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PERSPECTIVES

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Mycobacterial Membrane Domain, or a Primordial Organelle?

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Mycobacteria, like many other prokaryotic organisms, do not appear to have membrane-bound organelles to organize the subcellular space. Nevertheless, mycobacteria and related bacteria grow their cell envelope in a spatially controlled manner, restricting cell elongation to the polar regions of the rod-shaped cell. This spatial organization demands that *de novo* synthesized cell envelope components must be supplied to the polar ends of the cell. Because many cell envelope components are either lipids or built as lipid-anchored precursors, the plasma membrane is the major site of the biosynthesis. Thus, there are logistical questions of where in the plasma membrane these lipids and lipid precursors are made and how they are subsequently delivered to the growing poles of the cell. Our discovery of an intracellular membrane domain (IMD†) fills in this gap. Currently available data suggest that the IMD is a membrane domain within the plasma membrane of mycobacteria, which mediates key biosynthetic reactions for cell envelope and other lipid biosynthetic reactions. Consistent with its role in polar growth, the IMD is enriched in the polar regions of actively growing cells and becomes less polarized when the cells experience non-growing conditions. We discuss how such membrane compartmentalization may be generated and maintained in a mycobacterial cell and why it has not evolved into a *bona fide* organelle. In a broader perspective, we suggest that segregation of biosynthetic pathways into different domains of a planar membrane could be more widespread than we currently think.

ORGANELLES VERSUS MEMBRANE DOMAINS

Membrane-bound organelles have a functional space, or lumen, created by a surrounding lipid bilayer. Eukaryotic cells are full of such organelles featuring a vast array of specialized functions. It is conceivable that such structures are needed to maintain the working order of highly complex eukaryotic cells. Comparatively, prokaryotic cells are generally smaller than their eukaryotic counterparts, implying that there is less of a need for membrane-bound organelles. However, there is a wealth of examples for such functional compartmentalization in prokaryotic cells as well [1-4]. It appears that cell size is not always the determinant for creating organelles; rather the complexity or the special needs of the cell might de-

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†Abbreviations: DMK9, demethylmenaquinone; GPI, glycosylphosphatidylinositol; IMD, intracellular membrane domain; MK9, menaquinone; PA, phosphatidic acid; PIM, phosphatidylinositol mannoside; PMf, plasma membrane free of cell wall components; PM-CW, plasma membrane tightly associated with the cell wall; RIF, region of increased fluidity.

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mand organelle formation and many bacteria are likely as complex or specialized as eukaryotic cells in executing their biological functions.

One could imagine that the evolution of an organelle can be driven either by the need for the segregated, functionally distinct lumenal space, or by the need for increased, isolated membrane surface, or both. When neither lumen nor increased surface is needed, but separate areas are required for organization within a membrane, distinct domains in a planar membrane might suffice. Sterol- and sphingolipid-enriched nanodomains, often referred as membrane rafts, are the most prominent and well-known example of lateral membrane heterogeneities in eukaryotes as well as, more recently, in prokaryotes [5-9]. Such membrane domains are often involved in membrane trafficking and signal transduction. In contrast, little is known about the function of membrane domains in organizing the lateral flow of metabolic reactions within a plane. In *Mycobacterium smegmatis*, we identified a membrane domain that mediates various segments of biosynthetic pathways (Figure 1A and B). Given the presence of lateral heterogeneities in diverse biological membranes, we speculate that such metabolic partitions in membranes are likely widespread. However, the experimental evidence for the compartmentalized biosynthesis mediated by membrane-bound enzymes differentially localized in a planar membrane is scarce. We are aware of one example: mammalian glycosylphosphatidylinositol (GPI) biosynthesis, where a part of the pathway takes place in a mitochondria-associated membrane of the endoplasmic reticulum while the rest of the reactions take place in the regular endoplasmic reticulum membrane [10,11]. In the case of *Bacillus subtilis* plasma membrane, although enzymes involved in the phospholipid biosynthesis are not compartmentalized into multiple membrane regions, they are all enriched in the septal membrane [12]. In these cases, perhaps, the evolutionary pressure was such that membrane compartmentalization was needed without the demand for the creation of separate organelles. In this perspective, we will describe how we discovered a membrane domain in mycobacteria and discuss the implications of compartmentalization.

