Chapter 7

Pathophysiological Implications of Cell Envelope Structure in *Mycobacterium tuberculosis* and Related Taxa

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Additional information is available at the end of the chapter

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1. Introduction

Members of the *Mycobacterium tuberculosis* complex are successful pathogens due in a large part to the complex interactions of an array of very special lipid molecular classes and associated macromolecules that have been known for many years [1-6]. The cooperative assembly of these components results in the presence of very robust cell envelopes. In particular there is a specialised outer membrane; this can be termed a "mycomembrane", but in this review the abbreviation, MOM, for "mycobacterial outer membrane" will be used. Subtle structural variations in mycobacterial lipid components point to important roles in the integrity and function of the mycobacterial cell envelope. Over three decades ago, an attempt was made to rationalise the role of the known constituent molecules with a proposal for a "chemical" model of the cell envelope [4]. This general model has been supported in subsequent studies but a wealth of new knowledge suggests that a significant upgrade is needed. Each class of cell envelope structural units will be introduced, reasons for "why" such structures are produced will be explored and an updated cell envelope model presented as a working hypothesis. Close comparisons of the cell envelope compositions of *M. tuberculosis* and related taxa will be interpreted in terms of evolution and pathogenicity. In various diseases, the term "pathophysiology" is usually used to describe physiological changes in affected hosts; here its meaning is turned around to explore the impact of aspects of mycobacterial cell physiology in pathogenic members of the *M. tuberculosis* complex.

Precise structures of the lipid moieties displayed in members of the *M. tuberculosis* complex have become established during the past five decades, so it is now possible to explore the significance of structure in the pathophysiological role of such lipids. Attention will focus on lipids, whose regular occurrence in significant proportions indicates that they are integral structural members of the mycobacterial cell envelope. Details of general biosynthetic pathways will not be considered, nor will particular biological effects of individual lipid classes. Recent cryo-electron microscopy studies have allowed the absolute dimensions of the mycobacterial cell envelope to be estimated [7-9] so all components must adopt conformations that allow their integration into an effective organelle. Tubercle bacilli also have a less clearly defined capsule external to the more coherent cell envelope [10,11]; a detailed discussion of this important region will not be attempted here. Similarly, a detailed exploration of protein content is not given, even for structurally vital proteins such as well-characterised porins [12]. The main chemical structures that must be accommodated in the cell envelope of *M. tuberculosis* are considered in the following sections, commencing with the long-chain mycolic acids that are mycobacterial signature components [4,13-18].

2. Mycolic acids

The 70 to 90 carbon mycolic acids (MAs) are very characteristic chemical components in the genus *Mycobacterium*. Members of the *M. tuberculosis* complex have three classes, the so-called α-mycolates, methoxymycolates and ketomycolates (Figure 1) [4,13-18]. The latter two varieties (Figure 1B) are comprised of subclasses having either *cis*-cyclopropane rings or *trans*-cyclopropane rings with an adjacent methyl branch. In the case of the α-mycolates (Figure 1A), representative C_{80} mycolates from *Mycobacterium kansasii* and the *M. tuberculosis* complex are compared to highlight the importance of key structural differences, whose importance will be discussed later. All these MA classes occur naturally with at least five homologues and variations in the numbers of carbons between the various functional groups. The majority of MAs are covalently bound to arabinose termini of a mycoloylarabinogalactan-peptidoglycan (mAGP) macromolecule to form a lipid monolayer inner leaflet of the MOM [4,19], as will be described in detail later.

Recent physiochemical investigations have clearly demonstrated that mycolic acids characteristically adopt distinctly different folded conformations depending on structural niceties [20-24]. Ketomycolates in *M. tuberculosis* predominately fold to yield a compact "W" conformation, with four chains in parallel [20,21,23,24]. Such tight packing can provide the foundation for an effective hydrophobic permeability barrier in the inner leaflet of the MOM. In contrast, α- and methoxymycolates can form W-conformations but also more readily inhabit a range of more extended conformations [20-23], some of which can be visualised as "U" or "Z" shaped [24]. The α-mycolates tend towards an open fully extended U-conformation with the two distal chains extended and methoxymycolates show intermediate behaviour between the two other classes [22,24]. For the purpose of illustrating the importance of mycolate conformation in MOM inner leaflet function (see later), ketomycolates are restricted to W-

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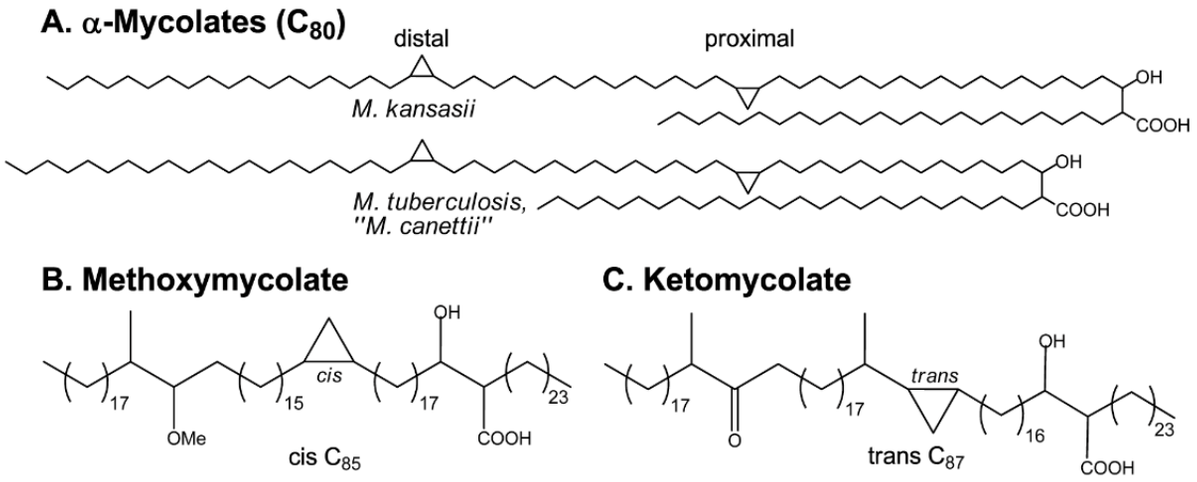

Figure 1. Representative structures of mycolic acids. **A.** α-Mycolates (C_{80}), comparing *M. kansasii* and *M. tuberculosis* complex. **B, C.** The main methoxy- and ketomycolates from *M. tuberculosis*.

conformations, methoxymycolates are shown with both semi-folded sZ and fully folded W-shapes [24] and α-mycolates have extended eU-conformations [24] (Figure 2).

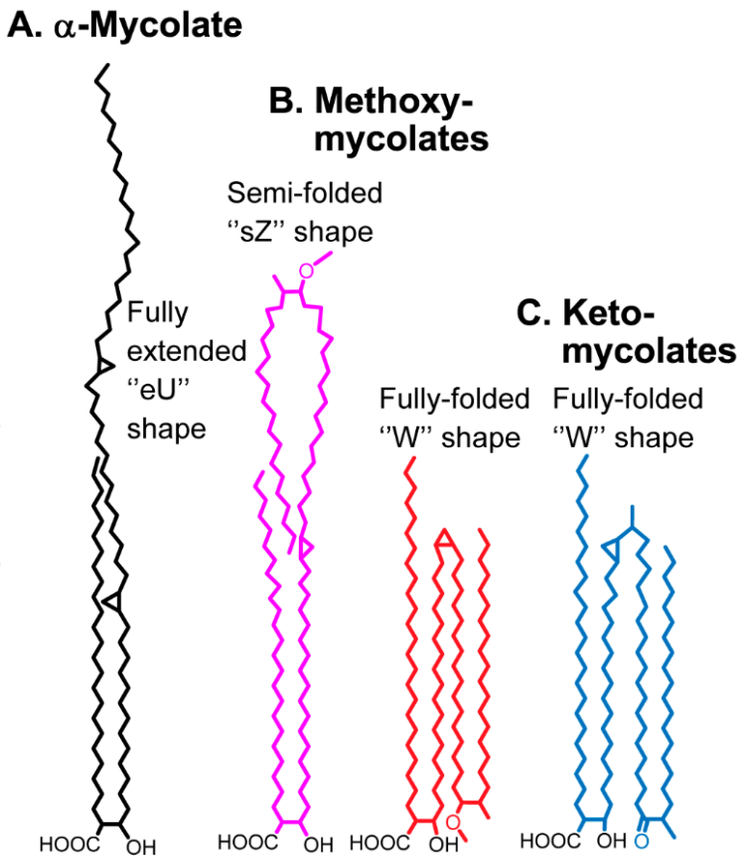

Figure 2. Two-dimensional representative conformations of *M. tuberculosis* mycolic acids. **A.** Extended α-mycolate. **B.** Two alternatives for methoxymycolates. **C.** Fully folded ketomycolate.

3. Esters of mycolic acids

The so-called "cord factors" are the best known mycolic acid esters in mycobacteria; they are principally trehalose dimycolates (TDMs) with trehalose monomycolates (TMMs) also being encountered (Figure 3). The proportions of TDMs and TMMs vary widely in mycobacteria so an integral structural role is not indicated, their main importance lying in the key role as intermediates in the transfer of mycolic acids on to arabinosyl units in the cell envelope [13]. Glucose monomycolates (GMMs) are common in mycobacteria, but in highly variable proportions [25]. Consistent proportions, however, are recorded for monomycoloyl glycerols (MMGs) (Figure 3) in the *Mycobacterium bovis* members of the *M. tuberculosis* complex [26,27], thereby suggesting some cell envelope structural involvement. Mycobacteria also produce very complex mixtures of di- and triacylglycerols, some of which contain non-hydroxylated fatty acids that correspond to the meromycolate portion of mycolic acids [28,29], the so-called "mycobacteric" acids [2]. Triacylglycerols have a storage role in "lipid bodies" [30] but they have also been suggested as contributors to the MOM outer leaflet [31]; the complex mixtures of di- and triacylglycerols must be fully unravelled before precise roles can be properly defined.

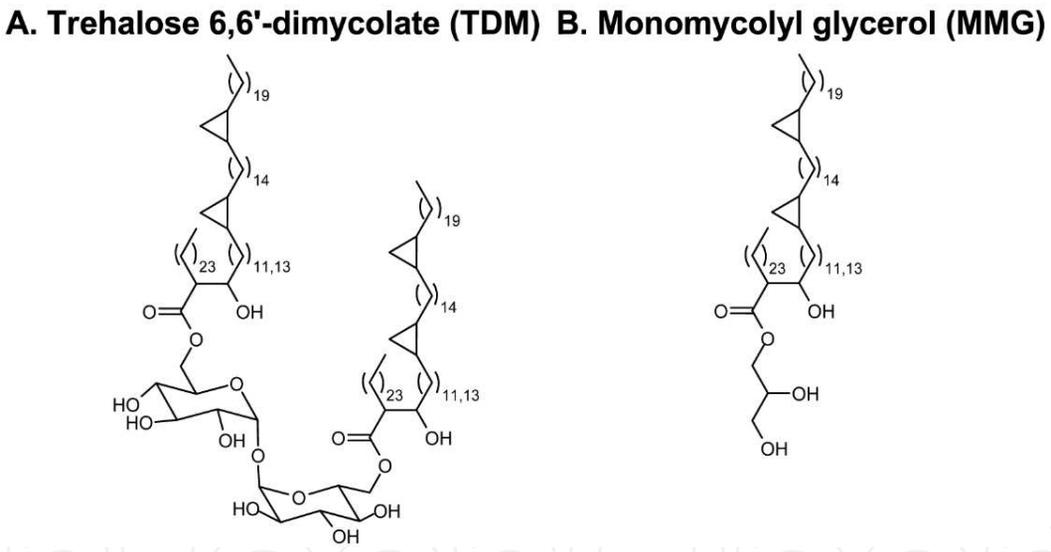

Figure 3. Mycolic acid esters. **A.** Trehalose dimycolate (TDM). **B.** Monomycoloyl glycerol (MMG).

4. Phthiocerol and phenolphthiocerol dimycocerosate families

The phthiocerol and phenolphthiocerol long-chain diols are esterified by multimethyl-branched "mycocerosic" acids whose chiral centres have ***R*** absolute configuration in members of the *M. tuberculosis* complex and *M. kansasii* (Figure 4) [32-35]. Phthiocerol dimycocerosates (PDIMs) are mainly based on long-chain diols, the phthiocerol As and phthiodiolones (Figure 4). Related families of the phenolphthiocerol dimycocerosates have characteristic antigenic oligosaccharides linked to the phenolic residue and these are commonly known as "phenolic

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glycolipids" (PGLs) (Figure 4) [33-36]. PDIMs are large (> 90 carbons) hydrophobic molecules that are considered to be "free lipid" constituents of the outer leaflet of the external MOM, interacting with the chains of the covalently bound MAs of the inner leaflet. The PDIMs from the *M. tuberculosis* complex are relatively large in comparison with those of other taxa. Other interesting cases are the so-called "Beijing" variants of *M. tuberculosis* [37] and *M. kansasii* [32, 33,35] where only restricted selections of members of the phthiocerol family are encountered.

