (Maes et al. 2007). Because of its structural similarity to LAM, it is reasonable to speculate that capsular arabinomannan shares functionality with LAM, such as the ability to bind to macrophages. Although over 90% of capsule glycans are neutral, there are small amounts of phosphorylated species of mannan and arabinomannan bearing a negative charge in the mycobacterial capsule (Maes et al. 2007).

In addition to glycans and glycolipids, many proteins are embedded within the mycobacterial capsular matrix (Sani et al. 2010). Many of these capsular proteins are transported to the capsule via secretion systems such as the type VII secretion system ESX-1. Among the various substrates of ESX-1 are T cell antigens that promote the escape of engulfed *M. tuberculosis* from phagosome into the cytosol (Sani et al. 2010; van der Wel et al. 2007). Capsular proteins in some *Mycobacterium* species appear to be cytoplasmic proteins, as they lack secretion signals (Daffe and Etienne 1999). The mechanism for their transport to the capsule thus seems to be independent of the general secretory pathway, but this mechanism remains unknown. The ESX-5 secretion system also appears to support capsular maintenance through transport of key capsular proteins such as PPE10, without which the capsule has altered composition and physical/morphological properties (Ates et al. 2016). Capsule defects caused by ESX-5 deficiency result in reduced pathogenicity in a zebrafish model of tuberculosis, once again implicating the capsule in pathogenesis.

Other mycolata organisms also produce capsules and extracellular polysaccharides. *Corynebacterium*, one of the closest genera to *Mycobacterium*, possesses an outer layer similar to the mycobacterial capsule. This 35–40 nm thick carbohydrate-rich outer layer is composed mostly of neutral polymers of Ara (10–20%), Man (20–35%), and Glc (50–70%) (Puech et al. 2001). Lectins with specificity to GlcNAc, *N*-acetyl-D-galactosamine, D-Gal, and sialic acid bind to the corynebacterial surface possibly implicating these carbohydrates as additional capsule constituents (Mattos-Guaraldi et al. 1999). Glucan is the major polysaccharide, and comes in two apparent masses, 110 and 1.7 kDa, in *Corynebacterium xerosis*. Arabinomannan size distribution is also bimodal with a 13 kDa and a 1 kDa species (Puech et al. 2001). The smaller glucans and arabinomannans are notable, being absent in mycobacterial capsules. Proteins are generally minor components of corynebacterial capsules, accounting for less than 10% of the dry weight of the capsular material. However, some strains of *C. glutamicum* have an outermost paracrystalline S-layer composed almost exclusively of one protein, PS2, which appears to associate with the outer

membrane through hydrophobic interactions (Chami et al. 1997; Peyret et al. 1993). The gene encoding PS2 is located on a chromosomal island, suggesting that a horizontal gene transfer event led to the development of the proteinaceous surface layer in these *C. glutamicum* strains (Hansmeier et al. 2006).

Rhodococcus equi expresses an immunogenic and antigenically varied 50–100 nm thick polysaccharide-rich capsule layer, which confers a mucoid appearance when grown on nutrient agar (Sydor et al. 2008). The antigenic capsular polysaccharide of one of the 27 identified *R. equi* serovars is made up of equal amounts of D-Glc, D-Gal, D-glucuronic acid, 4-*O*-(1-carboxyethyl)-D-Man, and pyruvic acid (Severn and Richards 1992). FbpA, a homolog of the mycobacterial mycoloyltransferase Ag85, is important for maintaining the integrity of this capsule in *Rhodococcus*, implying an importance for the mycolic acid layer in capsule stability (Sydor et al. 2008). However, capsule deficiency by the disruption of this gene does not impair pathogenicity, implying that the capsule is not a major virulence factor in *Rhodococcus* (Sydor et al. 2008).