DISCOVERY OF THE IMD, A MYCOBACTERIAL MEMBRANE DOMAIN

The initial hint of mycobacterial membrane compartmentalization came from a biochemical observation [13]. Our laboratory was primarily interested in the biosynthetic pathway of unique bacterial glycosylated PIs known as phosphatidylinositol mannosides (PIMs), lipomannan and lipoarabinomannan. In the early 2000's, we worked to establish a cell-free system to examine the biosynthesis of these glycosylated PIs [14]. One strategy we took to improve the cell-free system was the fractionation of mycobacterial cell lysate by a sucrose density gradient. The expectation at the time was to isolate the plasma membrane away from other cellular contaminants such as cytoplasmic proteins. However, from this experiment, we unexpectedly found two distinct membrane fractions, which showed distinct biosynthetic activities involved in the PIM biosynthesis. The radioactive mannose donor, GDP-[^{3}H]mannose, was used to monitor PIM biosynthesis between the two fractions. We found that early intermediates such as PIM1 and AcPIM2 were produced in one fraction, while later intermediates such as AcPIM4, AcPIM5, and AcPIM6 were produced in another fraction (Figure 1B). The membrane fraction that migrated to a lighter density region was originally named the PMf because it did not contain any of the cell wall components (*i.e.* Plasma Membrane free of cell wall components). The other membrane fraction that sedimented further down into a denser part of the sucrose gradient contained cell wall components including galactose, a component of arabinogalactan layer, and glycopeptidolipids, outer membrane glycolipids. Therefore, this fraction was named the PM-CW (*i.e.* Plasma Membrane tightly associated with the Cell Wall). Indeed, negative stain electron microscopy of the PM-CW fraction visualized large fragments and debris of cells, consistent with containing both the cell wall and the plasma membrane. In contrast, the PMf appeared to be homogenous vesicles of about 50 nm diameter [13]. Such a compartmentalized biosynthesis of PIMs was surprising because mycobacteria were not known to have membrane-bound organelles or membrane domains. As described below, evidence from more recent studies support the idea that the PMf is a membrane domain rather than a *bona fide* organelle, and we recently renamed the PMf to the Intracellular Membrane Domain (IMD) to highlight its functional context in the live mycobacterial cell rather than the original name PMf, which was based on its biochemical composition.

IS THE IMD FUNCTIONAL?

We have accumulated several pieces of evidence arguing in favor of the idea that the IMD is a metabolically active and dynamic structure, rather than a biochemical artifact.

PIM Biosynthesis

Our initial analysis of PIM biosynthetic activities, using a GDP-[^{3}H]mannose radiolabeling probe, suggested that the early and late portions of PIM biosynthesis are segregated between the IMD and the PM-CW (Figure 1) [13]. To provide further evidence, live, actively growing *M. smegmatis* cell was metabolically labeled with [^{3}H]