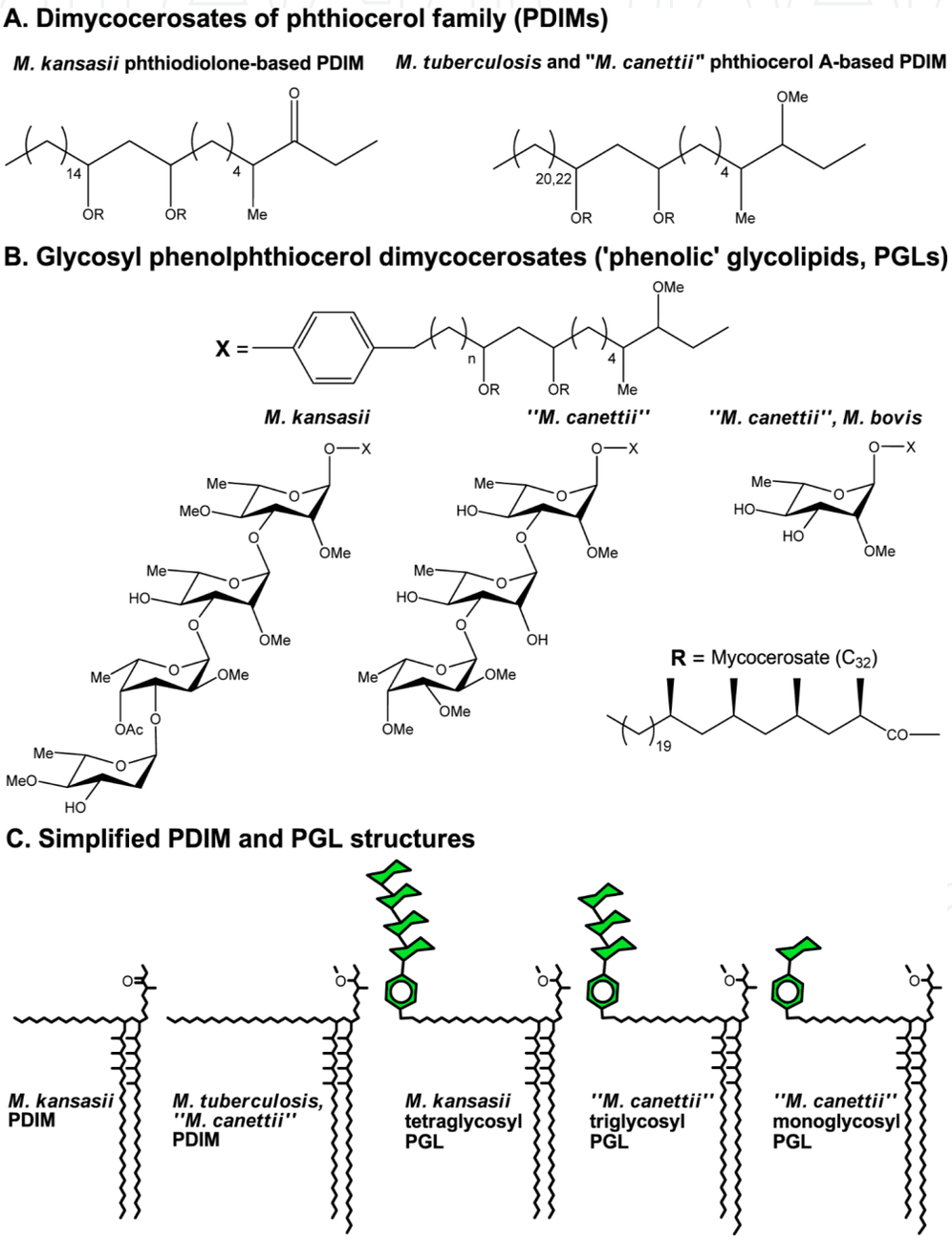

Figure 4. Representative structures of mycocerosate esters of phthiocerols and glycosyl phenophthiocerols. **A.** Phthiocerol family. **B.** Glycosyl phenophthiocerol (phenolic glycolipids). **C.** Simplified structures for illustrative use in Figures 10 and 11.

The oligosaccharide substituents of PGLs have demonstrable antigenicity [38] but the individual sugar units are relatively hydrophobic. Certain members of the *M. tuberculosis* complex, such as the rare smooth morphology tubercle bacilli termed "*Mycobacterium canettii*", have two PGL types with different oligosaccharides but *M. bovis*, for example, has only a single type (Figure 4). The PGLs from *M. kansasii* have close structural similarities to those from *"M. canettii"* (Figure 4), but not to those from *Mycobacterium marinum* [33-36]. The chiral centres in the multimethyl-branched fatty acid substituents in both the PGLs and PDIMs from *M. marinum* and *Mycobacterium ulcerans* are of ***S*** absolute configuration [33-36]. Significantly, the 1,3-diol units in the phthiocerol and phenolphthiocerol moieties (Figure 4) from *M. marinum* and *M. ulcerans* have *erythro* geometric configuration in contrast to the more common *threo* stereochemistry [33-36].

5. Acyl trehaloses

In addition to TDMs and TMMs, there are families of trehalose-based glycolipids acylated with multimethyl branched fatty acids with ***S*** absolute configuration of their chiral centres (Figure 5) [34,39,40]. The main fatty acids encountered are C_{24} mycosanoic, C_{27} mycolipenic, C_{27} mycolipanolic, C_{37} phthioceranic and C_{40} hydroxyphthioceranic acids (Figure 5). Diacyl trehaloses (DATs) are the simplest representatives, based on C_{24} mycosanoic and C_{27} mycolipanolic acids. The C_{27} mycolipenates are the characteristic acyl components of pentaacyl trehaloses (PATs) (Figure 5). The exceptionally long phthioceranic and hydroxyphthioceranic acids are the fatty acids found in a family of sulfated trehalose glycolipids (SGLs) (Figure 5) [3,41].

6. Lipooligosaccharides

A highly polar series of lipids, which include trehalose in their saccharide core, are termed lipooligosaccharides (LOSs) [42,43]. Such lipids are absent in many modern *M. tuberculosis* isolates but they are characteristic of "*M. canettii*" and *M. kansasii* (Figure 6). Lipooligosaccharides are associated with biofilms and motility [44]. Indeed, it has been shown that smooth variants of *M. kansasii*, containing LOSs, are rapidly cleared from the organs of infected animals, but rough variants, lacking all LOSs, produce chronic systemic infections [45].

7. Phosphatidylinositol mannosides and other polar lipids

The mycobacterial plasma membrane incorporates conventional polar lipids, such as phosphatidylethanolamine (PE), phosphatidylinositol (PI) and diphosphatidylglycerol (DPG) (Figure 7), which can interact together to form the basis of a typical membrane bilayer. However, most mycobacteria have a remarkably consistent family of four phosphatidylinosi-

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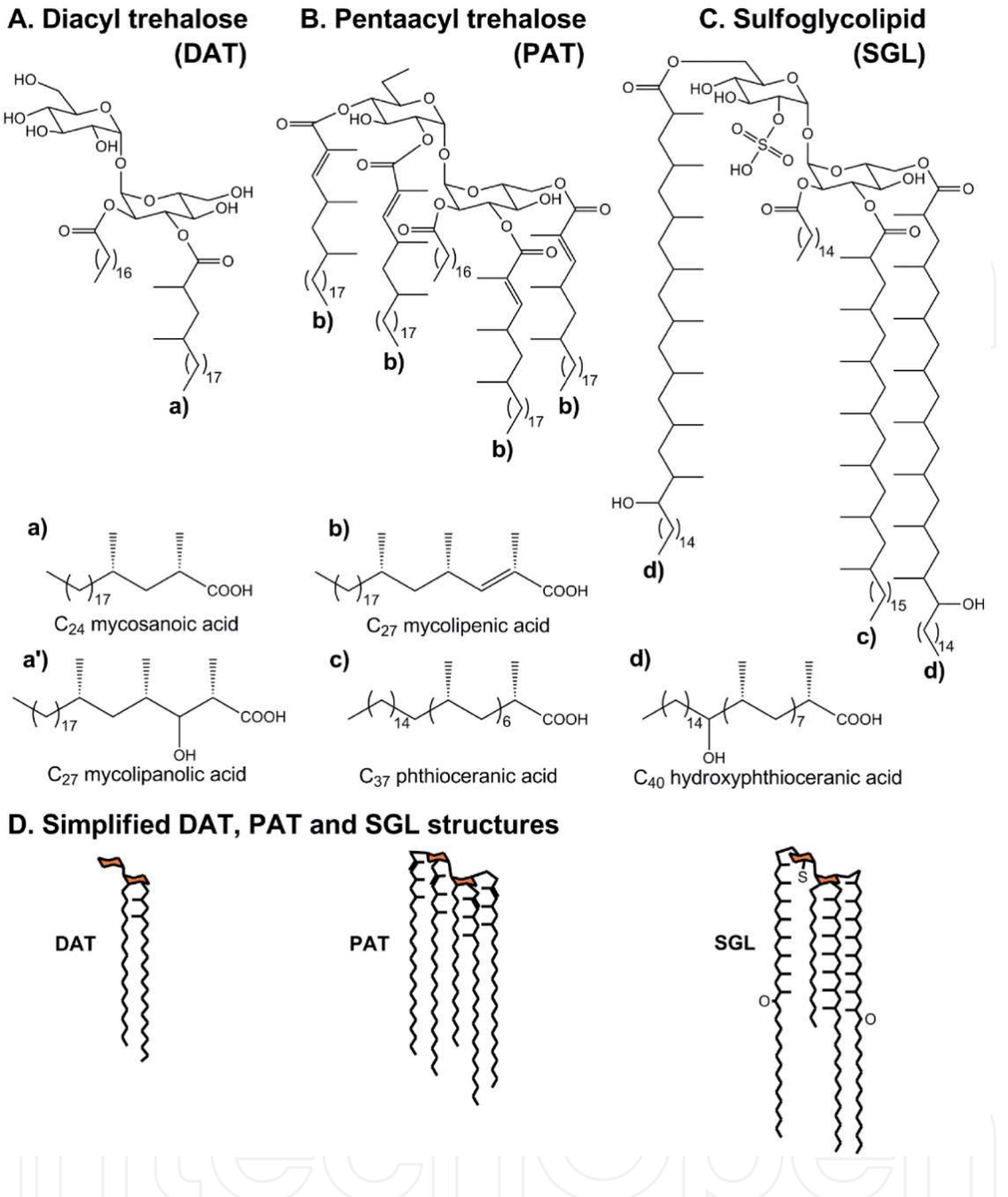

Figure 5. Acyl trehaloses. **A.** Diacyl trehalose (DAT). **B.** Pentaacyl trehalose (PAT). **C.** Sulfoglycolipid (SGL). **D.** Simplified structures for illustrative use in Figures 10 and 11.

tol mannosides (PIMs); these comprise mono- ($AcPIM_2$) and diacyl phosphatidylinositol dimannosides (Ac_2PIM_2) and mono- ($AcPIM_6$) and diacyl phosphatidylinositol hexamannosides (Ac_2PIM_6) (Figure 7) [46-49]. Recent research has provided evidence that PIM_2 and PIM_6 classes may be unevenly distributed over the two leaflets of the mycobacterial plasma membrane [31]. These findings will be interpreted, later, as showing that PIMs may act to reinforce the plasma membrane, perhaps adding a further level of selective permeability to the mycobacterial cell envelope.

