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Unraveling the structure of the Mycobacterial Envelope

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Running title: The Mycobacterial cell envelope

The mycobacterial cell envelope consists of a typical plasma membrane of lipid and protein, surrounded by a complex cell wall composed of carbohydrate and lipid. In pathogenic species, such as *Mycobacterium tuberculosis*, an outermost ‘capsule’ layer surrounds the cell wall. This wall embraces a fundamental, covalently linked ‘cell-wall skeleton’ composed of peptidoglycan, solidly attached to arabinogalactan whose penta-saccharide termini are esterified by very long-chain fatty acids (mycolic acids). These fatty acids form the inner leaflet of an outer membrane, called mycomembrane, whose outer leaflet consists of a great variety of non-covalently linked lipids and glycolipids. The thickness of the mycomembrane, which is similar to that of the plasma membrane, is surprising in view of the length of mycoloyl residues implying dedicated conformations of these fatty acids. Finally, a periplasmic space also exists in mycobacteria, between the membrane and the peptidoglycan. The present chapter provides a comprehensive overview of this biologically important and structurally unique mycobacterial cell compartment.

Keywords: tuberculosis; mycobacteria; cell envelope; outer membrane; mycolic acid.

INTRODUCTION

The bacterial cell envelope, defined as the structure that surrounds the cytosol, is critical for the bacterial physiology because many crucial processes take place in this compartment. In fact, the cell wall contributes to the bacterial shape, mechanical resistance of the cells and their protection against hostile environments. In addition, the envelope constituents are involved in cell division, transport of molecules (solutes and ions) and macromolecules (proteins), cell motility, adhesion and protection, being in direct contact with the environment, etc. The genus *Mycobacterium* belongs to the *Corynebacteriales* order, which also includes *Corynebacteria*, *Nocardia*, *Rhodococci* and other related microorganisms. Mycobacteria are probably the most successful microorganisms to parasitize animals and humans. Among the more than 200 valid species described to date in the genus *Mycobacterium*, only three are strict pathogens for humans: *Mycobacterium tuberculosis* (the Koch's bacillus), *M. leprae* and *M. lepromatosis*. Tuberculosis still represents a major public health problem worldwide, remaining one of the world leading causes of death from an infectious agent; about one quarter of the world's population is infected by the Koch's bacillus and thus susceptible to develop the disease. In addition, two-thirds of mycobacteria species are opportunistic pathogens for human and all mycobacteria produce granulomatous lesions in experimental animals with large enough inoculum (1). Most mycobacterial pathogenic species grow very slowly (*e.g.* generation times of 24 hours and 13 days for the tubercle and leprosy bacilli, respectively), whereas saprophytes are relatively rapid growers (*e.g.* doubling time of 6 hours for the widely used genetically tractable *M. smegmatis*).

Mycobacteria and related genera are classified among the *Actinobacteria*, a division of Gram-positive bacteria, although they do not reliably retain the stain, a clear indication of the uniqueness of their cell envelope composition (2). The dissection of their fascinating coat reveals an atypical architecture of their envelope, different from those of both Gram-positive and Gram-

negative bacteria (Fig. 1B). Unlike those of the former group of bacteria, with a reputed lipid-poor cell envelope (less than 10%), mycobacterial cell envelopes are extremely rich in lipids, representing up to 40% of their cell dry mass, more than the recorded 20% in Gram-negative bacteria (3). This high lipid content is in agreement with the huge number of genes devoted to the synthesis and regulation of this class of compounds (4). This hallmark would explain both the tendency of mycobacteria to grow in clumps, their distinctive property of acid-fastness, but also their impermeability to nutrients and small molecules and their resistance to alkali, dilute acid and infected mammalian cells (3).

Reviews have been recently published on the biogenesis (5), structures (6) and transport (2, 7-8) of mycobacterial cell envelope constituents. The present chapter highlights major advances in deciphering key biological and structural features of this unique and fascinating mycobacterial coat.

