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Mycobacterial outer membranes: in search of proteins

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Summary

The cell wall is a major virulence factor of *Mycobacterium tuberculosis* and contributes to its intrinsic drug resistance. Recently, cryo-electron microscopy showed that the mycobacterial cell wall lipids form an unusual outer membrane. Identification of the components of the uptake and secretion machinery across this membrane is critical for understanding the physiology and pathogenicity of *M. tuberculosis* and for the development of better anti-tuberculosis drugs. Although the genome of *M. tuberculosis* appears to encode over 100 putative outer membrane proteins, only a few have been identified and characterized. Here, we summarize the current knowledge on the structure of the mycobacterial outer membrane and its known proteins. Through comparison to transport processes in Gram-negative bacteria, we highlight several hypothetical outer membrane proteins of *M. tuberculosis* awaiting discovery.

Mycobacteria have a complex cell envelope

Scientific interest in mycobacteria has been sparked by the medical importance of *Mycobacterium tuberculosis* and by properties that distinguish them from other microorganisms. In particular, mycobacteria possess a remarkably complex cell envelope consisting of a cytoplasmic membrane and a cell wall, which constitutes an efficient permeability barrier and plays a crucial role in the intrinsic drug resistance and in survival under harsh conditions [1].

These microbes produce a fascinating diversity of lipids [1,2] such as the mycolic acids, exceptionally long fatty acids that account for 30% to 40% of the cell envelope mass [3,4]. Mycolic acids are covalently linked to peptidoglycan via an arabinogalactan polymer, a polysaccharide composed of arabinose and galactose subunits. In a typical arrangement, the peptidoglycan network is substituted by linear galactan molecules, which bear several branched arabinose chains [1,2]. These branches end in four arabinose dimers, each forming the head group for two mycolic acid molecules.

The mycolic acid-arabinogalactan-peptidoglycan polymer is arranged to form a hydrophobic layer with other lipids and the cytoplasmic membrane [5,6]. Recently, a model describing the

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complete primary structure of this complex cell wall has been published [7]. The observation of pore proteins in the mycobacterial cell wall [8,9] and their structural analysis [10] supported the model that the cell wall lipids are organized in an outer membrane despite the classification of mycobacteria as Gram-positive bacteria [11,12]. Tremendous progress has been recently made to elucidate the organization of lipids in the mycobacterial cell wall and to identify pore proteins that functionalize this unique compartment. These results have far-reaching implications for the physiology and virulence of *M. tuberculosis* and are reviewed here.

The mycobacterial outer membrane

In 1982, Minnikin proposed that mycobacteria have a second lipid bilayer formed by an inner leaflet of mycolic acids (covalently bound to the peptidoglycan) and an outer leaflet of free lipids [2]. This proposal was the basis for a variety of models, suggesting an asymmetric outer membrane-like lipid layer of exceptional thickness (≥10 nm) [1,13,14]. Although freeze-fracture experiments supported the existence of this second membrane [15], electron microscopy of ultrathin sections failed to demonstrate the lipid bilayer structure, which was readily observed for the cytoplasmic membrane [16,17].

A breakthrough was achieved by the use of cryo-electron tomography (CET) [18] and electron microscopy of ultrathin cryosections [18,19], techniques that abstain from harsh chemical treatment of biological samples (Box 1). CET revealed the native three-dimensional organization of the cell envelope of *Mycobacterium smegmatis* and *Mycobacterium bovis* BCG and disclosed the bilayer structure of the outer membrane (Figure 1). While the lipopolysaccharide-containing outer membrane of Gram-negative bacteria consists of leaflets of different thicknesses [20], cryo-electron microscopy showed that the mycobacterial outer membrane is approximately 8 nm thick and is morphologically symmetrical. This finding, in combination with the observation that the mild detergent octyl β-glucoside permeabilizes the outer membrane of *M. smegmatis*, suggests that free lipids with heterogeneous head groups are distributed over both leaflets in the mycobacterial outer membrane and are not restricted to the outer leaflet alone [18], in contrast to all previous models.