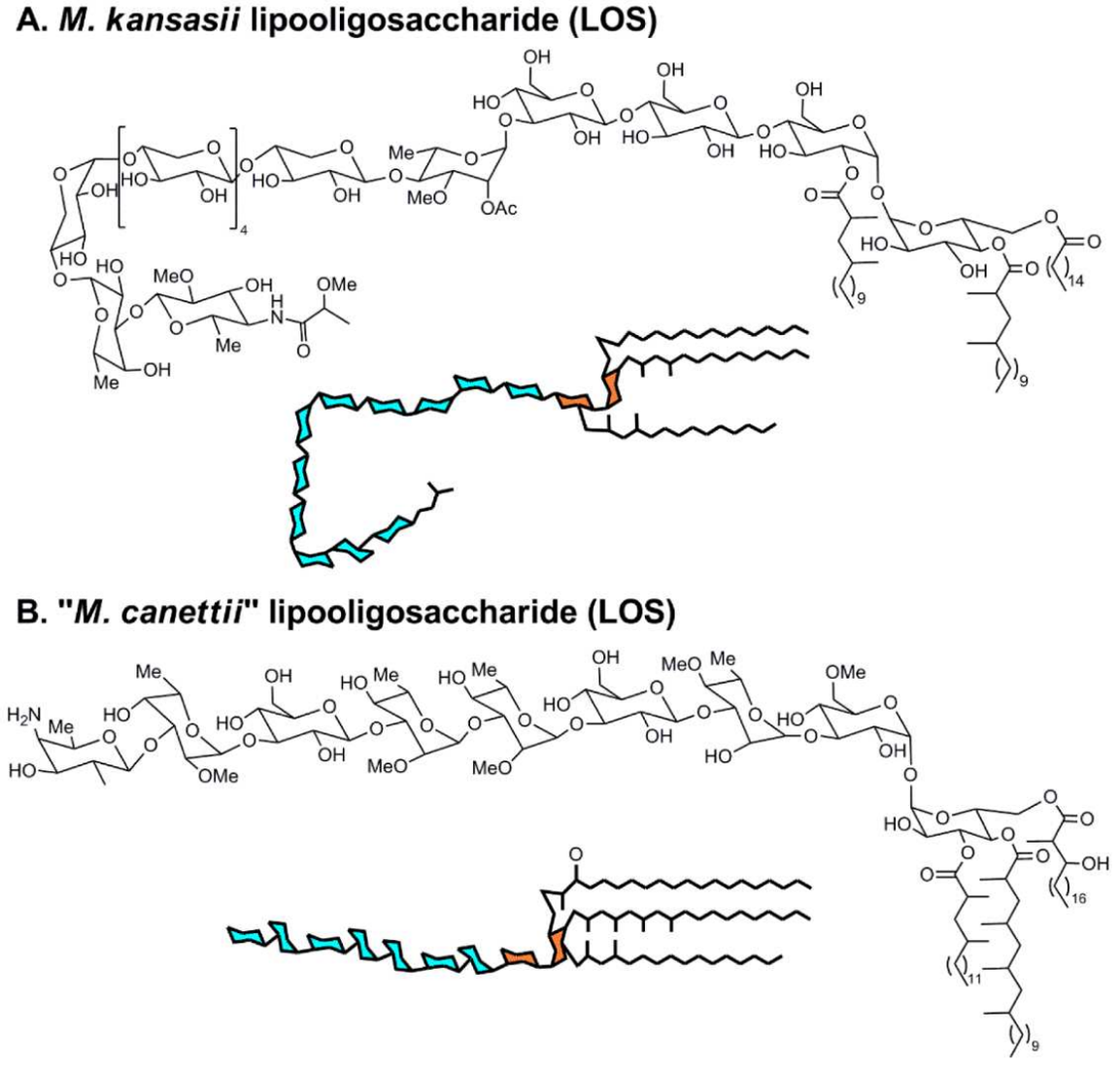

Figure 6. Lipooligosaccharides (LOSs). A. *M. kansasii*. B. *"M. canettii"*. Simplified structures are included.

8. Lipomannan and lipoarabinomannan

The basic structures of the PIMs polar lipid family (Figure 8) share the same manno-phosphatidylinositol (MPI) anchor with two classes of characteristic large lipoglycans, namely lipomannans (LMs) and lipoarabinomannans (LAMs) (Figure 8) [50-54].

9. Mycoloylarabinogalactan-peptidoglycan (mAGP)

The overall chemical structure of this complex macromolecule has been clarified during the past decade [55-58]. A specific linker unit covalently binds the proximal galactan portion of the arabinogalactan to peptidoglycan with the distal arabinose moieties providing anchorage for the 70 to 90 carbon long-chain mycolic acids (Figure 9) [19,55-58]. While chemical connec-

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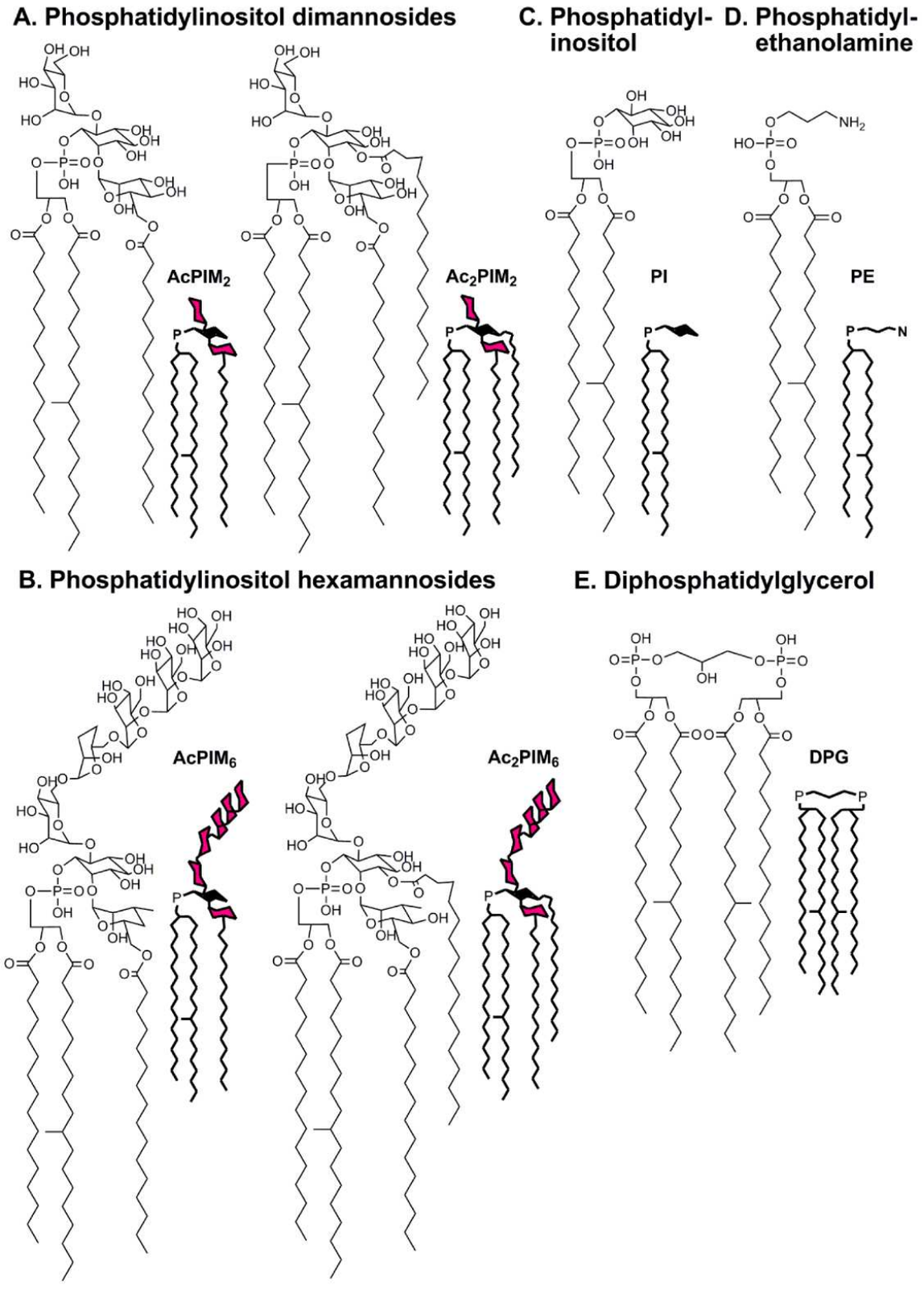

Figure 7. Phosphatidylinositol mannosides (PIMs). **A.** Phosphatidylinositol dimannosides. **B.** Phosphatidylinositol hexamannosides.**C.** Phosphatidylinositol. **D.** Phosphatidylethanolamine. **E.** Diphosphatidylglycerol. Simplified structures are included.

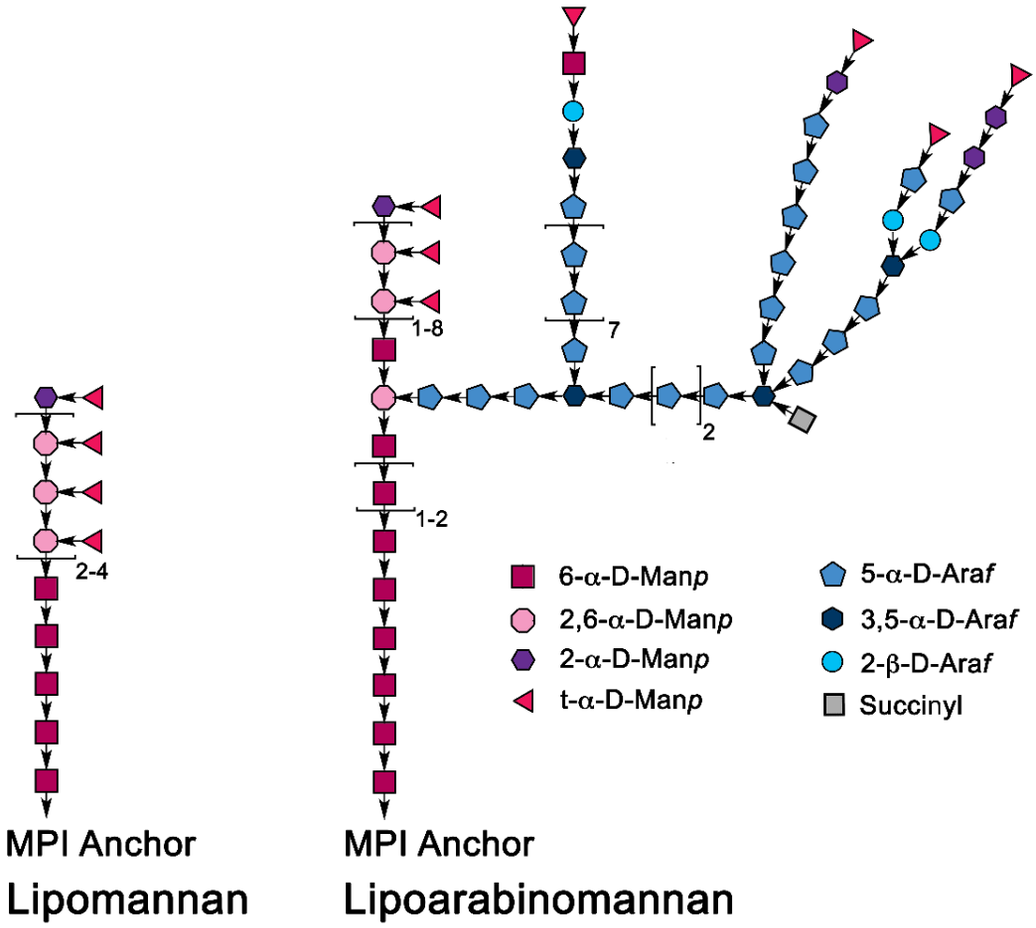

Figure 8. Essential structural topography of *M. tuberculosis* lipomannan and lipoarabinomannan. **MPI** is manno-phosphatidylinositol.

tivity is established, conformational preferences of the carbohydrate domains remain a matter of conjecture with diverse interpretations. It is becoming evident that versatile peptidoglycans can adopt different conformational arrangements, depending on the length of the polymeric disaccharide chains with helices being possible for shorter units [59]. It was shown that a synthetic peptidoglycan adopted a right-handed helical conformation [60]. A distinctive feature of mycobacterial peptidoglycan is the presence of a proportion of *N*-glycolyl muramic acid substituents, rather than the *N*-acetyl groups found in many other bacterial taxa [55-59]. The size and complexity of the mycoloylarabinogalactan- peptidoglycan, which is an extensive single macromolecule, provides a major challenge in perceiving how it can be coherently organised in three dimensions. This is not such a difficulty in many other bacterial taxa where no really major macromolecules are directly attached to peptidoglycan. It is now well established that the mycolic acids form a coherent inner leaflet of the MOM and this necessitates support from a well-integrated underlying platform.

The proposed mAGP arrangement (Figure 10) is based on a "scaffold" model [61,62], where peptide cross-linked helices are interspersed with helices of the galactan part of the arabinogalactan; the arabinan portion is then arranged to provide linkage points for mycolic acids. An

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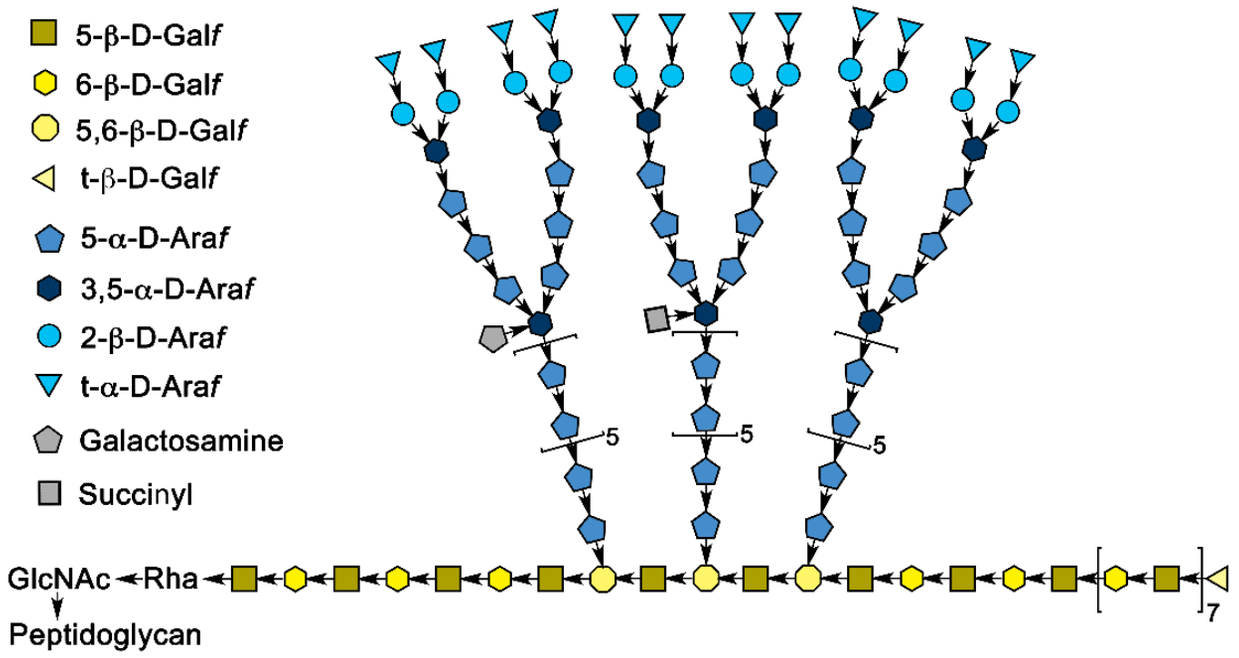

Figure 9. Essential structural topography of *M. tuberculosis* arabinogalactan-peptidoglycan.

attractive arrangement can be envisaged with the galactan extending to a level similar to that of the peptidoglycan helix to produce an essentially level "mosaic platform" as a stable anchorage for mycolic acids. While the helical galactan can provide a relatively rigid base unit, the arabinan may be more flexible so that the bound mycolic acids can jostle for position and occupy optimal locations. Indeed, arabinan flexibility may be an important factor in allowing hydrophobic interactions to govern the relative location of mycolic acid chains and associated free lipids. Calculations [63] indicate that the arabinofuranose polymer is reluctant to adopt a rigid compact helical conformation, thereby allowing a degree of flexibility.