THE MICROSCOPIC APPEARANCE OF THE MYCOBACTERIAL CELL ENVELOPE

When studied by *transmission electron microscopy* (TEM), the mycobacterial cell envelope recurrently displays a multi-layered structure (Fig. 1A). In TEM, the specimen is fixed prior to being embedded in a hydrophobic resin, followed by ultrathin sectioning and staining with heavy-metal salts. Consequently, fixation protocols and the use of different solvents can lead to extraction and shrinkage of its different constituents. Lipids non-covalently attached to *cell wall* (CW) polymers may be extracted by solvents during the dehydration step and water-rich structures may collapse (9, 10). In ultrathin conventional sections (11), the cell envelope of mycobacteria looks more like that of Gram-positive (Fig. 1). It consists of a *plasma membrane* (PM), a thick *electron-dense layer* (EDL), an *electron-transparent layer* (ETL), and an *outer layer* (OL) of variable density and thickness (Fig. 1). The PM appears asymmetrical in images of some conventionally

prepared cells, with the accumulation of material in its outer leaflet (12-14). This asymmetry, however, is dependent upon conditions of fixation (15). To circumvent these disadvantages, freeze-substitution techniques have been used (16, 17), but with little success, as the resulting images were diffuse and not resolved as expected (Fig. 1A).

More recently, cryo-electron microscopy of vitreous sections (CEMOVIS) and cryo-electron microscopy (cryoEM) of whole-mount cells have been applied to mycobacteria (Fig. 1B). In these techniques, the specimens are observed at liquid nitrogen temperature, which allows them to be imaged in their fully hydrated and unstained state. Expectedly, images observed for the mycobacterial cell envelope look significantly different from those seen by TEM (11, 18, 19). They revealed a symmetrical PM, a periplasm containing a granular layer (GL) presumably consisting of proteins and, for the first time, the presence of a long-predicted outer membrane (OM) (Fig. 1B). The existence of an OM was supported by data from freeze-fracture electron microscopy, which clearly showed the occurrence of a major fracture plane (FP) in the outer part of the envelope, in addition to the expected plane that typifies the PM (Fig. 1B) (20-24). However, no direct observations of the hypothetical OM was available using TEM. The demonstration of the existence of an OM nicely fits with the very low permeability of mycobacteria to nutrients and antibacterial drugs, 10 to 100-fold lower than that of the notably impermeable bacillus *Pseudomonas aeruginosa* (25). Consistently, mycobacteria and other members of the *Corynebacteriales* order contain pore-forming proteins (porins) in their cell walls (26).

During eukaryotic cell infection, a space between the wall of *M. lepraemurium* and the phagosomal membrane was observed in electron microscopic pictures, called the “capsular space” (27). Almost concomitantly, when studying the permeability of mycobacteria to dyes using light microscopic techniques, a tentative correlation was made between the “capsule space” and an

unstainable ‘halo’ observed only around some pathogenic mycobacteria (28-30). According to Hanks (31), this halo would also correspond to the ‘electron-transparent zone’ (ETZ) that is seen in several published electron micrographs surrounding intracellular mycobacterial pathogens, but not around non-pathogenic mycobacteria or pathogenic mycobacteria grown *in vitro* under standard conditions (32, 33). Consistently, a capsule-like structure was observed around *in-vitro*-grown pathogenic mycobacteria that were first either coated with specific antibodies or pre-embedded in gelatin prior to the different drastic treatments involved in conventional electron microscopy studies (34, 35). Under these conditions, the capsule-like structure was not seen around the non-pathogenic *M. smegmatis* and *M. aurum* (35). Definitive proof of the existence of a capsule in pathogenic mycobacteria came from whole-mount plunge-frozen mycobacteria imaged by cryoEM (Fig. 1C), which does not require the use of a cryo-protectant and under conditions where they were grown without detergent or shaking (19). The layer was not observed in CEMOVIS pictures (Fig. 1B; 11, 18), probably due to the fact that the density of the capsule matches that of the cryo-protectant-containing freezing solution. Likewise, the capsule was not observed in pictures of mycobacteria grown in detergent-containing culture media, routinely used to prevent mycobacterial cell clumping (19). When the same bacteria are instead processed by conventional methods commonly used to observe mycobacteria by TEM, the outer layer probably collapses by dehydration to give a dark layer (OL, Fig. 1A).