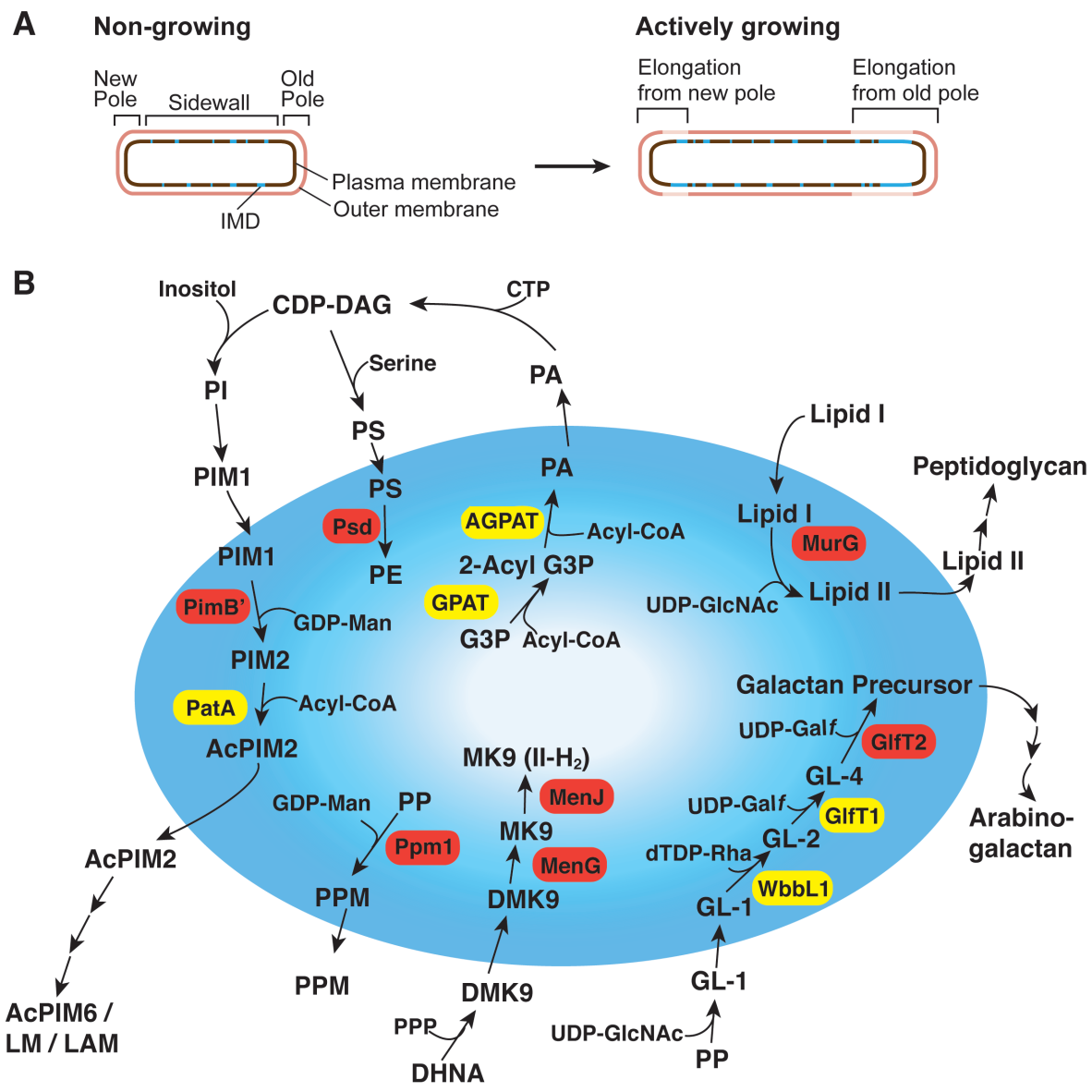

Figure 1. The IMD and associated metabolic reactions. (**A**) The IMD is proposed as a membrane domain in the plasma membrane of mycobacterial cells. It is distributed as patches along the sidewall of non-growing cells, but becomes enriched in the polar ends of actively growing cells. Note that mycobacteria grow from the polar ends by inserting new cell wall material in a spatially controlled manner. (**B**) Pathways compartmentalized in the IMD. Blue oval conceptualizes the IMD. Enzymes in red rounded rectangles are the ones for which either biochemical or microscopic experimental data are available. Enzymes in yellow rounded rectangles are the ones for which only proteomic data are available. Reactions outside of the IMD may take place in the PM-CW, or there may be no currently available data on their subcellular localization. See text for details. AGPAT, 2-acylglycerol 3-phosphate *O*-acyltransferase; DAG, diacylglycerol; DHNA, 1,4-dihydroxy-2-naphthoic acid; DMK, demethylmenaquinone; G3P, glycerol 3-phosphate; Galf, galactofuranose; GlcNAc, *N*-acetyl glucosamine; GlfT1, GL-2:UDP-Gal*f* galactofuranosyltransferase; GlfT2, GL-4:UDP-Gal*f* galactofuranosyltransferase; GPAT, G3P *O*-acyltransferase; LM, lipomannan; LAM, lipoarabinomannan; Man, mannose; MenG, DMK:*S*-adenosylmethionine methyltransferase; MenJ, MK reductase; WbbL1, GL-1:dTDP-Rha rhamnosyltransferase; MK, menaquinone; MurG, lipid I:UDP-GlcNAc GlcNAc transferase, PA, phosphatidic acid; PatA, PIM2:acyl-CoA acyltransferase; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIM, phosphatidylinositol mannoside; PimB', PIM1:GDP-Man mannosyltransferase; PP, polyprenol phosphate; PPM, polyprenol-phosphate-mannose; Ppm1, PPM synthase; PPP, polyprenol pyrophosphate; PS, phosphatidylserine; Psd, PS decarboxylase; Rha, rhamnose.