Another mycolata genus, *Gordonia*, like mycobacteria, forms biofilms that are held together by a matrix of bacterially derived macromolecules, and therefore highly suggestive of capsule and exopolysaccharide production (Linos et al. 2000). *Gordonia polyisoprenivorans* and another *Gordonia* strain Y-102 produce exopolysaccharides (Kondo et al. 2000). The acidic polysaccharide from Y-102, termed gordonan, has a molecular weight of about 5,000 kDa and a repeating [-3-4-*O*-(1-carboxyethyl)-Man*p*-β1,4-D-GlcA*p*-β1,4-D-Glc*p*-β1-] trisaccharide structure. A nearly identical exopolysaccharide with GlcA*p* in α configuration is reported in *Gordonia rubripertincta* (formerly *Mycobacterium lacticolum*) (Kochetkov et al. 1979). Notably, the antigenic capsular polysaccharides of *R. equi* referred to above also share similar sugar composition, namely 4-*O*-(1-carboxyethyl)-D-Man, D-Glc, and D-glucuronic acid residues (Severn and Richards 1992).

Exopolysaccharides have also been described in Actinobacteria outside of the mycolata, and some of these may form capsular polysaccharide surface layers. While there is no direct evidence for capsular structures, *Streptomyces* species do secrete exopolysaccharides into their environment (Selim et al. 2018; Wang et al. 2003). One example is ebosin, composed of Gal, Ara, Man, fucose, xylose, Rha, galacturonic acid, and Glc (Wang et al. 2003). The biosynthesis of ebosin remains sketchy, but carbohydrates are proposed to be built on a lipid-linked precursor (Wang et al. 2003). *Bifidobacterium* is also a notable producer of exopolysaccharides. Exopolysaccharides produced by this genera are thought to facilitate numerous beneficial interactions for the host including immune modulation, host protection, and antagonizing pathogens (Castro-Bravo et al. 2018; Hidalgo-Cantabrana et al. 2014). The soil bacterium *Brevibacterium otitidis* also produces exopolysaccharides, which are ~127 kDa in size, containing Ara, Man, Glc, and mannouronic acid (Asker and Shawky 2010). While this is not an exhaustive review, their ubiquity and structural/compositional variation suggest that capsules have many critical functions for Actinobacteria beyond their traditionally defined role in pathogenesis. These extracellular materials likely provide a convenient yet non-essential and modifiable matrix to modulate interactions with diverse environments.

Conclusions and Outlook

While there have been leaps and bounds in understanding the structure and biosynthesis of the actinobacterial cell envelope, there are still numerous questions remaining. With the medical importance of *Mycobacterium* species, the cell envelope biosynthesis in this genus will continue to be an important focus of future research. Given the successful uses of ethambutol and isoniazid as frontline anti-TB drugs, it would not be surprising to find many more drug targets from cell envelope biosynthetic pathways. Indeed, the mycolic acid transporter MmpL3 is emerging as a promising target for chemotherapy against nontuberculous mycobacteria diseases (Viljoen et al. 2017; Li et al. 2018; Kozikowski et al. 2017). It will also continue to be exciting to discover many more lipids and glycans from Actinobacteria. One recent prominent example is a discovery in *M. abscessus* of a glycosyl diacylated nonadecyl diol alcohol, which is transported by MmpL8 ortholog in this bacterium (Dubois et al. 2018). We also have a limited understanding of spatiotemporal regulation of cell envelope biosynthesis. At the transcriptional and post-transcriptional levels, cells must have mechanisms to sense and respond to environmental changes. Polar restricted growth of many rod-shaped bacteria within the Actinobacteria class implies tight spatial subcellular regulation as well. Finally, how mycolata build this unique diderm cell envelope continues to be enigmatic. Such a complex macromolecular assembly seems like a highly demanding task, and yet mycolata are highly successful bacteria found in diverse environmental niches. An insight that both mycobacterial arabinogalactan and Gram-positive wall teichoic acids are similarly linked to the peptidoglycan makes us wonder how mycolata evolved this unique outer membrane. Continued research on the diverse repertoire of Actinobacteria will bring an in-depth understanding of how these amazing bacteria evolved their cell envelope and succeeded in their own way.

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