Cryo-electron microscopy images do not give clues about the conformation of mycolic acids in the outer membrane. Mycolic acids consist of up to 90 carbon atoms, forming a long branch, called meromycolate, and a shorter α-branch (Figure 2a). Meromycolate contains cyclopropane rings, substitutions, or double bonds that are characteristic for mycobacteria [21]. However, the conformation of mycolic acids in the outer membrane is not known. If meromycolate had an elongated conformation [5, 22], it would span the complete hydrophobic matrix, leaving space for free lipids to intercalate (model I in Figure 2b). Results of monolayer experiments and simulation data are indeed consistent with a folded conformation for meromycolate (model II in Figure 2b) at moderate temperature and low lateral membrane pressure, with kinks at positions of cis double bonds or trans cyclopropane rings [23, 24]. This conformation might be stabilized by keto or methoxy groups that were recently proposed to interact with lipid head groups [19]. A sketch of a folded keto-mycolic acid is depicted in Figure 2a and shown as part of the mycobacterial outer membrane in Figure 2b (model II). However, direct experimental evidence for the conformation of mycolic acids *in situ* does not exist.

The outer membrane organization represented by these two models, as well as the structures of mycolic acids, are likely functionally important. Mutations in mycobacteria that either affect modifications [25-27] or length of the mycolic acids [28] result in phenotypes with altered colony morphology or persistence in host cells, or in a labile outer-membrane structure with changed permeability properties [29]. While mutations that lead to a complete loss of mycolic acids are lethal to mycobacteria, the corresponding mutants of closely related corynebacteria are viable [30]. Cryo-electron microscopy of a mycolic-acid deficient mutant of

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Corynebacterium glutamicum showed that the outer membrane bilayer is missing [18,19]. The cell envelope of this mutant is also more permeable to drugs [31], highlighting the significance of the outer membrane as a permeability barrier.

These observations define the periplasmic space and the outer membrane as two new subcellular compartments [32,33] in mycobacteria (Figure 2c). The arabinogalactan-peptidoglycan polymer resides within the periplasm and likely corresponds to the layered but still unidentified structures in the cryo-electron microscopy images (Figure 1). Of importance for the physiology of mycobacteria is how these two compartments are functionalized for uptake and secretion processes. For example, Gram-negative bacteria such as *Escherichia coli* employ more than 60 outer membrane proteins [34], the majority of which form channels to enable transport across the outer membrane [35]. The genome of *M. tuberculosis* appears to encode more than 140 putative outer membrane proteins [36]. However, only very few have been identified and characterized thus far.

The porin pathway across mycobacterial outer membranes

While hydrophobic compounds can penetrate membranes by temporarily dissolving in the lipid bilayer, direct diffusion of water-soluble compounds across any lipid bilayer is too slow to support bacterial growth. Thus, uptake of most hydrophilic solutes across the mycobacterial outer membrane likely requires some kind of transport proteins. A strong argument in favor of this hypothesis is provided by the existence of porins such as MspA in mycobacteria. Porins are defined as non-specific protein channels in bacterial outer membranes which enable the influx of hydrophilic solutes [35]. MspA was discovered as the major porin [9] and the most abundant protein of *M. smegmatis* [37]. Deletion of *mspA* reduced the outer membrane permeability towards glucose [38], phosphate [39], metal ions and amino acids [40], indicating that MspA represents the major general diffusion pathway in *M. smegmatis*. The loss of several Msp porins reduced the growth rate of *M. smegmatis* [39,40], indicating that the influx of hydrophilic nutrients through porins is required for normal growth.

The requirement for fast nutrient uptake is likely not as stringent for *M. tuberculosis*, with a generation time of 24 hours compared to 3 hours for *M. smegmatis*. This might be the reason why *M. tuberculosis* does not have MspA homologues [11]. To assess the role of the porin pathway in *M. tuberculosis* and *M. bovis* BCG, the *mspA* gene of *M. smegmatis* was expressed in these species [41,42]. Even a very low number of MspA pores increased glucose uptake and accelerated growth of *M. bovis* BCG [41]. Further, both *M. bovis* BCG and *M. tuberculosis* became more susceptible to β-lactam antibiotics, isoniazid, ethambutol and streptomycin. These results indicate that the very low efficiency and/or low number of endogenous pores contribute to the slow nutrient uptake and the intrinsic resistance of these organisms to drugs.

Nevertheless, pore-forming proteins have been detected in *M. tuberculosis* and *M. bovis* BCG [43-46], further supporting that diffusion across the lipid bilayer of the outer membrane is too slow, at least for some solutes. The C-terminal domain of one of these pore-forming proteins, OmpATb (Rv0899), has weak similarity to outer membrane proteins of the OmpA family of Gram-negative bacteria. Purified recombinant OmpATb was shown to form channels in lipid membranes [43,47], and uptake of serine (but not of glycine) was reduced in the mutant of *M. tuberculosis* lacking *ompATb* [48]. However, at pH 5.5, a 30-fold increase in transcription of *ompATb* was associated with decreased permeability of *M. tuberculosis* to serine [48]. Considering these conflicting results, it appears doubtful that the primary function of OmpATb in *M. tuberculosis* is that of a major porin [11].