mannose for a short 10 min period, and the lysate was fractionated by density gradient sedimentation. Similar to the cell-free assay of gradient fractions, we detected the [^{3}H]mannose-labeled AcPIM2 in the IMD while more mature species such as AcPIM6 were found in the PM-CW fractions [13]. These enzymatic activities correlated well with the localization of the enzymes: PimB', which mediates the second mannose transfer of PIM biosynthesis, was enriched in the IMD (Figure 1B), while PimE, which mediate the fifth mannose transfer, was enriched in the PM-CW [15,16]. Comparative proteomic analysis of the IMD and the PM-CW further identified PatA, the acyltransferase involved in AcPIM2 biosynthesis, as an IMD-enriched protein [17]. These observations supported that both of these membrane fractions are metabolically active and associated with different metabolic activities.

Phospholipid Biosynthesis

Comparative proteomics suggested that segments of phospholipid biosynthesis also take place in the IMD (Figure 1). Synthesis of phosphatidic acid (PA), a key intermediate of phospholipid biosynthesis, is mediated by two acyltransferases that use glycerol 3-phosphate as a starting substrate. According to the proteomic analysis, these two enzymes are both enriched in the IMD [17]. If these enzymes actively produced PA in the IMD, we considered that PA would be more enriched in the IMD. Lipidomic analysis indicated that this is indeed the case [17]. PA is then activated to CDP-diacylglycerol, which can be used for the synthesis of various phospholipids. Phosphatidylethanolamine is one of the major structural phospholipids, and is produced by sequential actions of phosphatidylserine synthase, Pss, and phosphatidylserine decarboxylase, Psd. Using a cell-free radiolabeling assay, we have shown that the enzymatic activity of Pss is enriched in the PM-CW while that of Psd is amassed in the IMD [13]. Furthermore, the proteomic analysis showed the enrichment of Psd in the IMD [17], supporting the idea that the key enzymes associated with the IMD are actively engaged in phospholipid biosynthesis.

Peptidoglycan Biosynthesis

Lipid II is the complete lipid-anchored precursor for peptidoglycan synthesis, anchoring a disaccharide-pentapeptide unit onto a polyprenol lipid linked through a pyrophosphate. The final step of lipid II synthesis is mediated by the glycosyltransferase MurG, which transfers *N*-acetyl glucosamine from UDP-*N*-acetyl glucosamine to lipid I, forming lipid II. This step takes place on the cytoplasmic side of the plasma membrane and is followed by the flippase MurJ translocation of lipid II from the cytoplasmic to the periplasmic side of the plasma membrane [18]. The *de novo* synthesized disaccharide-pentapeptide unit is then transferred from lipid II to the growing chain of the peptidoglycan by transglycosylases such as PonA1 [19]. We recently demonstrated that enzymatically functional epitope-tagged MurG, as well as its product lipid II, are localized to the IMD (Figure 1) [20]. Strikingly, genetic knockdown of MurJ flippase resulted in a massive accumulation of lipid II in the IMD, supporting the notion that the IMD is where lipid II is produced. Lipid II is an example of an IMD substrate that is possibly transported to the PM-CW by the flippase MurJ. Such a system for simultaneous flipping and lateral translocation may be used by other substrates as well. As detailed below, the IMD may have a different fluidity from the PM-CW. It would be interesting to examine if membrane fluidity plays a role in facilitating the flipping of large polar lipids across the membrane bilayer.