From the combination of chemical and microscopic data, it is now well established that the mycobacterial envelope consists of a PM surrounded by a complex CW composed of peptidoglycan (PG) covalently linked to arabinogalactan (AG) esterified by mycolic acids (MA), which form the inner leaflet of an OM bilayer. MA are very long-chain, up to 100 carbons, 2-alkyl 3-hydroxylated fatty acids. An outermost layer, called ‘capsule’ in the case of pathogenic species (9), in turn surrounds the CW (Fig. 1C). A periplasmic space, defined as

the region located between the PM and the CW peptidoglycan exists in mycobacteria, as revealed by CEMOVIS (Fig. 1B).

THE PLASMA MEMBRANE

The bilayer structure of the mycobacterial PM is similar to that of other Gram-positive and -negative microorganisms (Fig. 1), consistent with the published chemical composition, comprising phospholipids and proteins, with specific metabolic functions (9). Its asymmetrical appearance in images of some conventionally prepared cells (12, 15) has been attributed to the presence of excess glycoconjugates, *e.g.* phosphatidylinositol mannosides (PIM), lipoarabinomannan (LAM) and lipomannan (LM), in the thicker outer leaflet. Although glycoconjugates were identified in the PM (see below), their distribution between the two leaflets is not known. Alternatively, the observed apparent thicker outer leaflet in some conventional preparations may be due to the collapse of the GL, presumably composed of proteins, of a few nm beyond the outer leaflet of the PM (11), as shown by CEMOVIS (see Fig. 1).

The native PM is typically and easily obtained by breaking the cells by mechanical stress, *e.g.* sonication or shearing in the French pressure cell, followed by fractionation using differential centrifugation or density gradients (36, 37). The determination of specific enzyme activities, *e.g.* NADH oxidase (36) is generally used to follow the purification process. Further chemical and biochemical analyses of the fractions are necessary to check their purity (36, 37). As far as lipid composition of the purified PM is concerned, no significant difference between those of fast- and slow-growing *Mycobacterium* species examined have been observed. Polar lipids, mainly phospholipids, assemble, in association with proteins, into a lipid bilayer (see 38). The polar lipids of the mycobacterial PM are composed of hydrophilic head groups and fatty acid chains that usually consist of mixtures of straight chain, unsaturated and mono-methyl branched fatty acid residues having less than 20 carbons. Palmitic (C_{16:0}), oleic (cis Δ^9 C_{18:1}) and 10-

methyloctadecanoic (called tuberculostearic) acids are the major fatty acid constituents of the isolated PMs. The main phospholipids of the PM are PIM, *phosphatidyl glycerol* (PG), *cardiolipid* (CL) and *phosphatidyl ethanolamine* (PE), while *phosphatidyl inositol* (PI) occurs in small amounts. Incidentally, PIM and PE have also been identified among the lipids extracted from the mycobacterial cell surface by means of gentle mechanical treatment of cells with glass beads (39), yet the function of phospholipids in those layers is still unknown. Besides phospholipids, other lipids, such as menaquinones, may be present in the PM (see 38). The mycobacterial lipopolysaccharides LAM and LM are partly located in the PM, and the relative contents of both lipopolysaccharides are growth-phase dependent (36, 40). The lipid composition of the PM of *M. smegmatis* has been also determined using detergent-extraction, namely with dioctylsulfosuccinate sodium (41). The composition of the PM of *M. smegmatis* isolated by the latter method differs significantly from that obtained by breaking the cells by mechanical stress, followed by fractionation using differential centrifugation or density gradients (36, 37). Notably, *trehalose monomycolate* (TMM), a lipid never reported in isolated mycobacterial PMs (9), was found in the PM using the detergent-extraction method (41). Rather, TMM has been isolated from the cell surface of several mycobacterial species by mechanical treatment (39).