10. Cell envelope organisation

The original model [4], with an inner and outer membrane, was based mainly on chemical principles, supported by freeze-etching results [64] that showed two clear distinct parallel cleavage planes in the mycobacterial cell envelope. The dual membrane proposal was confirmed by a confocal microscopy study that showed differential location of two fluorescent dyes of different lipophilicity [65]. The outer membrane was visualised directly by cryo-electron microscopy and the essential dimensions of the mycobacterial cell envelope were revealed [7-9]. An updated model for the cell envelope organisation for tubercle bacilli is proposed in Figure 10. Justification for the details of the proposal will build outwards from the plasma membrane.

The inner plasma membrane in mycobacteria has been traditionally regarded as conventional, even though a significant role was lacking for the unusual phosphatidylinositol mannosides

(PIMs) (Figure 7). A resolution of this conundrum has been indicated in a study [31], which showed strong evidence for locating Ac_2PIM_2 (Figure 7) as the sole polar component of the inner leaflet of the inner membrane. It was suggested that PIM_6 would be present in the outer leaflet of the inner membrane, projecting into the periplasm. It is not clear why there are two versions of PIM_2 and PIM_6, with either three or four fatty acid chains (Figure 7), but as a working hypothesis both PIM_2 lipids are placed in the inner leaflet and both PIM_6 lipids in the outer leaflet of the plasma membrane (Figure 10). As demonstrated by two-dimensional thin-layer chromatography [26], the proportions of the principal four PIM types are remarkably consistent, as is the proportion of PI. It is possible that equal proportions of PIMs with three and four fatty acid constituents are optimal for close packing in membranes; detailed physical studies on these lipids would be instructive. The proportions of PIM_2 exceed those of PIM_6 so if PIM_2 lipids are considered to predominate in the inner leaflet [31], then PI, PE and DPG (Figure 7) may complete the outer leaflet along with PIM_6. There is a distinct possibility that mycobacterial inner plasma membranes, rich in PIMs with three and four fatty acid chain anchors, have special physical properties that enhance its stability and perhaps governs permeability. Indeed, it has been suggested that this inner membrane may be "a bilayer environment of unusually low fluidity" [31] contributing to drug resistance. It was also noted [31] that the behaviour of PIM_2 liposomes had been found [66] to have behaviour suggestive of exceptional stability. It is now apparent that the inner mycobacterial plasma membrane is a highly specialised organelle, worthy of being distinguished with special nomenclature. Given the developing popularity of "MOM" for the mycobacterial outer membrane, a related simple suggestion might be "MIM" for the "mycobacterial inner membrane". It was found that disruption of PIM_2 production causes growth arrest [67,68] but the higher PIMs were dispensable [69], thereby indicating an important structural role for PIM_2. It has also been indicated that the acylation state of PIMs is also significant [70].

The outer leaflet of the MIM inner plasma membrane is also a suggested location for the PIM-related LM and LAM (Figure 10), but unequivocal evidence is elusive with alternative MOM location being a possibility. In a well-balanced objective analysis [71], it was concluded that LAM had at least an initial anchorage in MIM. However, in some cases [72,73], the undoubted presence of LAM at the cell surface required invoking specific lipoglycan transport mechanisms that need to be fully defined. At least a transient MIM location for LAM is supported by the presence of related lipoglycans in other actinomycetes, which do not have mycolic acids and an outer membrane, as summarised recently [72]. The basic fact that the lipid anchors of LM and LAM are identical to those in PIMs (Figures 7,8) suggests very strongly that all these components have a common anchorage in MIM. This should not rule out possible interactions with the hydrophobic MOM surface, but such lipophilic binding is predictably less specific and it is very difficult to envisage LM and LAM as important integral components of the MOM outer leaflet. The PIM-based lipid anchors appear to be all very similar for related LM and LAM lipoglycans across the genus *Mycobacterium* and related mycolata; however, enormous variations in the surface of the MOM in such taxa would militate strongly against any specific incorporation of LM and LAM into the MOM outer leaflet. Immunogold atomic force microscopy failed to detect cell surface LAM in *M. bovis* BCG, but LAM was revealed after treatment with drugs that attack cell envelope targets [74]. For *Corynebacterium glutamicum*, it was found

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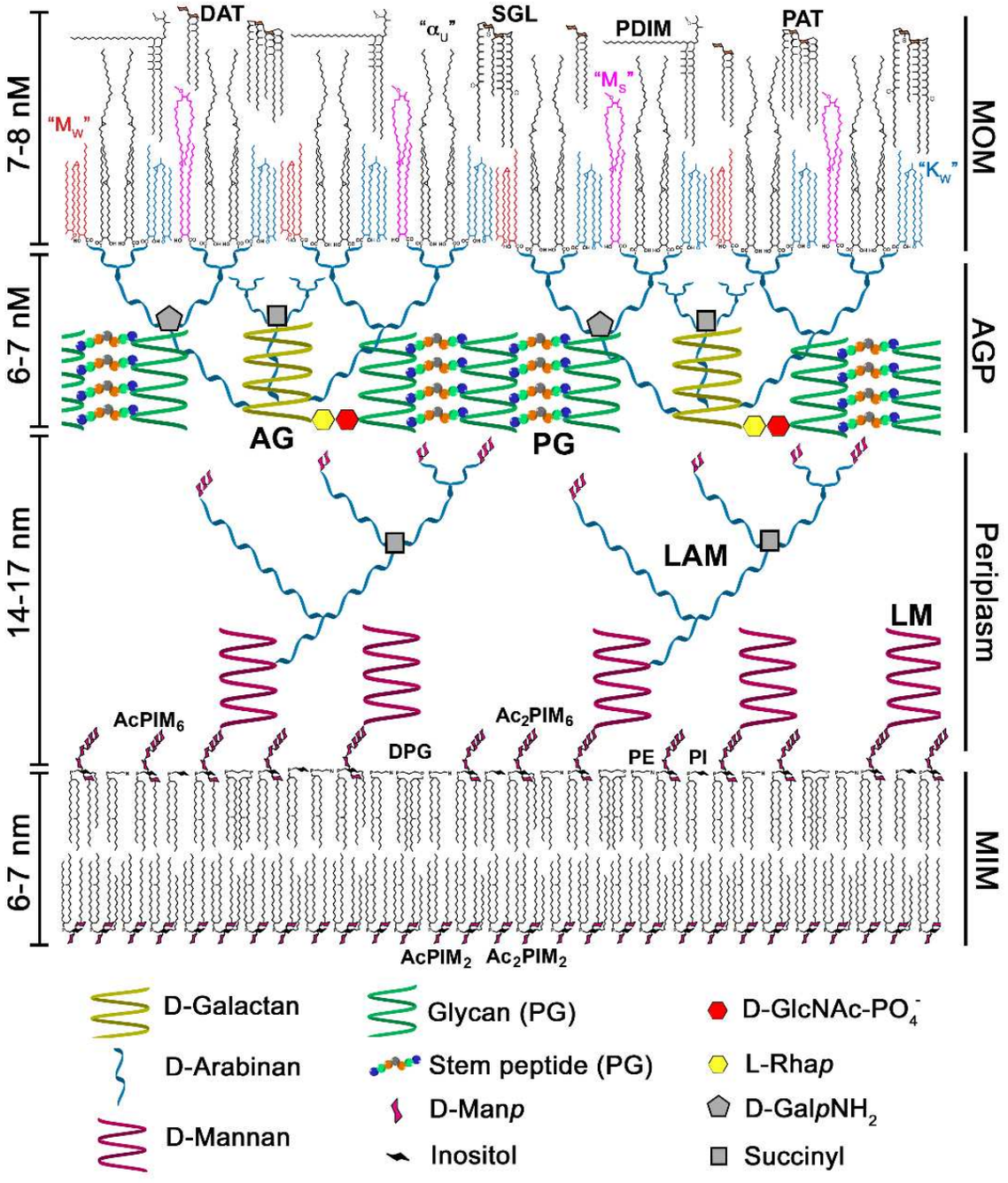

Figure 10. Two-dimensional representation of the location and interaction of structural components in the cell envelope of *M. tuberculosis*. MOM and MIM are "mycobacterial outer membrane" and "mycobacterial inner membrane", respectively. AGP is the arabinogalactan-peptidoglycan. Mycolic acid conformations (Figure 2) in the MOM are labelled "α_u" for α-mycolate for fully extended "eU" shape, "K_w" and "M_w" for fully folded keto- and methoxymycolate "W" shape, respectively, and "M_s" for semi-folded "sZ" shape. Correlating dimensions of all components are drawn to fit within the spatial constraints imposed by cryo-electron microscopy [8,9]. Dimensions of peptidoglycan (PG) helices are derived from synthetic material [60]. Helices of the mannan sections of lipomannan (LM) and lipoarabinomannan (LAM) and both the arabinan and galactan components of arabinogalactan (AG) were modelled using GLYCAM. Woods Group. (2005-2014) GLYCAM Web. Complex Carbohydrate Research Center, University of Georgia, Athens, GA. (http://www.glycam.com) [63]. Details of simplified lipid structures are in Figures 4-6.

that LM and LAM are embedded in the plasma membrane, along with the PIMs [75]. Overall, the importance of lipophilic anchors in external LM and LAM is diminished by the observation that their lipid-free glycan counterparts, mannan and arabinomannan, may be one destination of these components [72].

The extensive supposed "periplasmic space" (Figure 10) will, in fact, be an area of intense activity as all cellular components of the MOM whose synthesis is initiated in the cytoplasm, and continued through MIM, may be assembled and organised within [51-54]. The fact that many of these cell envelope components are relatively large may explain why such a relatively extensive compartment is needed. Cryo-electron microscopy studies [7-9,76] gave indications of some rather indistinct structural elements within the periplasm, labelled as layers L1 and L2 [7,76]. The internal L1 layer is most probably associated with "granular" material of protein origin, with the outer L2 layer corresponding to some of the peptidoglycan-arabinogalactan matrix [76]. It has been proposed that the maintenance of the relatively low-density periplasmic space could be facilitated by the presence of large polymeric material [9]. The helical mannan polysaccharide moiety in LM (Figure 10) may have such a role, but it could also act as a scaffold or template to compartmentalise various biochemical activities. In *M. smegmatis* it was shown that LAM was dramatically reduced as the bacteria approached stationary phase, but LM, mycolic acids and arabinogalactan were unchanged; this indicates that all these latter three components have important structural roles [73].

The accommodation and conformation of the mycoloyl arabinogalactan peptidoglycan macromolecular structure is a major challenge in the relatively limited space available (Figure 10). An informed choice has been made to use the "scaffold" approach of [61,62], with a helical peptidoglycan network interspersed with helical galactan units, as detailed in Figure 10. The relatively heavy peptidoglycan peptide cross-linking [52] may be a factor in favouring the scaffold arrangement in mycobacteria. This proposal echoes a previously advanced arrangement [52] that did not attempt to make precise spatial correlations and neglected to include the extensive periplasm. It would be interesting to explore the possibility that the *N*-glycolyl muramic acid substituents may have some influence on mycobacterial peptidoglycan conformation. The proposed coherent peptidoglycan – galactan "mosaic platform" layer (Figure 10) could provide a coherent anchorage for the arabinan moieties, some of which are esterified with mycolic acids. As noted previously, conformations of MAs vary with structural type and these are illustrated with W-conformations for keto-MAs, an extended U-conformation for α-MAs and equal numbers of W- and U-conformations for methoxy-MAs (Figure 2, 10). The MAs are comprised of approximately 50% α-MAs [17,18] as reflected in Figure 10. Such covalently bound MAs are instrumental in providing a stable MOM inner leaflet with potential for interaction with diverse free lipids to produce an effective outer membrane permeability barrier. Detailed spatial calculations attempted to simulate the accommodation of W-folded α-MAs into the MOM [77]; however, other MA conformations must be incorporated to provide a full picture. In a detailed quantitative study [31] of the cell envelope of *M. smegmatis* it was demonstrated that there were sufficient hydrocarbon chains in both inner and outer membranes to confirm the viability of the original dual membrane model [4].