Menaquinone Biosynthesis

The final pathway we analyzed for membrane compartmentalization was the biosynthesis of menaquinones, which is a lipidic electron carrier in the respiratory chain in mycobacteria. Membrane-associated enzymes, MenA, MenG, and MenJ, mediate the last three steps of the biosynthesis (Figure 1). First, MenA transfers a polyprenyl (mainly nonaprenyl) lipid onto 1,4-dihydroxy-2-naphthoic acid, making demethylmenaquinone (DMK9). Second, the methyltransferase MenG methylates DMK9 to produce menaquinone (MK9). Finally, the reductase MenJ reduces a single double bond in the β-isoprene unit of the prenyl chain, producing the mature species termed MK9 (II-H_2). The proteomic analysis suggested that MenG and MenJ are enriched in the IMD (Figure 1) [17]. Lipidomics reproducibly showed slight enrichment of mature menaquinones in the IMD, supporting the notion that it is produced there [21]. The final maturation of menaquinone in the IMD could imply the functional need for the mature species within the IMD. By density gradient sedimentation of epitope-tagged MenG and MenJ, we confirmed the localization of these two enzymes to the IMD. In contrast, the first enzyme MenA was localized to the PM-CW. The MenA product, DMK9, is an electron carrier known to be functional in other bacteria [22]. However, an accumulation of DMK9 appears toxic to mycobacteria, resulting in metabolic dysregulation and the cessation of growth [21]. The accumulation of DMK9 was achieved by inducible knockdown of the downstream enzyme MenG. Since MenG is an IMD-associated enzyme, we speculate that the toxicity is due to the abnormal enrichment of DMK9 in the IMD.

These pieces of experimental evidence from biochemical assays support that the IMD is a multi-functional membrane domain with numerous metabolic processes. The proteomic analysis revealed more than 300 proteins associated with the IMD, including the ones involved in

many other metabolic pathways, for which future biochemical confirmations await [17]. As mentioned above, the IMD appears as vesicles in the purified fraction under electron microscopy. But, are these IMD-associated proteins found on the same vesicle-like structures? We used gold-labeled antibodies to identify vesicles containing the endogenous protein PimB' and epitope-tagged fusion of GlfT2, an enzyme involved in galactan biosynthesis (see below and Figure 1B); both IMD proteins were found on the same vesicle-like structure. This observation was further supported by immunoprecipitation experiments in which the epitope-tagged GlfT2 pulled down endogenous PimB', but this interaction was ablated in the presence of mild detergents [17]. Collectively, these data reinforce the idea that the IMD is a distinct multifunctional membrane domain.

In contrast to the IMD, the PM-CW is purified as membrane tightly associated with the cell wall. Many cell envelope biosynthetic precursors produced in the IMD are presumably released into the PM-CW in a locally enriched manner and integrated into the supramolecular cell envelope complex. These final integration processes take place on the periplasmic side of the plasma membrane while the IMD reactions are primarily sequestered on the cytoplasmic side. This topology implies that the IMD supports cell envelope biosynthesis in the PM-CW by locally supplying necessary precursors from the opposite side of the membrane.

VISUALIZING THE SUBCELLULAR LOCALIZATION OF THE IMD

The initial biochemical analyses of metabolic compartmentalization led us to ask where in the cell the IMD is localized. Unlike many rod-shaped bacteria, which add new cell wall materials along the length of the sidewall, mycobacteria elongate primarily at the polar ends (Figure 1A) [23-25]. The metabolic potential of the IMD posed as a possible platform for coordinating the many processes needed for the polar cell envelope synthesis. Therefore, we sought to study the IMD in living cells to better understand its relationship to cellular growth.

Based on our proteomic data set, we tagged relatively abundant IMD proteins with a fluorescent protein and monitored their localization in actively growing cells. An IMD protein (GlfT2) and a PM-CW protein (PimE) were tagged with the fluorescent proteins mCherry and GFP, respectively. The mCherry-GlfT2 fusion protein localized as puncta particularly enriched at the polar ends of the cell while the PimE-GFP fusion protein outlined the cell membrane and septum. These localizations suggest a specific subcellular localization of the IMD, separate from the PM-CW [17]. A previous study established GlfT2 as a subpolar protein relative to DivIVA that defines the cell pole [26]. Even in chemically induced ectopic cell poles, this subpolar localization of the IMD protein was observed [17], suggesting that the IMD is critical for polar elongation of mycobacteria.

Due to the close proximity of the IMD to the cell poles, we further investigated the relationship of the IMD and cell growth. Click-iT chemistry was used to investigate the spatial relationship of the IMD to nascent peptidoglycan synthesis. Actively growing cells had enrichments of bioorthogonal peptidoglycan biosynthetic precursor incorporated intensely at the pole and weakly along the sidewall, distinct from the IMD fluorescent protein localization [27,28]. These observations were consistent with the idea that the IMD is not necessarily the site of the final peptidoglycan elongation and led us to suggest that the IMD may assist in restricting the spatial localization of cell envelope synthesis.