Bottom-up quantitative proteomics analysis allowed the identification of more than 2,000 membrane proteins in the purified PM of *M. smegmatis* (36). These include the canonical membrane ATP synthase, Cytochrome P450 and ABC transporter Yjff, as well as enzymes involved in the biosynthesis of PIM, *e.g.* the mannosyltransferase PimB (42), galactofuranosyltransferase GlfT2 (43). To assemble their cell wall components, which include peptidoglycan, arabinogalactan, lipids, lipoproteins as well as surface and outer membrane proteins (see below), mycobacteria must transport them or their precursors. Although little is known about the transport of these constituents from the cytosolic compartment to the exterior of the cell, mechanisms similar to those functioning in Gram-negative bacteria have been postulated (2, 7).

Consistently, several transmembrane proteins such as MmpL (*mycobacterial membrane protein large*), involved in the transport of various lipids (2, 7) have been located, at least partly, in the PM (Fig. 2). Among the MmpL of *M. tuberculosis*, MmpL3 is involved in the transport of TMM and MmpL7 mediates the transport of *phthiocerol dimycocerosate* (PDIM) whereas MmpL10 was reported to participate in the translocation of acylated trehaloses. All these lipids contribute to the integrity of the cell envelope, regulate membrane permeability and play important physiological functions. The PM contains also the sophisticated secretion systems, named 6 kDa *early secretory antigenic target* (ESAT6) protein family secretion (ESX) systems (also known as type VII secretion systems, 44), used to export a set of effector proteins that help the pathogen resist or evade the host immune response (8).

THE CELL WALL

The CW of mycobacteria and related genera differs in composition from those of both Gram-positive and Gram-negative bacteria. It is a giant complex constituted of the OM bilayer, AG and PG. The mycobacterial OM, also known as “*mycomembrane*” (MM), exhibits a 7-8 nm thickness (Fig. 1B), similar to that of the PM, and contains MAs. MAs form the inner leaflet of the MM, covalently linked to AG, which is in turn is covalently attached to PG, a complex polymer classified as A1 γ , as in *Escherichia coli* and *Bacillus spp.* This represents the so-called mAGP (*mycoloyl-ArabinoGalactan-Peptidoglycan*), formerly known as the *cell wall skeleton* (CWS), defined as the material remaining after removal of all non-covalently bound wall-associated substances (45). The outer leaflet of the MM is presumably composed of various lipids (36).

Mycobacterial walls are readily isolated and purified from fragments of PM and cytosolic material by breaking the cells with mechanical stress, followed by purification using differential centrifugation or density gradients to remove unbroken cells (see 36, 37). The mycobacterial CW appears in conventional TEM (46) as an EDL surrounded more externally by an ETL (Fig. 1A).

The occurrence of the latter in sections of *Corynebacteriales* devoid of mycolic acids, e.g. *Corynebacterium amycolatum* (47), questions the long-assumed interpretation that the low density of the ETL is attributed to lipids, notably MA.

The CW can be dissected into its constituent parts by relatively gentle methods, so that each part may be studied separately (see 48-50). The PG is composed of repeating units of *N*-acetylglucosamine and *N*-acetyl/glycolylmuramic acid cross-linked with a peptide side-chain that may be cross-linked to that of other glycan strands. The tetrapeptide side chains of PG consist of L-alanyl-D-isoglutaminyl-*meso*-diaminopimelyl-D-alanine. These peptide chains are heavily cross-linked, up to 80% cross-linking compared with 50% in *E. coli* (3). Interestingly, the PG of *M. leprae* has a glycine residue substituted for the L-alanine residue found in the PG of *Mycobacterium* spp. examined to date.

The AG is a branched heteropolysaccharide that contains a galactan chain composed of alternating 5- and 6-linked D-galactofuranosyl residues; three D-arabinan chains substituted the D-galactan chain in *M. tuberculosis*, of which MAs (51) esterify two thirds of the non-reducing termini of the penta-arabinosyl motifs. In fact, MAs (Fig. 2) esterify the four-hydroxyl groups at position 5 of both terminal and 2-linked D-arabinofuranosyl of the penta-arabinosyl motifs of AG. The CW of the *in-vivo*-grown leprosy bacillus (a yet *in-vitro* non-cultivable bacterium) was found to contain significantly more AG-MA attached to PG than that of *in-vitro*-grown *M. tuberculosis*, only half of these are occupied by mycoloyl residues (52).