Another porin candidate, Rv1698, was identified as an outer membrane protein of *M. tuberculosis* with channel activity [36,46]. Heterologous expression of *rv1698* in an *M. smegmatis* porin mutant partially complemented the permeability defects of the strain.

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However, recent experiments with *M. tuberculosis* and *M. smegmatis* mutants show that facilitation of nutrient uptake is not the physiological function of the Rv1698 pore, but rather an overexpression artifact (Wolschendorf and Niederweis, unpublished). Thus, despite long-term efforts by several groups, a doubtless identification of *M. tuberculosis* porins that mediate uptake of small, hydrophilic solutes across the outer membrane has not yet been achieved. Further studies of pore-forming proteins of this bacterium would provide important clues for its physiology by revealing which solutes utilize these pathways. In addition, porins play a major role in antibiotic uptake in *M. smegmatis* [49] and Gram-negative bacteria [35]. Hence, functional and structural data might also indicate how these pores could be exploited to more efficiently transport drugs into the cell and improve anti-tuberculosis chemotherapy.

Structure of mycobacterial outer membrane proteins

MspA is the only mycobacterial protein whose crystal structure has been solved [10]. The structure has proven to be of immense value not only as a paradigm for a new class of proteins, but also for understanding the function of MspA [50], for elucidating its membrane topology [12], and for applications in nanotechnology [51-53]. The porin has an octameric goblet-like conformation with a single central channel 10 nm in length (Figure 3). This structure is different from that of trimeric porins in Gram-negative bacteria which have one pore per monomer and are approximately 5 nm long [54]. The constriction zone of the octameric MspA channel consists of 16 aspartate residues (D90/D91) creating a high density of negative charges [10], which likely explain the cation preference of this porin [9]. Due to its novel protein architecture, MspA became the founding member of a new class of outer membrane proteins which has more than 30 homologues in mycobacteria [55]. More high-resolution structures are needed to identify unique characteristics of mycobacterial outer membrane proteins as well as features that are shared with proteins of Gram-negative bacteria.

Energy-dependent uptake of nutrients across outer membranes

Despite its important role in the uptake of some hydrophilic nutrients, the porin pathway is not efficient enough for (i) solutes of very low abundance (below 1 μM), such as iron, because small concentration gradients result in very low diffusion rates; and (ii) large solutes such as vitamin B_{12} exceed the size exclusion limit of most porin channels. Hence, uptake of these solutes across the outer membrane of Gram-negative bacteria requires active transport [56]. Substrates of energy-dependent transport systems bind with high affinity to surface receptors, many with dissociation constants in the sub-nanomolar range. In *E. coli*, energy is provided by the inner membrane complex ExbBD via the periplasmic protein TonB to multiple outer membrane receptors [56].

Iron is highly limited to bacterial pathogens due to sequestration by the host [57] and, therefore, needs to be acquired by active transport. This is achieved by high affinity siderophores which are specifically taken up by outer membrane receptor proteins in Gram-negative bacteria [58]. *E. coli* contains three major independent siderophore receptors in the outer membrane: FhuA, FepA and FecA. Upon binding an iron-loaded siderophore, energy transferred by TonB initiates structural rearrangements in the transporter, releasing the siderophore into the periplasm where a substrate binding protein shuttles it to a specific transporter of the ATP-binding cassette transporter family in the inner membrane [56,58].

What is the situation in mycobacteria? *M. tuberculosis* produces two salicylate-derived siderophores. The more polar carboxymycobactin is released into the medium, whereas the less polar mycobactin remains associated with the cell [59]. In this bacterium, an ABC transporter composed of the proteins IrtA and IrtB is required for export of siderophores across the inner membrane [60]. Uptake of iron-loaded carboxymycobactin is not energy-dependent [61], leading to the hypothesis that siderophores might diffuse through porins [59]. However,

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in other microbes such as *E. coli*, the concentration of extracellular iron-loaded siderophores is not high enough to support passive diffusion during infection [62]. Computer models suggest that even the smaller siderophore exochelin of *M. smegmatis* [63] in its iron-bound form appears to be too large to pass through the MspA pore (Jones and Niederweis, unpublished). This argues for the existence of energy-dependent receptors in mycobacteria for active uptake of iron-loaded siderophores across the outer membrane. Uptake of vitamin B_{12} [64] and other scarce or large solutes provides a similar challenge for *M. tuberculosis* and probably also requires outer membrane receptors.