STRESS RESPONSE OF THE IMD

In actively growing cells, we established the IMD as a polarly enriched membrane domain, that continuously associates with the cellular growth pole. These observations supported the idea that the IMD synthesized precursors of the cell envelope, and thusly remained in close proximity to the location of their final incorporation at the polar ends. We wondered what would happen to the IMD when the cells cease their growth. Therefore, we examined the IMD in *M. smegmatis* during various stresses such as stationary growth phase, starvation, and antibiotic perturbation.

During stationary phase, cells continue to replicate in the absence of cell elongation, providing an ideal opportunity to investigate the IMD when polar elongation is not occurring. Indeed, when cells enter stationary phase, peptidoglycan synthesis is no longer at the polar ends, and is concentrated at the septal region [28]. Subsequently, this lack of elongation led to shorter cells when entering deeper into stationary phase. Similarly, when nutrients were removed from the environment, cell wall synthesis was refocused towards the septum of the cells, leading to the appearance of multi-septal cells. Both stationary phase and starvation saw the decrease in the IMD enrichment at the polar ends, correlating with the alteration in peptidoglycan synthesis [28].

To confirm this relationship between the IMD and the cell wall synthesis, chemical and genetic probing determined the correlative localization of these two entities. D-cycloserine is a chemical inhibitor of peptidoglycan precursor biosynthesis. A diaminopimelate auxotroph is a genetic mutant unable to produce the amino acid diaminopimelate, a key component of the mycobacterial peptidoglycan precursor. Upon stress by D-cycloserine or diaminopimelate starvation in the auxotroph, pepti-

doglycan synthesis halted, and the IMD localized away from the polar ends [28]. These data support that the polar localization of IMD-associated proteins is dependent on peptidoglycan synthesis.

The localization of the IMD-associated proteins correlated to that of cell wall synthesis, even upon removal of the stress, when cells transitioned back to polar elongation. Cells affected by a stress had higher IMD localization to the sidewall, but upon recovery due to removal of the stress, relocalization of the IMD-associated proteins to the polar end was evident. Incorporation of nascent cell wall precursors was initially slower in cells recovering from stress than those logarithmically growing. This delay in elongation correlates to the transition from a sidewall-distributed IMD to a pole-enriched IMD [28]. These data suggest that the polarly focused IMD corresponds with optimally efficient cell elongation, acting as supply generator for cell envelope synthesis. Such a dynamic role of the IMD is relevant for the infection cycle of *Mycobacterium tuberculosis*, during which the bacterium is exposed to extreme environmental changes such as low nutrients and low oxygen in host immune cells. Our unpublished analysis indicates that *M. tuberculosis* has the IMD, implying a critical role of a similar membrane organization in the pathogenic species.

Notably, the delay in elongation during recovery is similar to that of cells undergoing normal cell division in which alternator and accelerator cells have been identified [23,24]. The elongation of alternator cell, which inherits slow-growing new pole from the mother cell, is delayed in comparison to that of accelerator cell, which inherits preexisting old pole from the mother cell. The maturation of the IMD at the alternator cell's new pole poses a potential explanation for the growth delay. Since the accelerator cell inherits the mature growth pole from the mother cell, it does not require additional time to reform an IMD for elongation. Similarly, the IMD localizes to ectopic growth poles that are induced by a chemical treatment [17], and this localization takes place shortly after the ectopic growth pole formation. As discussed above, the PM-CW plays the critical roles in actual integration of the materials supplied from the IMD into the cell envelope. Thus, coordination of enzymes associated with the IMD and the PM-CW is pivotal in normal growth as well as during the recovery from stress conditions.

MORPHOLOGY OF THE IMD

Biochemically purified IMD appeared as vesicles under electron microscopy. But, does the IMD membrane vesicle exist in the cell or is this formation a consequence of cell lysis? Currently, available data suggest that the latter is likely.

First, a number of recent studies visualized the internal structures of mycobacterial cells using cryo-electron microscopy. Rapid freezing of live samples reduces the formation of artificial structures in the cell that are often produced during chemical fixation. In these recent cryo-electron microscopy images, there is no clear evidence for the presence of cytoplasmic vesicles [29-32]. Second, we created a number of strains of *M. smegmatis*, in which the IMD-associated proteins were fused to a fluorescent protein. We have confirmed that despite the attachment of a fluorescent protein, these enzymes remain functional. In our recent analysis using structured illumination microscopy, two such proteins, MurG-Dendra2 and mCherry-GlfT2, were closely co-localized with each other in a single cell, and both proteins appeared as patches along the cell perimeter rather than intracytoplasmic vesicles [20]. Nonetheless, we acknowledge that fluorescent microscopy is limited in resolution and more detailed electron microscopic analysis is needed to firmly define the morphological nature of the IMD.