In 1982, Minnikin has proposed an original model of an outer membrane analogous to that found in Gram-negative bacteria. In this model, the very long chains MA that esterified the AG would arrange themselves to form the inner leaflet of an asymmetric outer membrane, the outer leaflet being constituted of free polar mycolate trehaloses (38). This implied the existence of a second hydrophobic layer in addition to the PM and could explain the very low permeability to polar molecules. It could also provide a good explanation for the additional and main fracture

plane observed in freeze fractures of mycobacteria (20-24): the fracture would occur between the two leaflets of the outer membrane (Fig. 1B). This model was later supported by experimental data (see 53), notably the work of Nikaido and his colleagues who showed that an aqueous suspension of purified walls of *M. chelonae* produced an X-ray diffraction pattern with reflections characteristic of close-packed hydrocarbon chains in a semi-crystalline monolayer (54). However, the complete lack of observation of this outer membrane bilayer in transmission electron micrographs of sectioned mycobacterial cells seriously questioned the validity of this model.

Using CEMOVIS, three research groups have independently clearly visualized a symmetrical 7-8 nm-thick bilayer whose thickness was comparable to that of PM in the mycobacterial species analysed (11, 18, 19). The thickness of the outer membrane MM was even less in corynebacteria, around 4-5 nm (11, 18). The fact that the outer membrane contains MA was proved by the absence of this structure in a mycolate-less *Corynebacterium* mutant. LAM and LM located in the MM would presumably be associated with its outer leaflet (Fig. 3), comparable to the location of LPS in Gram-negative organisms (36, 40). Among the lipids proposed to compose the outer leaflet of the MM in various models, TMM, trehalose *dimycolate* (TDM, Fig. 2) and phospholipids (CL/PG, PE and PI, plus a few PIM) have been clearly identified in the MM-containing fractions of *M. smegmatis* (36). The 7-8 nm thickness of the outer membrane layer contradicts the arrangement of MA whose long alkyl chains would be organized parallel to one another to yield a > 40 nm-thick asymmetrical bilayer (38). In view of the 7-8 nm-thickness of the MM in the observed micrographs (Fig. 1B), the alkyl chains of MA have to be arranged in a folded manner (as shown for TMM and TDM in Fig. 2). Consistent with the published chemical structures and fold of mycobacterial MAs (55, 56), a model was proposed in which mycoloyl chains are intercalated in a zipper-like manner and where the long mycoloyl chains are folded in an ω -shape (11).

Like Gram-negative bacteria, mycobacteria need to transport and assemble their outer membrane and to import small polar molecules across their outer membrane, notably those needed for nutrition. In Gram-negative bacteria, the transport of lipids, lipoproteins and outer membrane proteins (OMP) involves a combination of ATP-dependent transporters in the PM, chaperones in the periplasm, and β -barrel proteins that facilitate the insertion and translocation/folding into the outer membrane (2). The nutrition issue is solved by producing specialized proteins called porins, which form hydrophilic pores through the structure (Fig. 3). These pore-forming proteins have been identified in the walls of both fast-growing mycobacterial species such as *M. chelonae* and *M. smegmatis* (26, 57, 58) and *M. tuberculosis* (59, 60). However, as Rv0899 (OmpA-Tb) adopts a mixed alpha/beta-structure and does not form a transmembrane beta-barrel (61), its status as a porin is a matter of debate (26). Expectedly, the crystal structure of the major OMP of *M. smegmatis*, MspA, reveals a β -barrel transmembrane channel (62). MspA forms an octameric composite β -barrel where each monomer comprises two β -strands. In Gram-negative bacteria, such as *E. coli*, there are about 60 OMP. Using *in silico* analysis, two independent studies have proposed a list of putative OMP of *M. tuberculosis* based essentially on β -barrel computational predictions (26). Out of 100 putative mycobacterial OMP, only a few have been identified and characterized. These include mycoloyl transferases (originally called Fbp for fibronectin-binding proteins or antigen85, 63), some glycosylated proteins (Rpf proteins) (64), the outer membrane channel protein CpnT (65) and sphingomyelinase Rv0888 SpmT protein (66). Proteomic analysis confirmed the presence of Fbps and porins (MspA in the case of *M. smegmatis*) in the cell wall fraction, but also putative transporters, *e.g.* mycobacterial cell entry (MCE) protein family (36).