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A wide range of free lipid types are considered to form the outer leaflet of the outer mycomembrane of members of the *M. tuberculosis* complex. These range in polarity from the hydrophilic LOSs (Figure 6), through PGLs (Figure 4), DATs, SGLs and PATs (Figure 5), to the highly apolar hydrophobic PDIMs (Figure 4). All these unusual lipids have specialised fatty acid components, incorporating varying numbers of methyl branches located near to the carboxyl group (Figures 4-6). For the original chemical model of the mycobacterial cell envelope [4], it was conjectured that the presence of methyl branches might moderate the depth of insertion of the fatty acids into the MOM bilayer. This remains an attractive hypothesis, though detailed physical studies would be of value. The proximal multimethyl branches would favour a coiled conformation, leaving the distal straight-chain portion of such fatty acids to interact with mycolic acid chains. The straight-chain fatty acids in DATs, PATs (Figure 5) and LOSs (Figure 6) could possibly be layered parallel to the MOM surface providing elements of lateral stabilisation. In certain previous cell envelope models [9,78] there has been an unjustified tendency to include inverted TDMs as major components in the MOM outer leaflet. A proven role of TDMs is as an agent for the transfer of mycolic acids onto the arabinan matrix, presumably in the assembly of the inner leaflet of the MOM. It is more likely, therefore that TDMs would align themselves with the trehalose unit adjacent to the arabinan, as previously indicated [4]. Similarly, monomycoloyl glycerols (MMGs) (Figure 3) in *M. bovis* would be more readily accommodated with an internal glycerol unit. Such a location might possibly act to modify MOM fluidity in *M. bovis* whose mycolic acid composition has high proportions of *cis*-cyclopropyl keto-MAs, in comparison with the predominant *trans*-cyclopropyl keto-MAs in *M. tuberculosis* [17,18]. However, in related taxa, such as *Corynebacterium glutamicum*, it is probable that the structurally much simpler mycolic acids, and perhaps their trehalose esters, are able to participate as members of the outer leaflet of the outer membrane [8,78].

11. Evolutionary and pathogenicity aspects of cell envelope composition

It is of particular interest to attempt to obtain an understanding of the influence and importance of cell envelope composition in mycobacterial pathogenicity and evolution. A consensus is developing that an attractive evolutionary pathway can be envisaged from environmental *Mycobacterium kansasii*, through *"M. canettii"* to all the modern biotypes of the *M. tuberculosis* complex [79-83]. *M. kansasii* is the environmental organism that phenotypically resembles *M. tuberculosis* most closely and this relationship has been supported by genomic comparisons [79-81]. Cogent arguments have been advanced to associate the evolution of ancient tubercle bacilli, such as *"M. canettii"*, with bacteria similar to *M. kansasii*, including indications of horizontal gene transfer between these taxa [80,81]. Key genes acquired by horizontal gene transfer include those coding for mycobacterial lipids, transferases and proteins related to adaptation to anaerobic conditions [80,81]. *M. kansasii* continues to cause pulmonary disease in Silesian and South African miners, the bacterium being contracted from water in showers [81]. A detailed study has shown that the unusual smooth morphology *"M. canettii"* strains appear to form a pivotal role in the evolution of tuberculosis [84]. Although extant strains of

"M. canettii" still cause human tuberculosis, they differ significantly in infectivity and appear to be relatively ancestral [85,86]. Genomic studies indicate that the very diverse *"M. canettii"* isolates appear to coalesce into a form of bottleneck after which all the modern human and animal biotypes evolved in a relatively linear manner [83,84,87,88]. A plausible working hypothesis for the evolution of *M. tuberculosis sensu stricto* is outlined in Figure 11, highlighting the possible contribution of cell envelope lipid composition.

The most fundamental underlying difference between members of the *M. tuberculosis* complex, broadly including *"M. canettii"*, and *M. kansasii* and related environmental taxa is seen in the mycolic acids. The α-mycolic acids from *M. kansasii* have the regular spacing of the proximal and distal *cis*-cyclopropyl groups (Figure 1), common to a wide range of mycobacteria [17,18]. In clear contrast, α-mycolates from *"M. canettii"* and members of the *M. tuberculosis* complex have the chain between the hydroxyl group and the proximal cyclopropyl group shortened most significantly from 17 methylene groups in, for example, *M. kansasii* to 11 and 13 methylenes in members of the *M. tuberculosis* complex [4,17,18,79]. Additionally, the chains in 2-position and the terminal meromycolate chain are both relatively extended by two carbons. As noted above [20,22,24,79], *M. tuberculosis* α-mycolic acids extend more readily in molecular dynamics simulations with apparent interaction of the chain in 2-position with the chain between the two cyclopropyl groups, in a "fully extended shape" (Figure 2) [22] or "eU" conformation [24]. The methoxymycolates and ketomycolates of *"M. canettii"* and *M. tuberculosis* (Figure 1) conform to the general pattern of these components in related mycobacteria, such as *M. kansasii,* but, significantly, these oxygenated mycolates are slightly larger than any others [17,18]. The particular ability of α-mycolates to adopt extended flexible U-conformations is probably significant for interactions with free lipid components of the MOM outer leaflet. Indeed it is possible that the exceptionally long terminal chain in *M. tuberculosis* α-mycolic acids may penetrate right to the MOM outer leaflet to contribute to the hydrophobicity of the cell envelope surface. In this context, the balance of the three main types of mycolates is probably significant; the ratios of the α-, methoxy- and ketomycolates are, respectively, ~10:5:8 for *M. kansasii*, ~10:6:8 for "*M. canettii"* and ~10:5:5 for *M. tuberculosis* [17,18,79]. It is conceivable that having 50% α-mycolates may optimise hydrophobic interactions with the particular range of free lipids in the MOM outer leaflet in *M. tuberculosis.* In a detailed consideration [79], it was found possible to discern quite a range of distinct differences in the overall mycolic acid composition of these three taxa; the overall conclusion was an apparent simplification and tightening up of mycolate composition in modern *M. tuberculosis*.

There are also very significant changes in cell envelope MOM free lipid composition between all the taxa, shown in Figure 11. *M. kansasii* has characteristic polar LOSs (Figure 6), relatively polar PGLs (Figure 4) and PDIMs based only on phthiodiolones (Figure 4). These three lipid families appear to be the principal components of the outer leaflet of the MOM of *M. kansasii*, possibly contributing to a relatively polar cell surface compatible with a native hydrophilic environment. The PGLs of *M. kansasii* and *"M. canettii"* provide an appealing phenotypic link, with a loss of a single sugar to give a triglycosyl PGL and three sugar units to give a monoglycosyl PGL (Figures 4 and 11), as also highlighted previously [81]. However, some most significant new lipid structural principles are introduced in *"M. canettii"* (Figure 11). Two

major classes of acyl trehalose glycolipids, the DATs and PATs (Figure 5), are encountered [86]. The relatively polar DATs are good antigens, which probably contribute to the cell surface properties of tubercle bacilli [38]. In contrast, the barely antigenic PATs are very non-polar and are likely to increase cell surface hydrophobicity quite significantly. The reduced 9-sugar oligosaccharide in the LOSs of *"M. canettii"*, compared with the 13-sugar oligosaccharide in *M. kansasii* might also result in reduced hydrophilicity. It is not known if all the diverse isolates of *"M. canettii"* have similar lipid profiles, but it certainly appears that the MOM free lipid composition of this taxon has some redundancy with perhaps an over-generous provision of lipid types (Figure 11). This redundancy might correlate with a high propensity for horizontal gene transfer, positively accumulating new mycobacterial lipid principles at the expense of a rather overblown excess of lipid biosynthesis. This might support a concept that *"M. canettii"* is a low pathogenicity intermediate taxon in evolutionary transit, rather than an efficient pathogen with a particular niche. In contrast, as will be detailed below, the modern *M. tuberculosis* complex has emerged out the ancestral melange as a group of efficient pathogens with a range of specialised hosts.

Aspects of the cell envelope lipid composition of *"M. canettii"* are essentially the same as for *M. tuberculosis sensu stricto*, including mycolic acid composition, but there are a number of key differences. Significantly, the LOSs and PGLs present in *"M. canettii"* are absent in *M. tuberculosis,* but the latter is uniquely characterised by the presence of SGLs [89]. As noted above, the highly polar LOSs are associated with hydrophilic interactions, such as motility and biofilm formation [44]. The PGLs are also relatively polar, but in addition to having a structural role in the outer leaflet of the MOM, particularly specific functions are elusive. SGLs behave on chromatography as quite polar lipids, presumably due to the presence of the sulfated trehalose unit. However, SGLs have very large nonpolar phthioceranic and hydroxyphthioceranic fatty acid acyl chains (Figure 5), which are likely to provide enhanced hydrophobicity. In two informative studies, it was shown that deficiencies in SGLs [90] and DATs and PATs [91] did not have decisive effects on replication and persistence. In bacterial disease, clear distinctions must be made between transmissibility and the pathogenic process once the infection has been established. It would appear that the very hydrophobic SGLs and PATs (Figure 5) may have primary value in the transmission process, perhaps accompanied by some secondary roles in pathogenicity.

Previous studies have shown an important link between hydrophobicity and aerosol performance in *Mycobacterium avium* [92]. Preliminary studies are under way to compare the hydrophobicity of *"M. canettii"* and *M. tuberculosis.* For cultures grown on solid media in the presence of Congo Red [93], the smooth colonies of *M. kansasii* and *"M. canettii"* resisted staining by the dye, but two different rough strains of *M. tuberculosis* absorbed so much dye that they almost merged into the background (Figure 12). In preliminary standard partitioning experiments between hexadecane and water [94], it was found that *M. tuberculosis* was approximately one fifth more hydrophobic than "*M. canettii"* (details not shown). These simple experiments indicate a distinct difference in hydrophobicity between one member of the smooth supposed ancestral "Cannetti" taxon and rough modern human tubercle bacilli. It follows, therefore, that *M. tuberculosis sensu stricto* may be specifically adapted for aerosol

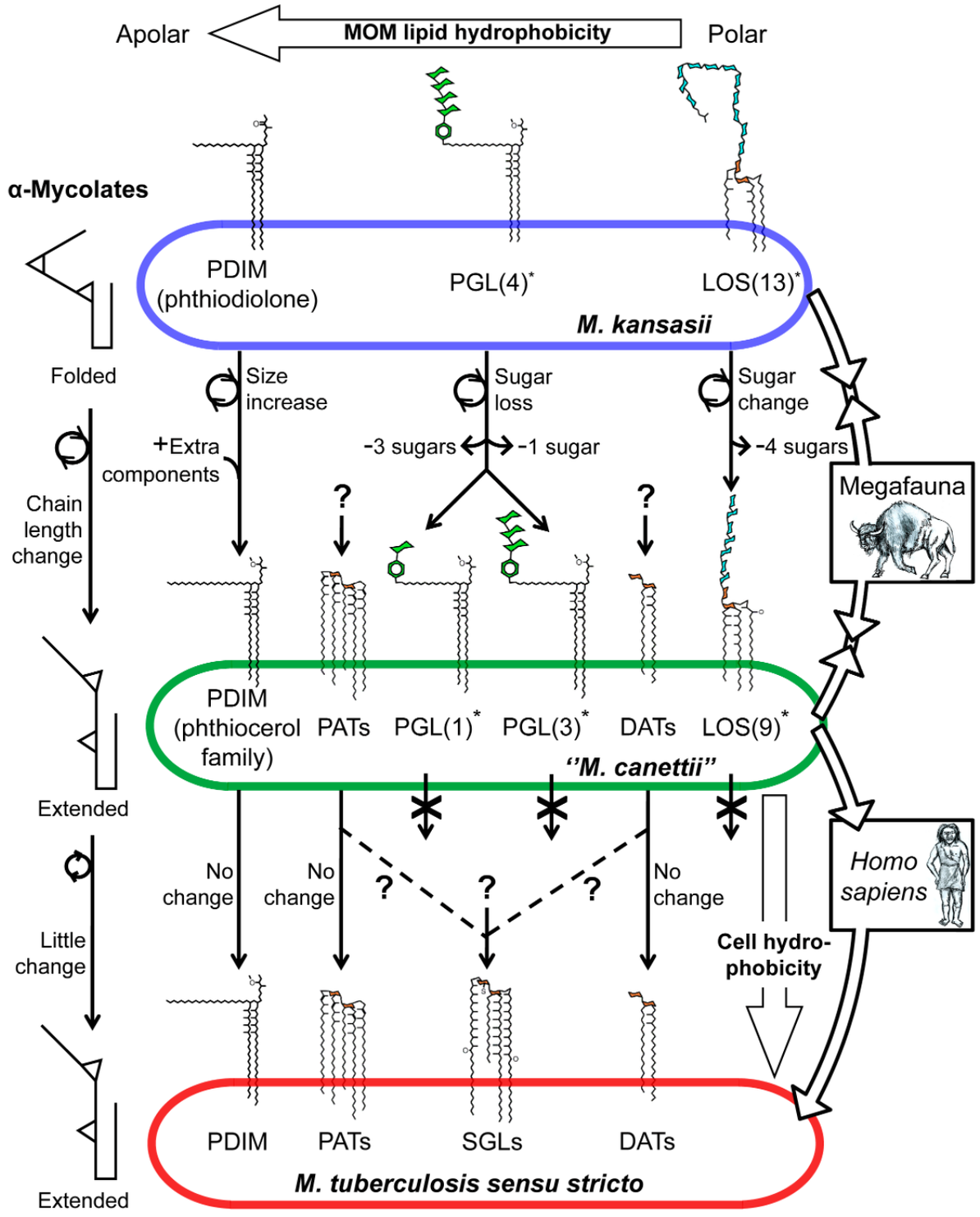

Figure 11. A generalised scenario for the significance of lipid composition in the evolution of *M. tuberculosis*. Details of the simplified lipid structures are in Figures 4-6. The numbers of sugars in particular LOSs and PGLs are shown in brackets with an asterisk, e.g. LOS (13)*.