Uptake of hydrophobic compounds across outer membranes

Nikaido and co-workers have shown that diffusion rates through the water-filled channels of porins drop drastically with increasing solute hydrophobicity [35]. Both direct diffusion of anionic fatty acids through lipid membranes [65] and an alternative 'flip-flop' movement of protonated fatty acids through membranes [66] are slow. These findings explain why bacteria and eukaryotes have evolved proteins for fatty acid uptake across membranes [67]. For example, the outer membrane protein FadL mediates energy-independent uptake of fatty acids by *E. coli* [68].

Considerable circumstantial evidence suggests that *M. tuberculosis* uses lipids as a carbon source after the onset of the adaptive immune response in mice [69-72]. However, no FadL homologue is apparent in mycobacteria, and the mechanism by which fatty acids cross the mycobacterial outer membrane is unknown. Identification of an outer membrane fatty-acid transporter will be important for understanding the physiology of *M. tuberculosis* in the human host and might shed light on the types of lipids used by this microbe. In addition to fatty acids, cholesterol is another hydrophobic compound which appears to be used by *M. tuberculosis* as a carbon source [73-75]. Interestingly, the *mce4* operon is required for efficient uptake of cholesterol [75] and encodes some proteins (Mce4A, Mce4B, Mce4C, Mce4D and Mce4F) that have been proposed to be outer membrane proteins, based on secondary structure predictions and other characteristics [36]. They might form an outer membrane channel to enable cholesterol to enter the cell.

Efflux processes

M. tuberculosis is intrinsically resistant to many antibiotics due to the formidable permeability barrier established by the outer membrane, in synergy with other resistance mechanisms such as multi-drug efflux [76]. Considering that its genome encodes 69 putative drug efflux pumps [77], it is not surprising that all current tuberculosis drugs are substrates of efflux. In Gram-negative bacteria, only efflux across both membranes is an effective resistance mechanism [78], and we expect a similar situation to occur in mycobacteria. The major drug efflux system of *E. coli* is a tripartite pump consisting of an inner-membrane transporter protein (AcrB), a periplasmic adapter protein (AcrA) and an outer membrane channel (TolC). *E. coli tolC* mutants are highly susceptible to a wide variety of toxic compounds [79].

Although TolC homologues are ubiquitous among Gram-negative bacteria, they do not seem to exist in mycobacteria. Hence, we hypothesize that *M. tuberculosis* might have an outer membrane channel protein that connects to inner membrane pumps, allowing for efficient efflux across the two membranes. This hypothesis is supported by the observation that over-expression of *rv0194*, a gene encoding an inner-membrane ABC transporter of *M. tuberculosis*, increased resistance of *M. bovis* BCG to ampicillin [80]. Because targets of β-lactam antibiotics are located in the periplasm, increased resistance must result from efflux across the outer membrane. A connection of an outer membrane channel with an inner membrane efflux pump similar to the drug efflux systems in Gram-negative bacteria would also provide the energy required for efflux against the concentration gradient. Discovery of a

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TolC-like protein would represent a major breakthrough in our understanding of drug efflux in *M. tuberculosis*.

Other putative outer membrane proteins

In the previous sections, we have highlighted a few transport processes requiring outer membrane proteins. Yet, many other functions as described in Gram-negative bacteria are completed by proteins embedded in the outer membrane [34]. We propose that functionally equivalent proteins exist in mycobacteria.

For example, YaeT is required by *E. coli* to correctly insert proteins into the outer membrane [81,82]. Conditional depletion of the homologous Omp85 in *Neisseria gonorrhoeae* results in periplasmic accumulation of misfolded proteins [83]. Mycobacteria might possess a functional homologue of protein insertion machinery in the outer membrane. Similarly, Gram-negative bacteria require an outer membrane protein, Imp, to insert lipopolysaccharide (LPS) into the outer leaflet of the outer membrane [84]. Such membrane assembly proteins are likely also required for the many different lipids in the outer leaflet of mycobacterial outer membranes. For example, transport of the phthiocerol dimycocerosates (PDIMs) requires the inner membrane transporter MmpL7 [85,86] and the lipoprotein LppX [87], but it is unknown how PDIMs cross the outer membrane to reach the cell surface.