THE CAPSULE

The first mycobacterial capsules defined, namely those of *M. leprae* and *M. lepraemurium*, were composed of lipids, but this turns out to be the exception. The ‘fibrillar’ substance that surrounds *M. lepraemurium* inside the phagocytic vacuoles of the host cell was composed of glycopeptidolipids (GPL, 46) and massive amounts of phenolic glycolipids (PGL, Fig. 2) were isolated from cells infected with *M. leprae* (67, 68). However, both GPL-positive and GPL-negative strains of the *M. avium-M. intracellulare* complex elaborate capsules (69), indicating that the capsule is not always composed of lipids. Moreover, most strains of *M. tuberculosis* are devoid of PGL (70).

Knowing that *in vitro*-grown pathogenic mycobacterial species secrete abundant amounts of polysaccharides and proteins (71, 72), we postulate that the material shed into the culture medium of *in vitro*-grown mycobacteria is retained by the phagosomal membrane to form the electron-transparent zone seen around pathogenic species in phagocytic cells (8). Consistently, more extracellular material was recovered from the culture filtrates of the pathogenic species, *e.g.* *M. tuberculosis* and *M. kansasii*, than from those of saprophytic and non-pathogenic strains such as *M. smegmatis* and *M. aurum* (71, 73). Accordingly, the nature of the capsular constituents of various mycobacterial species were determined by analyzing the composition of their extracellular materials and those extracted by gentle shaking of bacterial pellicles with glass beads and/or by detergent extraction for short periods of time (39, 73). Importantly, these treatments, aiming at de-clumping the cells, do not affect their viability, as judged by scanning electron microscopy (39, 74).

The main components of the outermost capsular layer of slow-growing mycobacterial species, most of which are pathogens (*e.g.* *M. tuberculosis* and *M. kansasii*), are polysaccharides (72) whereas the major components of the outer layer of rapid growers (*e.g.* *M. phlei* and *M. smegmatis*) are proteins (71). The major extracellular and capsular component of slow-growing mycobacterial species is a glucan (Fig. 3) that is composed of repeating units of five or six

α -(1→4) linked D-glucosyl residues substituted at position 6 with mono- or oligoglucosyl residues (71, 72, 75). The surface-exposed and extracellular materials also contain a D-arabino-D-mannan, a heteropolysaccharide that exhibits an apparent molecular mass of 13 kDa, and a mannan chain composed of a α -(1→6)-D-mannosyl core, with some units substituted with α -D-mannose at position 2. Importantly, glucan was identified in the capsule of *M. tuberculosis* inside the phagocytic vacuoles of the host cell (76).

The outermost part of the mycobacterial cell envelope contains only a tiny amount of lipid (2-3% of the surface-exposed material). Some of the species- and type-specific lipids and glycolipids, e.g. PDIM, PGL and GPL, can be found on the surface of the capsule, in agreement with serological and ultrastructural findings (see 9). Progressive extraction of the capsular material with glass beads and short-time treatment with Tween 80 detergent shows that most of the lipids are in the inner rather than in the outer part of the capsule (39).

The outermost capsular proteins of *in vitro*-grown *M. tuberculosis* are a complex mixture of polypeptides (72). Some proteins seem to correspond to secreted polypeptides found in short-term culture filtrates whereas other appear as cell wall-associated (34). In fact, most of the hundreds of proteins identified in the short-term culture filtrate of *M. tuberculosis* by 2-D SDS-PAGE (77) are also found in significant amounts in the surface-exposed material extracted by mild mechanical treatment of cells. This observation supports the concept that the compounds found in the culture filtrate of *in vitro*-grown cells is probably shed from the surface of the bacilli and, in an *in vivo* context, would be confined around the cells in the capsular layer (9). Components of the early secretory antigen transport system (ESX) were recently localized to the capsule by immuno-electron microscopy of cryosectioned mycobacteria (19).