transmission, whereas *"M. canettii"* is less favoured for this mode of transmission [85,86]. A link between cell envelope hydrophobicity and aerosol transmission is also probably signifi-

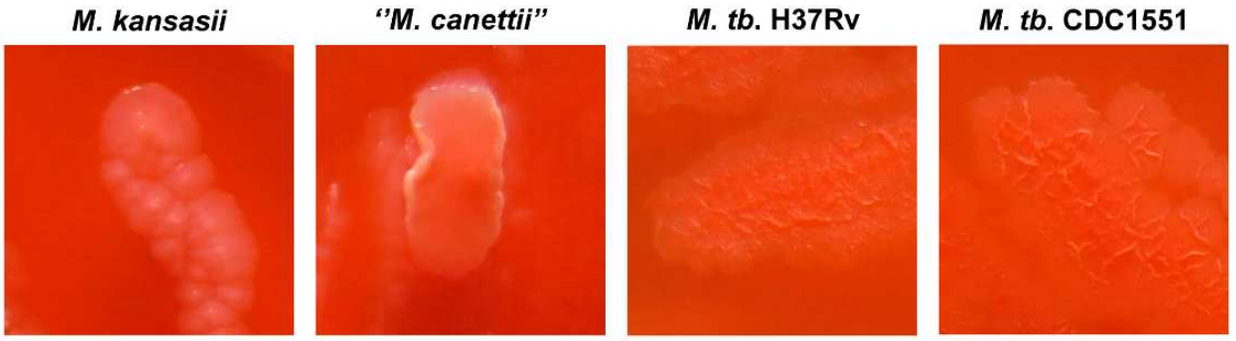

Figure 12. Differential uptake of Congo Red by *M. kansasii* ATCC12478, *"M. canettii"* CIPT 140010059 MNC1485, *M. tuberculosis* H37Rv and *M. tuberculosis* CDC1551. Strains were cultivated on 7H11 agar with 40μg/ml Congo Red.

cant in *Mycobacterium abscessus* infections [95,96]. Airway infections are often associated with rough variants that lack polar glycopeptidolipids and are likely to be more hydrophobic and aerosol transmissible than the usual smooth strains [95,96]. It was also shown that rough *Gordona* species were much more hydrophobic than smooth morphology variants of the same strain [97]. By use of chemical force microscopy the cell envelope surface of *M. bovis* BCG was found to be uniformly hydrophobic, but drugs disrupting mycolic acid synthesis destroyed this feature [74].

A jump from environmental *M. kansasii* to the obligate *M. tuberculosis* human pathogen requires the cooperation of suitable animal hosts, in which evolutionary changes can take place. The total dearth of any evidence for tuberculosis in *Homo sapiens*, prior to the Holocene, suggests zoonotic evolutionary hosts [79,98,99]. Characteristic skeletal lesions in a variety of Pleistocene megafauna are suggesting the presence of tuberculosis over a wide time period, going back to at least 120 ka BP [100,101]. In one well-documented example of a 17ka extinct bison, diagnosis by lesion was conclusively confirmed by amplification of *M. tuberculosis* complex DNA and recovery of pristine mycolipenate (Figure 5) and mycocerosate (Figure 4) tuberculosis lipid biomarkers [99,102,103]. The complex digestive organs in megafauna, such as bison and mastodons are potentially ideal vessels to facilitate evolutionary development, as the rich population of other microbial species present would be well situated to participate in horizontal gene transfer. There is substantial evidence for Pleistocene horizontal gene transfer in ancestral strains of the tubercle bacillus before linear evolution in the Holocene [80,81,104].

It is conceivable that, during the Pleistocene, thinly spread members of *Homo sapiens,* and also *Homo neanderthalensis,* may have contracted evolving ancestral tuberculosis from infected megafauna. However, there is no evidence to suggest that modern tuberculosis developed from ancestral strains solely in the human vehicles, mentioned above. Indeed, recent studies indicate that Neanderthals all became extinct before about 40ka BP [105], which precedes the perceived ~30 to 12ka BP evolutionary bottleneck in tubercle bacilli [84,87,106]. A feasible scenario, therefore, for the emergence of all the modern biotypes of the *M. tuberculosis* complex through the bottleneck would involve a complex web of interactions between *H. sapiens* and Pleistocene animal reservoirs until the dramatically ameliorated climatic conditions at the

beginning of the Holocene allowed humans to form settlements and promulgate communal tuberculosis. The apparent ready spread of human *M. tuberculosis* in early Holocene settlements was probably a result of the ability of the tubercle bacillus to fly in aerosols. This could be a consequence of the dramatically enhanced hydrophobicity of *M. tuberculosis sensu stricto* as compared with the most likely ancestral strains, labelled *"M. canettii"*. It will not be easy to pinpoint a precise event when modern *M. tuberculosis* made its decisive debut on the human stage. As noted above, the digestive systems of Pleistocene megafauna were probably very efficient vessels for the evolution of tubercle bacilli and the change from the relatively hydrophilic *"M. canettii"*, in the digestive tract, to the hydrophobic *M. tuberculosis* that could be expelled from the lung. The enormous diversity of the extant surviving *"M. canettii"* taxon [84] probably points to an even greater range of smooth ancestral strains being passaged, recycled and adapted through animals and the environment. Indeed it probably reasonable to suggest that the great genetic diversity of smooth strains of the tubercle bacilli may be a result of their emergence over a wide time scale and geographical area.

12. Conclusion

The cell envelope of *M. tuberculosis* and related taxa is an assemblage of efficiently coordinated and closely interacting macromolecules and exquisitely designed lipid moieties. One of the most characteristic organelles is the "mycobacterial outer membrane" or "MOM", but recent studies have shown that the "mycobacterial inner membrane", or "MIM", may be comparable in importance (Figure 10). Indeed the MIM, if it has a very characteristic inner leaflet composed predominately of PIM_2 phospholipids, may be a formidable barrier protecting the cytoplasm (Figure 10). The MOM is composed of an outer leaflet of "free lipids", which interacts with a monolayer of mycolic acids covalently bound to the relatively flexible arabinan portion of an arabinogalactan that, in turn, is linked to peptidoglycan. Utilising the hypothesis of the "scaffold" model, the peptidoglycan and the galactan section of the arabinogalactan form helices perpendicular to the cell surface resulting in a stable type of "mosaic platform" from which the MOM can be linked *via* an arabinan cushion (Figure 10). The space between the MIM and peptidoglycan, usually termed the "periplasm", is envisaged as being the initial place for the linkage of the characteristic lipoglycans, namely "lipomannan" (LM) and "lipoarabinomannan" (LAM) (Figure 10). However, LAM certainly is transported to the external capsular regions, but LM may linger in the periplasm affording a degree of organisation and stability. Overall, it is important to note that the mycobacterial cell envelope is a dynamic three-dimensional organelle; the hypothetical arrangement pictured in Figure 10 can only be a two-dimensional snapshot illustrating likely locations and interactions.

Given the hypothesis that tubercle bacilli evolved from an environmental organism, such as *M. kansasii, via* a mammalian adapted semi-environmental ancestral taxon, such as *"M. canettii"*, parallel linked changes in the lipid components of the MOM can be discerned (Figure 11). A clear phenotypic link between *M. kansasii* and *"M. canettii"* is provided by clearly comparable "phenolic glycolipids" (PGLs) (Figure 4). The introduction of distinct mycolic acid

(Figure 1) and acyl trehalose (DATs, PATs, Figure 5) structures shows that *"M. canettii"* has an indisputable link with modern members of the *M. tuberculosis* complex (Figure 11). Refinement of the MOM lipid composition of *"M. canettii"*, leads to the simplified MOM free lipids of *M. tuberculosis*, comprising PDIMs, DATs, PATs (Figures 4,5) and the very characteristic sulfoglycolipids (SGLs, Figure 5). The apparent enhanced hydrophobicity of the cell envelope of *M. tuberculosis sensu stricto* correlates well with the ability to be transmitted in aerosols. In broad evolutionary terms, a plausible scenario involves various Pleistocene megafauna passaging environmental mycobacteria, such as *M. kansasii*, until horizontal gene transfer resulted in the diverse family of smooth morphology strains labelled *"M. canettii"*. Further evolutionary development, with possible human involvement for the first time, produced modern *M. tuberculosis* whose novel ability to fly in aerosols probably coincided with congregating settled humans multiplying together along with tubercle bacilli as new devastating companions.

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References

[1] Anderson RJ. Structural peculiarities of acid-fast bacterial lipids. Chemical Reviews 1941;29: 225-243.

[2] Asselineau J. The Bacterial Lipids. Paris: Hermann; 1966.

[3] Goren MB, Brennan PJ. Mycobacterial lipids: Chemistry and biologic activities. In: Tuberculosis. Youmans G.P. (ed.) Philadelphia USA: W.B. Saunders Company; 1979. p63-193.

[4] Minnikin, DE. Lipids: Complex lipids, their chemistry, biosynthesis and role. In: Ratledge C, Stanford J. (eds.) The Biology of Mycobacteria. London, UK:, Academic Press; 1982. p95-184.

[5] Daffé M, Draper P. The Envelope Layers of Mycobacteria with Reference to their Pathogenicity. Advances in Microbial Physiology 1998;39: 131-203.

[6] Brennan PJ, Nikaido H. The Envelope of Mycobacteria. Annual Reviews of Biochemistry 1995; 64: 29-63.

[7] Hoffmann C, Leis A, Niederweis M., Plitzko JM, Engelhardt H. Disclosure of the mycobacterial outer membrane: Cryo-electron tomography and vitreous sections reveal the lipid bilayer structure. PNAS 2008;105: 3963-3967.

[8] Zuber B, Chami M, Houssin C, Dubochet J, Griffiths G, Daffé M. Direct visualization of the outer membrane of mycobacteria and corynebacteria in their native state. Journal of Bacteriology 2008;190: 5672-5680.

[9] Daffé M, Zuber B. The Fascinating Coat Surrounding Mycobacteria. In: Remaut H., Fronzes R. (eds.) Bacterial Membranes: Structural and Molecular Biology. Linton, Cambridge UK: Caister Academic Press; 2014. p179-192.

[10] Daffé M, Etienne G. The capsule of *Mycobacterium tuberculosis* and its implications for pathogenicity. Tubercle and Lung Disease 1999:79: 153–169.

[11] Draper P, Daffé M. The Cell Envelope of *Mycobacterium tuberculosis* with Special Reference to the Capsule and Outer Permeability Barrier. In: Cole ST, Eisenach KD, McMurray DN, Jacobs WR. (eds.) Tuberculosis and the Tubercle Bacillus. Washington, DC: American Society for Microbiology; 2005. p287-305.

[12] Niederweis M. Mycobacterial Porins. In: Daffé M., Reyrat, J-M. (eds.) The Mycobacterial Cell Envelope. Washington, DC: American Society for Microbiology; 2008. p153-165.

[13] Kremer L, Besra GS. A Waxy Tale, by *Mycobacterium tuberculosis*. In: Cole ST, Eisenach KD, McMurray DN, Jacobs WR. (eds.) Tuberculosis and the Tubercle Bacillus. Washington, DC: American Society for Microbiology; 2005. p287-305.

[14] Verschoor JA, Baird MS, Grooten J. Towards understanding the functional diversity of cell wall mycolic acids of *Mycobacterium tuberculosis*. Progress in Lipid Research 2012;51: 325–339.

[15] Marrakchi H, Bardou F, Laneélle M, Daffé M. A comprehensive overview of mycolic acid structure and biosynthesis. In: Daffé M, Reyrat JM. (eds). The Mycobacterial Cell Envelope. Washington, DC: American Society for Microbiology; 2008. p41–62.