DO LIPIDS DRIVE THE FORMATION OF THE IMD?

Lipid composition of the IMD is distinct from that of the PM-CW [17]. However, we currently do not know if lipids drive the creation and maintenance of the membrane domain. By examining biosynthetic pathways associated with the IMD, we found many enzymes that react on polyprenol lipids as substrates. Examples include MurG in peptidoglycan precursor synthesis, Ppm1 in polyprenol-phosphate-mannose biosynthesis, WbbL1, GlfT1, and GlfT2 in galactan precursor biosynthesis, as well as, MenG and MenJ in menaquinone biosynthesis [17,20,21]. Non-sterol isoprenoids such as polyprenols can destabilize lamellar lipid bilayer by inducing hexagonal II phase formation [33-35], can increase membrane fluidity in *in vitro* systems [33,36-39], and can induce lipid cluster formation while being incompatible with cholesterol-rich membrane rafts [37,40,41]. Therefore, it is tempting to speculate that the IMD is a polyprenol-rich membrane domain that is more fluid than the conventional plasma membrane (*i.e.* PM-CW). If the formation of the IMD is a lipid-driven process, proteins may be localized to the IMD by recognizing specific lipids within the IMD. Polyisoprenyl recognition sequences are found in the transmembrane domains of a variety of proteins in both eukaryotes and prokaryotes [42,43]. However, a large proportion of the IMD-associated proteins are peripheral membrane proteins without predicted transmembrane domains [17], and it remains unknown if similar lipid-binding motifs play roles in the localization of IMD proteins.

If the IMD formation is a lipid-driven process, molecules that disrupt membrane organization might have

profound impacts on the IMD localization of proteins. Benzyl alcohol is a well-documented membrane fluidizer, which can disrupt the membrane domain organization in various biological membranes [44,45]. In particular, there is a fluid membrane, termed Region of Increased Fluidity (RIF), in *Bacillus subtilis*, which is conceptually similar to the IMD [45]. Strikingly, the evolutionarily conserved peptidoglycan biosynthetic enzyme MurG is associated with the *Bacillus* RIF and the formation of RIF was disrupted by benzyl alcohol treatment [45]. When we tested the effect of benzyl alcohol in mycobacteria, it was not bactericidal but bacteriostatic at the concentration we used. We prepared a lysate from benzyl alcohol-treated mycobacterial cells, fractionated by the density gradient, and analyzed lipid profiles by thin layer chromatography. This experiment revealed altered distributions of major lipid species in the IMD and the PM-CW. Furthermore, less MurG-Dendra2 was enriched in the IMD density fraction. Consistent with the biochemical analysis, fluorescence microscopy revealed that the polar enrichment of MurG-Dendra2 in growing cells was severely diminished upon treatment with benzyl alcohol [20]. The reorganization of MurG-Dendra2 reflected to non-polar synthesis of peptidoglycan, suggesting a connection between lipid organization and polar cell envelope growth. While further studies are needed, these initial observations indicate that lipids play key roles in structurally organizing the IMD.

CONCLUSIONS

The IMD is a metabolically active membrane domain, which acts as a localized supply generator for cell envelope elongation. However, there are many other enzymes, which are not directly linked to cell envelope elongation, associated with the IMD as well. This is exemplified by the association of menaquinone biosynthesis with the IMD, implying that the IMD is tightly linked to the central energy metabolism. How the IMD is generated, maintained, and regulated in response to changing environment is an important question for the future. Our unpublished data suggest that the IMD exists in *M. tuberculosis*, but evolutionary conservation of the membrane compartmentalization in other *Mycobacterium* and related bacteria remains unknown. Is the IMD an organelle? Perhaps not, but it may be a way for mycobacteria to organize their subcellular environment to support efficient growth and proliferation, much like how eukaryotic cells utilize their biogenic organelles for metabolic coordination.

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