CONCLUDING REMARKS

The chemical structure of most of the core components of the mycobacterial cell envelope was determined a long time ago, establishing its extreme richness in lipid. However, it is only recently that combination of CEMOVIS and whole-cell cryoEM has revealed the true structure of the mycobacterial cell envelope, *i.e.* the existence of a Gram-negative-like (diderm) outer membrane in these Gram-positive bacteria, and an outermost capsular layer of protein and carbohydrate (Fig. 3). Determining the composition, localization and supramolecular organization of the capsular and CW constituents and understanding how these factors make the envelope essential to *M. tuberculosis* survival, virulence and pathogenesis remain a major challenge. This will certainly require synergistic expertise in analytical and structural biochemistry and biophysics to fill the gap in understanding the MM compartment by isolating and defining the full lipid and protein compositions of the capsule and CW. Deciphering the supramolecular organization of the capsule and MM-containing CW represent an additional exciting goal. The discovery of how mycobacteria select and organize their envelope constituents to resist killing should help decode the mechanisms involved in the mycobacterial pathogenicity and provide new potential targets for anti-tuberculosis chemotherapy. In this respect, the biogenesis of MM constitutes a highly relevant and validated pharmaceutical target for anti-tuberculous drug discovery.

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FIGURE LEGEND:

Figure 1: Diagrams (adapted from references 11 and 17-19) of the cell envelopes extracted from images obtained by (A) TEM), (B) CEMOVIS of various bacterial cells and (C) cryoEM of whole-mount mycobacteria.

AG, arabinogalactan; CEMOVIS, cryo-electron microscopy of vitreous sections; cryo-EM, cryo-Electron Microscopy; EDL, electron-dense layer; ETL, electron-transparent layer; FP, fracture plane; GL, granular layer; OL, outer layer; OM, outer membrane; PG, peptidoglycan; PM, plasma membrane. Peri, periplasm; TEM, transmission electron microscopy.

Figure 2: Chemical structures of representative mycobacterial lipids and their export systems through the plasma membrane to reach their final locations in the outer membrane (mycomembrane) or/and capsular compartment of the cell envelope. Some of the lipids are ubiquitous (*e.g.* TMM and TDM) whereas other are species- or type species-specific (*e.g.* PAT, PDIM, PGL-tb), found in selective mycobacterial species or strains. TMM is used to transfer its mycoloyl residue onto both the arabinan termini of AG and TMM to yield the cell wall skeleton mAGP and TDM, respectively.

Ac₂SGL, diacylated sulfoglycolipid; AG, arabinogalactan; DAT, diacyl trehalose; mAGP, mycoloyl-arabinogalactan-peptidoglycan; mAGP, mycoloyl-AG-peptidoglycan; MmpL, mycobacterial membrane protein large; PAT, polyacyltrehalose; PDIM, phthiocerol dimycocerosate; PGL-tb, phenol glycolipid of *M. tuberculosis*; SL-I, sulfolipid I, the major sulfolipid of *M. tuberculosis*; TDM, trehalose dimycolate; TMM, trehalose monomycolate.

Figure 3: Model of the mycobacterial cell envelope. The cell envelope consists of (i) a plasma membrane, (ii) a periplasm, (iii) the cell wall skeleton and (iv) the outermost layer, called capsule

in the case of pathogenic species. The plasma membrane is composed of phospholipids (*e.g.* PIM), lipopolysaccharides (*e.g.* LAM) and proteins, which include MmpL involved in the transport of lipids. The periplasmic space contains the GL and presumably proteins. The cell wall skeleton is made of peptidoglycan, arabinogalactan and mycolic acid residues that form the inner leaflet of the outer membrane (mycomembrane). The outer leaflet of the latter membrane is composed of various lipids, notably trehalose mycolates. The capsular layer is a matrix of glucan that contains proteins, (lipo)polysaccharides and small amounts of lipids.

AM, arabinomannan; LAM, lipoAM; GL, granular layer; MmpL, mycobacterial membrane protein large (PIM, phosphatidyl inositol mannosides; PL, phospholipids; TDM, trehalose dimycolate; TMM, trehalose monomycolate.