[16] Marrakchi H, Lanéelle M-A, Daffé M. Mycolic Acids: Structures, Biosynthesis, and Beyond. Chemistry & Biology 2014;21: 67- 85.

[17] Watanabe M, Aoyagi Y, Ridell M, Minnikin DE. Separation and characterization of individual mycolic acids in representative mycobacteria. Microbiology 2001;147: 1825–1837.

[18] Watanabe M., Aoyagi Y, Mitome H, Fujita T, Naoki H, Ridell M, Minnikin DE. Location of functional groups in mycobacterial meromycolate chains; the recognition of new structural principles in mycolic acids. Microbiology 2002;148: 1881-1902.

[19] McNeil M, Daffé M, Brennan PJ. Location of the Mycolyl Ester Substituents in the Cell Walls of Mycobacteria. Journal of Biological Chemistry 1991;266: 13217-13223.

[20] Villeneuve M., Kawai M, Kanashima H, Watanabe M, Minnikin DE, Nakahara H. Temperature dependence of the Langmuir monolayer packing of mycolic acids from *Mycobacterium tuberculosis*. Biochimica et Biophysica Acta Biomembranes 2005;1715: 71–80.

[21] Villeneuve M, Kawai M, Watanabe M, Aoyagi Y, Hitotsuyanagi Y, Takeya K, Gouda H, Hirono S, Minnikin DE, Nakahara H. Conformational behavior of oxygenated mycobacterial mycolic acids from *Mycobacterium bovis* BCG. Biochimica et Biophysica Acta Biomembranes 2007;1768: 1717–1726.

[22] Villeneuve M, Kawai M, Watanabe M, Aoyagi Y, Hitotsuyanagi Y, Takeya K, Gouda H, Hirono S, Minnikin DE, Nakahara H. Differential conformational behaviors of alpha-mycolic acids in Langmuir monolayers and computer simulations. Chemistry and Physics of Lipids 2010;163: 569–579.

[23] Villeneuve M, Kawai M, Horiuchi K, Watanabe M, Aoyagi Y, Hitotsuyanagi Y, Takeya K, Gouda H, Hirono S, Minnikin DE. Conformational folding of mycobacterial methoxy and ketomycolic acids facilitated by α-methyl *trans*-cyclopropane groups rather than *cis*-cyclopropane units. Microbiology 2013;159: 2405–2415.

[24] Groenewald W, Baird MS, Verschoor JA, Minnikin DE, Croft AK. Differential spontaneous folding of mycolic acids from *Mycobacterium tuberculosis*. Chemistry and Physics of Lipids 2014;180: 15–22.

[25] Moody DB, Reinhold BB, Guy MR, Beckman EM, Frederique DE, Furlong ST, Ye S, Reinhold VN, Sieling PA, Modlin RL, Besra GS, Porcelli SA. Structural requirements

for glycolipid antigen recognition by CD1b-restricted T cells. Science 1997;278: 283-286.

[26] Dobson G, Minnikin DE, Minnikin SM, Parlett JH, Goodfellow M, Ridell M, Magnusson M. Systematic analyses of complex mycobacterial lipids. In: Goodfellow M, Minnikin DE. (eds.) Chemical Methods in Bacterial Systematics. London, UK: Academic Press; 1985. p237- 265.

[27] Andersen CS, Agger EM, Rosenkrands I, Gomes JM, Bhowruth V, Gibson KJ, Petersen RV, Minnikin DE, Besra GS, Andersen P. A Simple Mycobacterial Monomycoloylated Glycerol Lipid has Potent Immunostimulatory Activity. Journal of Immunology 2009; 182: 424-432.

[28] Kremer L, de Chastellier C, Dobson G, Gibson KJ, Bifani P, Balor S, Gorvel J-P, Locht C, Minnikin DE, Besra GS. Identification and structural characterization of an unusual mycobacterial monomeromycoloyldiacylglycerol. Molecular Microbiology 2005;57: 1113–1126.

[29] Rafidinarivo E, Lanéelle M-A, Montrozier H, Valero-Guillén P, Astola J, Luquin M, Promé J-C, Daffé M. Trafficking pathways of mycolic acids: structures, origin, mechanism of formation, and storage form of mycobacteric acids. Journal of Lipid Research 2009;50: 477–490.

[30] Garton NJ, Christensen H, Minnikin DE, Adegbola RA, Barer MR. Intracellular lipophilic inclusions of mycobacteria *in vitro* and in sputum. Microbiology 2002;148: 2951–2958.

[31] Bansal-Mutalik R, Nikaido H. Mycobacterial outer membrane is a lipid bilayer and the inner membrane is unusually rich in diacyl phosphatidylinositol dimannosides. PNAS 2014;111: 4958-4963.

[32] Minnikin DE, Dobson G, Goodfellow M, Magnusson M, Ridell M. Distribution of Some Mycobacterial Waxes Based on the Phthiocerol Family. Journal of General Microbiology 1985;131: 1375-1381.

[33] Daffé M, Lanéelle M-A. Distribution of Phthiocerol Diester, Phenolic Mycosides and Related Compounds in Mycobacteria. Journal of General Microbiology 1988;134: 2049-2055.

[34] Minnikin DE, Kremer L, Dover LG, Besra GS. The methyl-branched fortifications of *Mycobacterium tuberculosis*. Chemistry and Biology 2002; 9: 545-553.

[35] Onwueme KC, Cheryl J, Vos CJ, Zurita J, Ferreras JA, Quadri LEN. The dimycocerosate ester polyketide virulence factors of mycobacteria. Progress in Lipid Research 2005;44: 259–302.

[36] Guilhot C, Chalut C, Daffé M. Biosynthesis and Roles of Phenolic Glycolipids and Related Molecules in *Mycobacterium tuberculosis*. In: Daffé M, Reyrat J-M. (eds.) The

Mycobacterial Cell Envelope. Washington, DC: American Society for Microbiology; 2008. p273-289.

[37] Huet SG, Constant C, Malaga W, Lanéelle M-A, Kremer K, van Soolingen D, Daffé M, Guilhot C. A lipid profile typifies the Beijing strains of *Mycobacterium tuberculosis*. Identification of a mutation responsible for a modification of the structures of phthiocerol dimycocerosates and phenolic glycolipids. Journal of Biological Chemistry 2009;284: 27101–27113.

[38] Ridell M, Wallerström G, Minnikin DE, Bolton RC, Magnusson M. A comparative serological study of antigenic glycolipids from *Mycobacterium tuberculosis*. Tubercle and Lung Disease 1992;73: 101-105.

[39] Jackson M, Stadthagen G, Gicquel B. Long-chain multiple methyl-branched fatty acid-containing lipids of *Mycobacterium tuberculosis*: Biosynthesis, transport, regulation and biological activities. Tuberculosis 2007;87: 78–86.

[40] Daffé M, Lacave C, Lanéelle M-A, Gillois M, Lanéelle G. Polyphthienoyl trehalose, glycolipids specific for virulent strains of the tubercle bacillus. European Journal of Biochemistry 1988;172: 579-584.

[41] Bertozzi CR, Schelle MW. Sulfated Metabolites from *M. tuberculosis*: Sulfolipid-1 and Beyond. In: Daffé M, Reyrat J-M. (eds.) The Mycobacterial Cell Envelope. Washington, DC: American Society for Microbiology; 2008. p291-304.

[42] Daffé M, McNeil M, Brennan PJ. Novel Type-Specific Lipooligosaccharides from *Mycobacterium tuberculosis*. Biochemistry 1991;30: 378-388.

[43] Gilleron M, Puzo G. Lipooligosaccharidic antigens from *Mycobacterium kansasii* and *Mycobacterium gastri*. Glycoconjugate Journal 1995;12: 298-308.

[44] Ren H, Dover LG, Islam ST, Alexander DC, Chen JM, Besra GS, Liu J. Identification of the lipooligosaccharide biosynthetic gene cluster from *Mycobacterium marinum*. Molecular Microbiology 2007;63: 1345-1359.

[45] Belisle JT, Brennan PJ. Chemical basis of rough and smooth variation in mycobacteria. Journal of Bacteriology 1989;171: 3465-3470.

[46] Gilleron M, Quesniaux VFJ, Puzo G. Acylation State of the Phosphatidylinositol Hexamannosides from *Mycobacterium bovis* Bacillus Calmette Guérin and *Mycobacterium tuberculosis* H37Rv and Its Implication in Toll-like Receptor Response. Journal of Biological Chemistry 2003; 278: 29880-29889.

[47] Gilleron M, Ronet C, Mempel M, Monsarrat B, Gachelin G, Puzo G. Acylation state of the phosphatidylinositol mannosides from *Mycobacterium bovis* bacillus Calmette Guérin and ability to induce granuloma and recruit natural killer T cells. Journal of Biological Chemistry 2001;276: 34896-34904.

[48] Guerin ME, Korduláková J, Alzari PM, Brennan PJ, Jackson M. Molecular Basis of Phosphatidyl-myo-inositol Mannoside Biosynthesis and Regulation in Mycobacteria. Journal of Biological Chemistry 2010;285: 33577-33583.

[49] Morita YS, Fukuda T, Sena CBC, Yamaryo-Botte Y, McConville MJ, Kinoshita T. Inositol lipid metabolism in mycobacteria: Biosynthesis and regulatory mechanisms. Biochimica et Biophysica Acta 2011;1810: 630–641.

[50] Nigou J, Gilleron M, Puzo G. Lipoarabinomannans: from structure to biosynthesis. Biochimie 2003;85: 153-166.

[51] Mishra AK, Driessen NN, Appelmelk BJ, Besra GS. Lipoarabinomannan and related glycoconjugates: structure, biogenesis and role in *Mycobacterium tuberculosis* physiology and host-pathogen interaction. FEMS Microbiology Reviews 2011;35: 1126–1157.

[52] Kaur D, Guerin ME, Škovierová H, Brennan PJ, Jackson M. Biogenesis of the cell wall and other glycoconjugates of *Mycobacterium tuberculosis*. Advances in Applied Microbiology 2009;69: 23-78.

[53] Jankute M, Grover S, Rana AK, Besra GS. Arabinogalactan and lipoarabinomannan biosynthesis: structure, biogenesis and their potential as drug targets. Future Microbiology 2012;7: 129-147.

[54] Jankute M, Grover S, Birch HL, Besra GS. Genetics of Mycobacterial Arabinogalactan and Lipoarabinomannan Assembly. In: Hatfull GF, Jacobs WR. (eds.) Molecular Genetics of Mycobacteria, 2nd Edition. Washington, DC: American Society for Microbiology; 2014. p535-557.

[55] Barry CE, Crick DC, McNeil MR. Targeting the formation of the cell wall core of *M. tuberculosis*. Infectious Disorders and Drug Targets 2007;7: 182-202.

[56] Crick DC, Brennan PJ. Biosynthesis of the Arabinogalactan-Peptidoglycan Complex of *Mycobacterium tuberculosis*. In: Daffé M., Reyrat, J-M. (eds.) The Mycobacterial Cell Envelope. Washington, DC: American Society for Microbiology; 2008. p25-40.

[57] Crick DC, Chatterjee D, Scherman MS, McNeil MR. Structure and Biosynthesis of the Mycobacterial Cell Wall. Comprehensive Natural Products II: Chemistry and Biology 2010;6: 381–406. DOI: 10.1016/B978-008045382-8.00173-8.

[58] Daffé M. The Global Architecture of the Mycobacterial Cell Envelope. In: Daffé M., Reyrat, J-M. (eds.) The Mycobacterial Cell Envelope. Washington, DC: American Society for Microbiology; 2008. p3-11.

[59] Turner RD, Vollmer W, Foster SJ. Different walls for rods and balls: the diversity of peptidoglycan. Molecular Microbiology 2014;91: 862-874.

[60] Meroueh SO, Bencze KZ, Hesek D, Lee M, Fisher JD, Timothy L. Stemmler TL, Mobashery S. Three-dimensional structure of the bacterial cell wall peptidoglycan. PNAS 2006;103: 4404–4409.

[61] Dmitriev BA, Ehlers S, Rietschel ET, Brennan PJ. Molecular mechanics of the mycobacterial cell wall: from horizontal layers to vertical scaffolds. International Journal of Medical Microbiology 2000;290: 251-258.

[62] Dmitriev B, Toukach F, Ehlers S. Towards a comprehensive view of the bacterial cell wall. Trends in Microbiology 2005;13: 569-574.

[63] Kirschner KN, Yongye AB, Tschampel SM, Daniels CR, Foley BL, Woods RJ. GLYCAM06: A Generalizable Biomolecular Force Field. Carbohydrates. Journal of Computational Chemistry 2008;29: 622-655.

[64] Barksdale L, Kim K-S. *Mycobacterium*. Bacteriological Reviews 1977; 41: 217–372.

[65] Christensen H, Garton NJ, Horobin RW, Minnikin DE, Barer MR. Lipid domains of mycobacteria studied with fluorescent molecular probes. Molecular Microbiology 1999;31: 1561–1572.

[66] Sprott GD, Dicaire CJ, Gurnani K, Sad S, Krishnan L. Activation of Dendritic Cells by Liposomes Prepared from Phosphatidylinositol Mannosides from *Mycobacterium bovis* Bacillus Calmette-Guérin and Adjuvant Activity *in vivo*. Infection and Immunity 2004;72: 5235–5246.