Mycobacteria produce capsules [88,89] and biofilms [90-92], but it is unknown how the materials for these extracellular structures are secreted to the cell surface. In *E. coli*, capsular material is translocated across the outer membrane by the Wza protein [93,94]. Given the requirement of extracellular structures for the survival of *M. tuberculosis* during infection [89], we propose that a secretion machinery for biofilm and capsular materials in the outer membrane also exists.

An area of intense research involves the proteinaceous virulence factors in *M. tuberculosis* culture filtrates. Proteins are transported across the inner membrane via the general (Sec), twin-arginine (Tat), or Esx secretion pathways. Importantly, *esx1* is required for the release of the virulence factors Esat-6 and Cfp-10 [95,96]. Yet, the outer membrane components of these secretion pathways await identification. Additionally, lipases [97], esterases [98], attachment and invasion proteins, and multiple other proteins [36] such as those of the enigmatic PE family [99] perform functions on the surface of the cell and are likely anchored or integrated in the outer membrane.

Concluding remarks and future directions

Outer membrane proteins of *M. tuberculosis* are intriguing for several reasons. First, considering that many nutrient molecules are hydrophilic and thus have inherently slow diffusion rates across lipid membranes, it is likely that proteins in the outer membrane are required for their uptake [100]. Hence, their identification is essential for understanding the physiology and pathogenicity of this microorganism. Second, outer membrane proteins of *M. tuberculosis* reside in an unusual lipid membrane distinct from that of Gram-negative bacteria. Therefore, their structures are probably novel, as shown for the porin MspA of *M. smegmatis* [10]. It is likely that they also function by novel mechanisms. Third, outer membrane proteins required for virulence such as OmpATb [48] represent attractive drug targets because inhibitors do not have to cross the notoriously impermeable outer membrane, a major determinant of intrinsic drug resistance [1]. Fourth, many outer membrane proteins of pathogenic Gram-negative bacteria are involved in interactions with host cells [101]. We assume that this will also be the case for *M. tuberculosis* and that these interactions are critical to the outcome of infection. In conclusion, the outer membrane represents a novel subcellular compartment in mycobacteria harboring proteins of unknown structures and functions. The

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visualization of mycobacterial outer membranes and the discovery and characterization of a few proteins that reside in this compartment have opened a new field in tuberculosis research of paramount scientific and medical importance.

However, considerable challenges remain to be overcome to fully understand the role of the outer membrane for the physiology and pathogenesis of *M. tuberculosis*. These challenges include:

- **i.** A complete ultrastructural characterization of the cell envelope of *M. tuberculosis*, whose capsule remains to be visualized [88]. Recent mutants with defects in capsular glucan biosynthesis offer the possibility to visualize this delicate structure [89].
- **ii.** The arrangement and configuration of lipids in the mycobacterial outer membrane is unknown, but is very likely a primary determinant of its permeability properties. The machinery required to assemble this complex structure is still a mystery.
- **iii.** The identification of the low abundance outer membrane proteins of *M. tuberculosis* represents a considerable experimental challenge. We have recently proposed a combinatorial protocol of simplified subcellular fractionation and whole-cell protease digestion to examine membrane association and surface accessibility as a novel approach to identify outer membrane proteins in mycobacteria [36]. However, both methods are prone to experimental errors and require proper controls and careful execution to yield reliable results.
- **iv.** The elucidation of the physiological function of novel proteins is difficult if the phenotype of a mutant is not apparent. This is likely the case for many outer membrane proteins, as was shown for porins with multiple functional orthologs [11]. Microarrays for phenotypic characterization have been recently established for mycobacteria and should prove helpful to overcome this hurdle [102].

In summary, the identification and characterization of outer membrane proteins of *M. tuberculosis* are critical for understanding the physiology and pathogenicity of this microorganism, and might lead to the development of new anti-tuberculosis drugs.

Box 1

The cryo-electron tomography (CET) technique

Cryo-electron tomography (CET) provides close-to-life structure analysis of intact (native) cells in 3-D at macromolecular resolution. Samples are prepared in such a way (cryo-preparation) that preserves the distribution and interaction of macromolecular complexes and allows the visualization of biological structures in situ. The technique is applicable to isolated macromolecular assemblies, viruses, and microbial and eukaryotic cells [94].

For cryo-preparation, cells are mounted on electron microscope specimen supports (copper grids covered with a film containing small holes of 1-2 μm in diameter) during growth in liquid medium. Then, they are subjected to rapid freezing (vitrification) by plunging into a cryogen (e.g., liquid ethane) at about −180 °C, without any dehydration, chemical fixation or staining. Thick biological samples (cells, tissues) are prepared by high pressure freezing to prevent crystallization of water and thinned by cryosectioning afterwards. Vitrified cells are structurally preserved down to the macromolecular level.

For cryosectioning, cell suspensions or small samples of tissue are transferred into small tubes which are mounted in a cryo-ultramicrotome under the atmosphere of liquid nitrogen following high pressure freezing. Cryosections of 30 to 300 nm thickness are collected and transferred to microscope grids. All samples are held frozen and are inspected in the electron microscope at liquid nitrogen temperature.

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A series of projections (tomographic data) is automatically recorded by tilting the specimen around one axis (or two perpendicular axes) in the transmission electron microscope. Finally, the data are three-dimensionally reconstructed and visualized using specific algorithms and software (e.g. *TOM toolbox* [104]).

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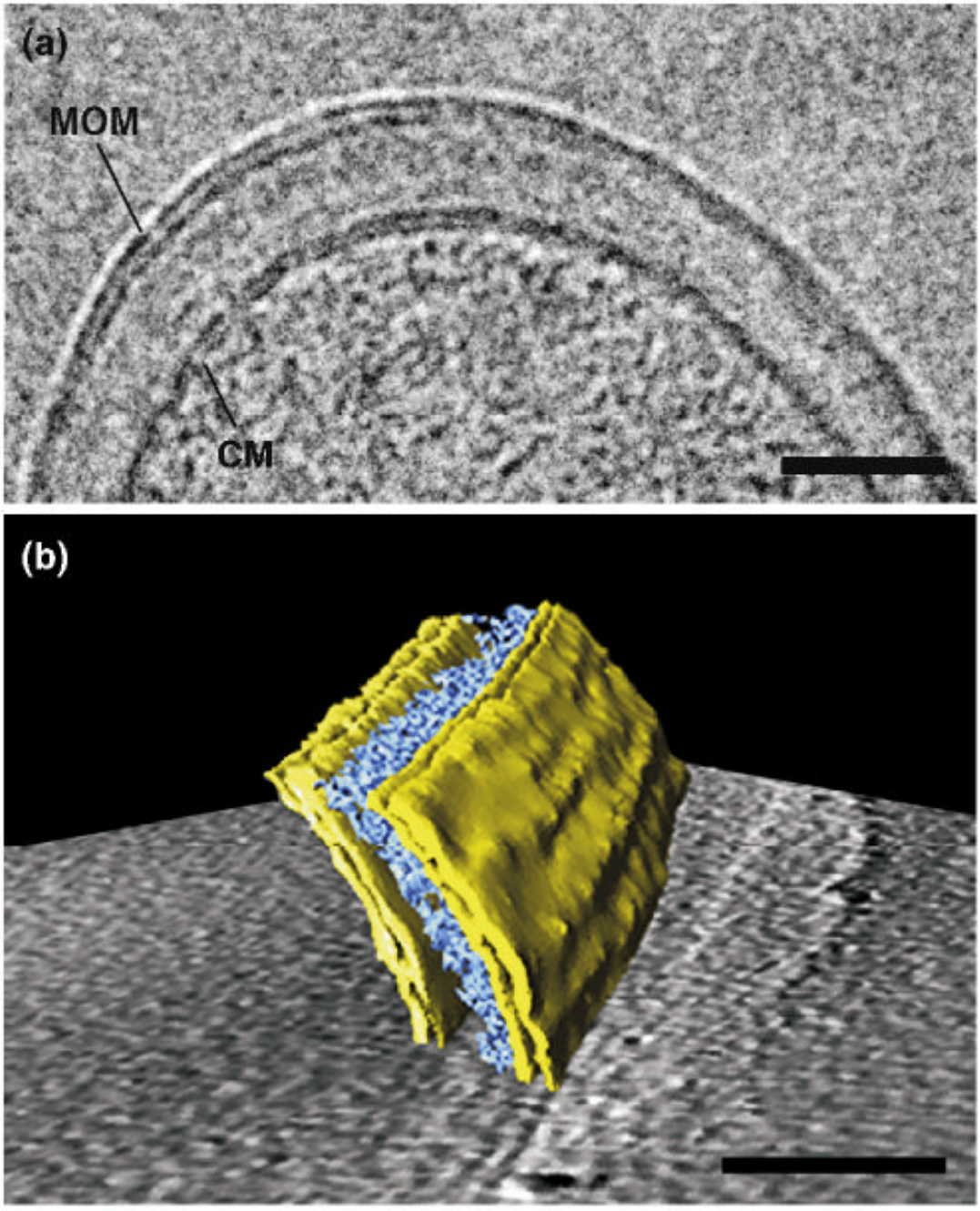