[67] Korduláková J, Gilleron M, Mikusova K, Puzo G, Brennan PJ, Gicquel B, Jackson, M. Definition of the First Mannosylation Step in Phosphatidylinositol Mannoside Synthesis. Journal of Biological Chemistry 2002;277: 31335–31344.

[68] Guerin ME, Kaur D, Somashekar BS, Gibbs S, Gest P, Chatterjee D, Brennan PJ, Jackson M. New insights into the early steps of phosphatidylinositol mannoside biosynthesis in mycobacteria: PimB' is an essential enzyme of *Mycobacterium smegmatis*. Journal of Biological Chemistry 2009;284: 25687–25696.

[69] Driessen NN, Ummels R, Maaskant JJ, Gurcha SS, Besra GS, Ainge GD, Larsen DS, Painter GF, Vandenbroucke-Grauls CM, Geurtsen J, Appelmelk BJ. Role of phosphatidylinositol mannosides in the interaction between mycobacteria and DC-SIGN. Infection and Immunity 2009;77: 4538–4547.

[70] Korduláková J, Gilleron M, Puzo G, Brennan PJ, Gicquel B, Mikusová K, Jackson M. Identification of the required acyltransferase step in the biosynthesis of the phosphatidylinositol mannosides of *Mycobacterium* species. Journal of Biological Chemistry 2003;278: 36285–36295.

[71] Gilleron M, Jackson M, Nigou J, Puzo G. Structure, Biosynthesis and Activities of the Phosphatidyl-*myo*-Inositol-based Lipoglycans. In: Daffé M, Reyrat, J-M. (eds.) The Mycobacterial Cell Envelope. Washington, DC: American Society for Microbiology; 2008. p75-105.

[72] Pitarque S, Larrouy-Maumus G, Payré B, Jackson M, Puzo G, Nigou J. The immunomodulatory lipoglycans, lipoarabinomannan and lipomannan, are exposed at the mycobacterial cell surface. Tuberculosis 2008;88: 560-565.

[73] Dhiman RK, Dinadayala P, Ryan GJ, Lenaerts AJ, Schenkel AR, Crick DC. Lipoarabinomannan Localization and Abundance during Growth of *Mycobacterium smegmatis*. Journal of Bacteriology 2011;193: 5802-5809.

[74] Alsteens D, Verbelen C, Dague E, Raze D, Baulard AR, Dufrêne YF. Organization of the mycobacterial cell wall: a nanoscale view. Pflugers Arch- European Journal of Physiology 2008;456: 117-125.

[75] Marchand CH, Salmeron C, Bou Raad R, Méniche X, Chami M, Masi M, Blanot D, Daffé M, Tropis M, Huc E, Le Maréchal P, Decottignies P, Bayan N. Biochemical Disclosure of the Mycolate Outer Membrane of *Corynebacterium glutamicum.* Journal of Bacteriology 2012;194: 587-597.

[76] Sani M, Houben ENG, Geurtsen J, Pierson J, de Punder K, van Zon M, Wever B, Piersma SR, Jiménez CR, Daffé M, Appelmelk BJ, Bitter W, van der Wel1 N, Peters PJ. Direct Visualization by Cryo-EM of the Mycobacterial Capsular Layer: A Labile Structure Containing ESX-1-Secreted Proteins. PLoS Pathogens 2010;6: e1000794.

[77] Bhamidi S, Scherman MS, Jones V, Crick DC, Belisle JT, Brennan PJ, McNeil MR. Detailed structural and quantitative analysis reveals the spatial organization of the cell walls of *in vivo* grown *Mycobacterium leprae* and *in vitro* grown *M. tuberculosis*. Journal of Biological Chemistry 2011;286: 23168-23177.

[78] Dover LG, Cerdeño-Tárraga AM, Pallen MJ, Parkhill J, Besra GS. Comparative cell wall core biosynthesis in the mycolated pathogens, *Mycobacterium tuberculosis* and *Corynebacterium diphtheriae*. FEMS Microbiology Reviews 2004:28: 225–250.

[79] Minnikin DE, Lee OY-C, Wu HHT, Besra GS, Bhatt A, Nataraj V, Rothschild BM, Spigelman M, Donoghue HD. Ancient mycobacterial lipids: Key reference biomarkers in charting the evolution of tuberculosis. Tuberculosis 2015;95: S133-S139.

[80] Veyrier F, Pletzer D, Turenne C, Behr MA. Phylogenetic detection of horizontal gene transfer during the step-wise genesis of *Mycobacterium tuberculosis*. BMC Evolutionary Biology 2009;9: 196.

[81] Veyrier FJ, Dufort A, Behr MA. The rise and fall of the *Mycobacterium tuberculosis* genome. Trends in Microbiology 2011;19: 156-161.

[82] Bottai D, Stinear TP, Supply P, Brosch R. Mycobacterial Pathogenomics and Evolution. In: Hatfull GF, Jacobs WR. (eds.) Molecular Genetics of Mycobacteria, 2nd Edition. Washington, DC: American Society for Microbiology; 2014. p27-47.

[83] Smith NH, Hewinson RG, Kremer K, Brosch R, Gordon SV. Myths and misconceptions: the origin and evolution of *Mycobacterium tuberculosis*. Nature Reviews in Microbiology 2009;7: 537-544.

[84] Supply P, Marceau M, Mangenot S, Roche D, Rouanet C, Khanna V, Majlessi L, Criscuolo A, Tap J, Pawlik A, Fiette L, Orgeur M, Fabre M, Parmentier C, Frigui W, Simeone R, Boritsch EC, Debrie AS, Willery E, Walker D, Quail MA, Ma L, Bouchier C,

Salvignol G, Sayes F, Cascioferro A, Seemann T, Barbe V, Locht C, Gutierrez MC, Leclerc C, Bentley SD, Stinear TP, Brisse S, Médigue C, Parkhill J, Cruveiller S, Brosch R. Genomic analysis of smooth tubercle bacilli provides insights into ancestry and pathoadaptation of *Mycobacterium tuberculosis*. Nature Genetics 2013;45: 172-179.

[85] Fabre M, Hauck Y, Soler C, Koeck J-L, van Ingen J, van Soolingen D, Vergnaud G, Pourcel C. Molecular characteristics of ''*Mycobacterium canettii*'' the smooth *Mycobacterium tuberculosis* bacilli. Infection, Genetics and Evolution 2010;10: 1165–1173.

[86] Koeck J-L, Fabre M, Simon F, Daffé M, Garnotel E, Matan AB, Gérôme P, Bernatas JJ, Buisson Y, Pourcel C. Clinical characteristics of the smooth tubercle bacilli '*Mycobacterium canettii*' infection suggest the existence of an environmental reservoir. Clinical Microbiology and Infection 2011;17: 1013–1019.

[87] Gutierrez MC, Brisse S, Brosch R, Fabre M, Omaïs B, Marmiesse M, Supply P, Vincent V. Ancient origin and gene mosaicism of the progenitor of *Mycobacterium tuberculosis*. PLoS Pathogens 2005;1(1): e5.

[88] Gordon SV, Bottai D, Simeone R, Stinear TP, Brosch R. Pathogenicity in the tubercle bacillus: molecular and evolutionary determinants. BioEssays 2009;31: 378–388.

[89] Soto CY, Cama M, Gibert I, Luquin M. Application of an easy and reliable method for sulfolipid-I detection in the study of its distribution in *Mycobacterium tuberculosis* strains. FEMS Microbiology Letters 2000;187: 103-107.

[90] Rousseau C, Turner OC, Rush E, Bordat Y, Sirakova TD, Kolattukudy PE, Ritter S, Orme IM, Gicquel B, Jackson M. Sulfolipid Deficiency Does Not Affect the Virulence of *Mycobacterium tuberculosis* H37Rv in Mice and Guinea Pigs. Infection and Immunity 2003; 71: 4684–4690.

[91] Rousseau C, Neyrolles O, Bordat Y, Giroux S, Sirakova TD, Prevost M-C, Kolattukudy PE, Gicquel B, Jackson M. Deficiency in mycolipenate- and mycosanoate-derived acyltrehaloses enhances early interactions of *Mycobacterium tuberculosis* with host cells. Cellular Microbiology 2003;5: 405–415.

[92] Falkinham JO. Mycobacterial Aerosols and Respiratory Disease. Emerging Infectious Diseases 2003;9: 763-767.

[93] Cangelosi GA, Palermo CO, Laurent J-P, Hamlin AM, Brabant WH. Colony morphotypes on Congo red agar segregate along species and drug susceptibility lines in the *Mycobacterium avium-intracellulare* complex. Microbiology 1999;145: 1317-1324.

[94] Stokes RW, Norris-Jones R, Brooks DE, Beveridge TJ, Doxsee D, Thorson LM. The Glycan-Rich Outer Layer of the Cell Wall of *Mycobacterium tuberculosis* Acts as an Antiphagocytic Capsule Limiting the Association of the Bacterium with Macrophages. Infection and Immunity 2004;72: 5676–5686.

[95] Jönsson B, Gilljam M, Lindblad A, Ridell M, Wold AE, Welinder-Olsson C. Molecular epidemiology of *Mycobacterium abscessus*, with focus on cystic fibrosis. Journal Clinical Microbiology 2007;45: 1497–1504.

[96] Jönsson B, Ridell M, Wold AE. Phagocytosis and cytokine response to rough and smooth colony variants of *Mycobacterium abscessus* by human peripheral blood mononuclear cells. APMIS 2013;121: 45–55.

[97] Bendinger B, Rijnaarts HHM, Altendorf K, Zehnder AJB. Physicochemical Cell Surface and Adhesive Properties of Coryneform Bacteria Related to the Presence and Chain Length of Mycolic Acids. Applied and Environmental Microbiology 1993;59: 3973-3977.

[98] Hershkovitz I, Donoghue HD, Minnikin DE, Besra GS, Lee OY-C, Gernaey AM, Galili E, Eshed V, Greenblatt CL, Lemma E, Kahila Bar-Gal G, Spigelman M. Detection and molecular characterization of 9000-year-old *Mycobacterium tuberculosis* from a Neolithic settlement in the Eastern Mediterranean. PLoS ONE 2008;3: e3426.

[99] Lee OY-C, Wu HHT, Donoghue HD, Spigelman, M, Greenblatt CL, Bull ID, Rothschild, BM, Martin, LD, Minnikin, DE, Besra, GS. *Mycobacterium tuberculosis* Complex Lipid Virulence Factors Preserved in the 17,000-Year-Old Skeleton of an Extinct Bison, *Bison antiquus*. PLoS ONE 2012;7: e41923.

[100] Rothschild BM, Martin LD. Did ice-age bovids spread tuberculosis? Naturwissenschaften 2006;93: 565-569.

[101] Rothschild BM, Laub R. Hyperdisease in the late Pleistocene: validation of an early 20th century hypothesis. Naturwissenschaften 2006;93: 557–564.

[102] Minnikin DE, Lee OY-C, Wu HHT, Besra GS, Donoghue HD. Molecular biomarkers for ancient tuberculosis. In: Cardona P-J. (ed.) Understanding Tuberculosis – Deciphering the Secret Life of the Bacilli. Rijeka, Croatia.: InTech -Open Access Publisher. 2012. p. 1-36. http://www.intechopen.com/books/understanding tuberculosis-deciphering-the-secret-life-of-the-bacilli

[103] Rothschild BM, Martin LD, Lev G, Bercovier H, Bar-Gal GK, Greenblatt C, Donoghue H, Spigelman M, Brittain D. *Mycobacterium tuberculosis* complex DNA from an extinct bison dated 17,000 years BP. Clinical and Infectious Diseases 2001;33: 305–311.

[104] Jang J, Becq J, Gicquel B, Deschavanne P, Neyrolles O. Horizontally acquired genomic islands in the tubercle bacilli. Trends in Microbiology 2008;16: 303-308.

[105] Higham T, Douka K, Wood R, Ramsey CB, Brock F, Basell L, Camps M, Arrizabalaga A, Baena J, Barroso-Ruíz C, Bergman C, Boitard C, Boscato P, Caparrós M, Conard NJ, Draily C, Froment A, Galván B, Gambassini P, Garcia-Moreno A, Grimaldi S, Haesaerts P, Holt B, Iriarte-Chiapusso MJ, Jelinek A, Jordá Pardo JF, Maíllo-Fernández JM, Marom A, Maroto J, Menéndez M, Metz L, Morin E, Moroni A, Negrino F, Panagopoulou E, Peresani M, Pirson S, de la Rasilla M, Riel-Salvatore J, Ronchitelli A, Santamaria D, Semal P, Slimak L, Soler J, Soler N, Villaluenga A, Pinhasi R, Jacobi

R. The timing and spatiotemporal patterning of Neanderthal disappearance. Nature 2014;512: 306-309.

[106] Djelouadji Z, Raoult D, Drancourt M. Palaeogenomics of *Mycobacterium tuberculosis*: epidemic bursts with a degrading genome. Lancet Infectious Diseases 2011;11: 641–650.

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