Figure 1.
Cryo-electron microscopy of the mycobacterial cell envelope. The mycobacterial outer membrane (MOM) from *Mycobacterium bovis* BCG is visualized by cryosectioning **(a)** and by cryo-electron tomography **(b)**. The periplasmic space between the MOM and the cytoplasmic membrane (CM) contains the layers of the arabinogalactan-peptidoglycan polymer (indicated in blue in the 3-D representation). Scale bars: 50 nm. Adapted, with permission, from Ref. [18].

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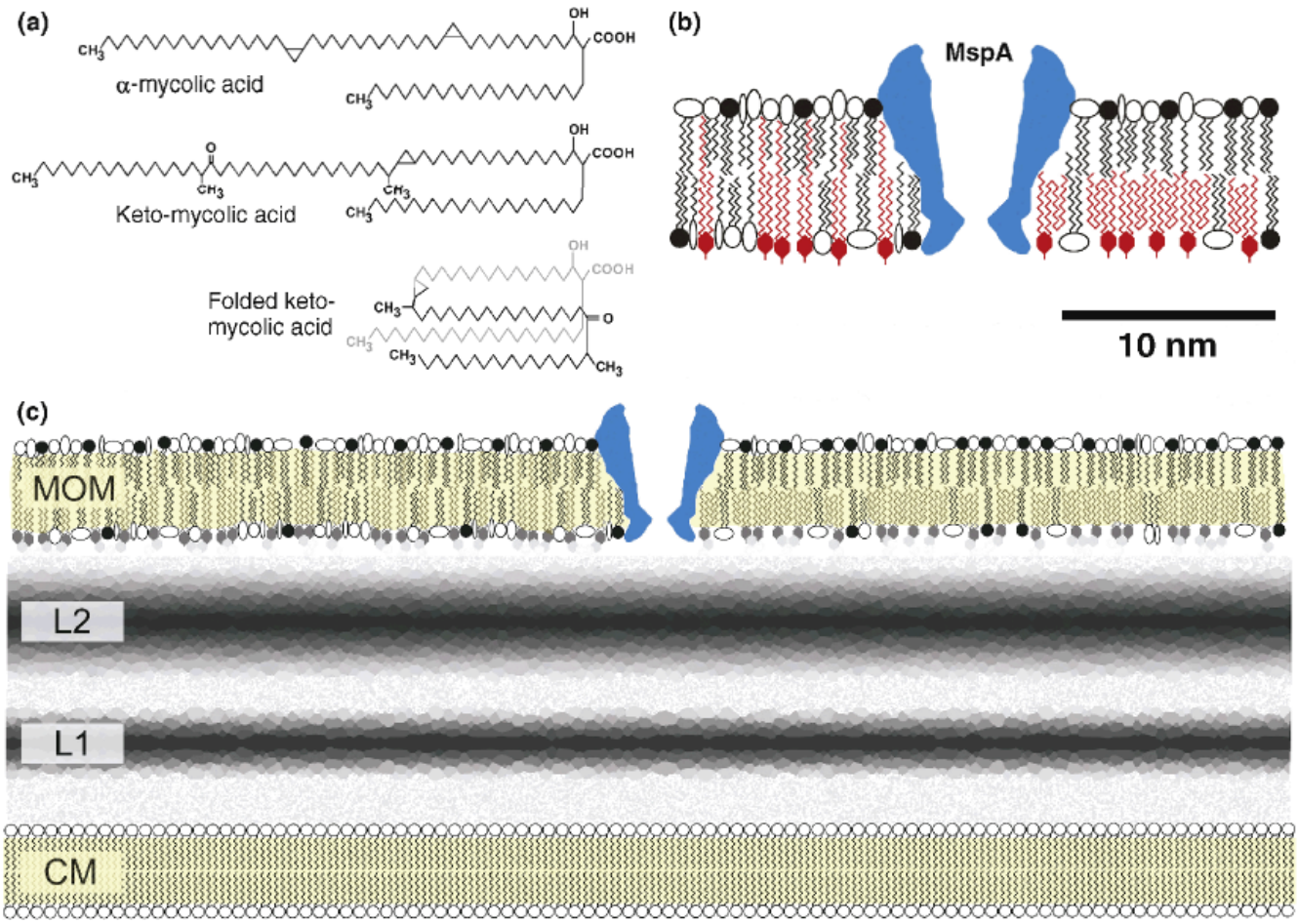

Figure 2.

Models of the mycobacterial outer membrane and cell envelope. **(a)** Schematic structure of mycolic acids in the elongated and folded conformation (based on the major components found in *M. tuberculosis* [24,103]). **(b)** Models of the mycobacterial outer membrane (adapted, with permission, from Refs. [18,19]). Mycolic acids are drawn in red and inserted either in the elongated conformation (model I, left side) or the folded conformation (model II, right side). A porin, here MspA from *M. smegmatis*, is represented in blue. The symbols of lipid head groups indicate that different free lipids may occur in both leaflets of the outer membrane. Scale bar: 10 nm. **(c)** Model of the mycobacterial cell envelope. The dimensions of the membranes and the periplasmic layers are taken from [18]. Abbreviations: CM, cytoplasmic membrane (membrane proteins are not shown); L1 and L2, periplasmic layers of still unknown identity (L2 represents at least part of the peptidoglycan-arabinogalactan polymer); MOM, mycobacterial outer membrane according to the representation in **(b)**. The hydrophobic matrix of membranes is indicated in yellow color. The models in **(b)** and **(c)** are approximately drawn to scale.

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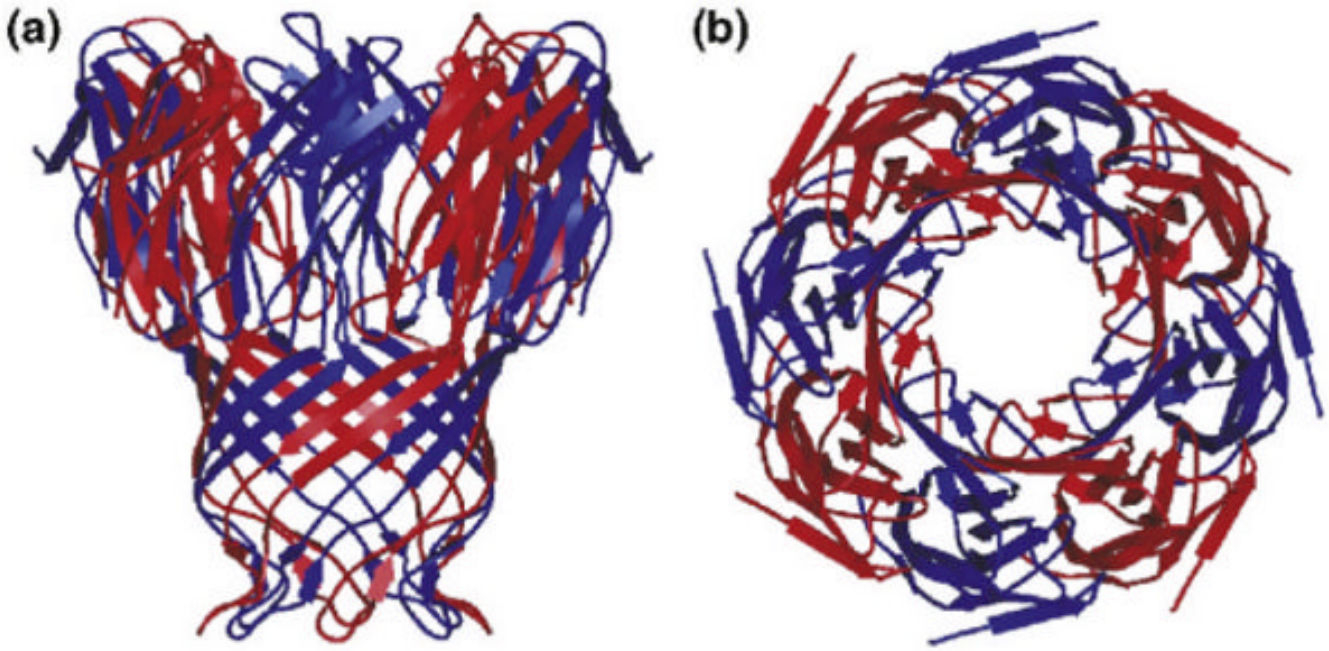

Figure 3.
Structure of the porin MspA of *M. smegmatis*. **(a)** Side view, **(b)** top view. The atomic coordinates of MspA were taken from the crystal structure (PDB accession code: 1UUN [10]). Alternating adjacent monomers are colored in red and blue.