Compartmentalized Cell Envelope Biosynthesis

in *Mycobacterium tuberculosis*

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Membrane domain in *Mycobacterium tuberculosis*

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Abstract (250 words)

The intracellular membrane domain (IMD) is a metabolically active and laterally discrete membrane domain initially discovered in *Mycobacterium smegmatis*. The IMD correlates both temporally and spatially with the polar cell envelope elongation in *M. smegmatis*. Whether or not a similar membrane domain exists in pathogenic species remains unknown. Here we show that the IMD is a conserved membrane structure found in *Mycobacterium tuberculosis*. We used two independent approaches, density gradient fractionation of membrane domains and visualization of IMD-associated proteins through fluorescence microscopy, to determine the characteristics of the plasma membrane compartmentalization in *M. tuberculosis*. Proteomic analysis revealed that the IMD is enriched in metabolic enzymes that are involved in the synthesis of conserved cell envelope components such as peptidoglycan, arabinogalactan, and phosphatidylinositol mannosides. Using a fluorescent protein fusion of IMD-associated proteins, we demonstrated that this domain is concentrated in the polar region of the rod-shaped cells, where active cell envelope biosynthesis is taking place. Proteomic analysis further revealed the enrichment of enzymes involved in synthesis of phthiocerol dimycocerosates and phenolic glycolipids in the IMD. We validated the IMD association of two enzymes, α1,3-fucosyltransferase and fucosyl 4-*O*-methyltransferase, which are involved in the final maturation steps of phenolic glycolipid biosynthesis. Taken together, these data indicate that functional compartmentalization of membrane is an evolutionarily conserved feature found in both *M. tuberculosis* and *M. smegmatis*, and *M. tuberculosis* utilizes this membrane location for the synthesis of its surface-exposed lipid virulence factors.

IMPORTANCE (150 words)

M. tuberculosis remains an important public health threat, with more than one million deaths every year. The pathogen's ability to survive in the human host for decades highlights the importance of understanding how this bacterium regulates and coordinates its metabolism, cell envelope elongation, and growth. The IMD is a membrane structure that associates with the subpolar growth zone of actively growing mycobacteria, but its existence is only known in a non-pathogenic model, *M. smegmatis*. Here, we demonstrated the presence of the IMD in *M. tuberculosis*, making the IMD an evolutionarily conserved plasma membrane compartment in mycobacteria. Furthermore, our study revealed that the IMD is the factory for synthesizing phenolic glycolipids, virulence factors produced by slow-growing pathogenic species.

INTRODUCTION

Mycobacteria grow by depositing *de novo* synthesized cell envelope materials near the pole rather than the main sidewall of the rod-shaped cell (1–5). The cell envelope of mycobacteria is complex, composed of plasma membrane, peptidoglycan, arabinogalactan, and mycomembrane (6–9), making the spatially restricted cell envelope elongation a unique challenge for these bacteria. Precise coordination of the synthesis of cell envelope components are likely crucial, as highlighted by numerous proteins localizing to specific subcellular regions in mycobacteria (10). Among them, DivIVA, a filamentous coiled-coil protein, is associated with the pole of the cell and its physical interaction with early cell elongation machinery is implicated in the determination of the site of cell envelope elongation (11, 12). The recruitment of other enzymes involved in cell envelope biosynthesis defines a subpolar growth zone where new envelope components are made and incorporated into the growing pole (5, 11, 13). Polar enrichment is observed not only for proteins but also for membrane lipids. Plasma membrane heterogeneity was first reported using fluorescent lipid probes (14), and a more recent work suggested an enrichment of cardiolipin to the pole of growing mycobacterial cells (15).

Previously, we reported the presence of a growth-pole associated membrane domain, the intracellular membrane domain (IMD), in the nonpathogenic model organism *Mycobacterium smegmatis* (16–18). This domain is separated from the conventional plasma membrane upon density gradient fractionation of mycobacterial cell lysate. Plasma membrane is copurified with the cell wall, apparently due to physical connections between the two (termed PM-CW). The IMD, in contrast, is purified as ~50 nm membrane vesicles without the association of cell wall components. Among the proteins associated with the IMD, we find essential enzymes involved in lipid biosynthetic reactions. For instance, phosphatidylinositol mannosides (PIMs) are a major component of mycobacterial plasma membrane, and the initial phase of its biosynthesis is enriched in the IMD (16, 17). The IMD is not only present for cell envelope biosynthesis but also for other membrane-associated metabolisms. Menaquinone is a major respiratory chain electron carrier in mycobacteria, and the final maturation steps of its biosynthesis take place in the IMD (19). These observations indicate the diversity of crucial processes required for polar cell envelope growth and central metabolism that are associated with this membrane domain.

In *Mycobacterium tuberculosis*, we know comparatively less about subcellular localizations of membrane proteins. DivIVA is associated to the poles in *M. tuberculosis* (11) and CwsA, which

plays a role in cell wall synthesis and interacts with DivIVA, also shows polar and mid-cell

localization (20). On the other hand, FtsZ and its interacting partner SepF localize to future

division sites (21, 22). Furthermore, the sensor kinase MtrB localizes to septa in *M. tuberculosis*

as well (23). These past studies show conserved features of pole- and septum-associated

membrane proteins. However, other types of membrane compartmentalization have not been

reported in pathogenic mycobacteria. In this study, we investigated the presence of the IMD in

M. tuberculosis. Using subcellular fractionation, we first examined the IMD proteome in

comparison to the PM-CW proteome. We then visualized the localization of the IMD in live

growing *M. tuberculosis*. Our results demonstrate that the IMD is a membrane domain

conserved in mycobacteria*.*

RESULTS

Density gradient fractionation of the *M. tuberculosis* cell lysate

We prepared a lysate of exponentially growing *M. tuberculosis* cells and fractionated by density

gradient sedimentation (**Fig. 1A**). As previously seen in *M. smegmatis*, the majority of proteins

were enriched in the top two fractions. In the density gradient of *M. smegmatis* lysate,

cytoplasmic proteins are retained in the top fractions and do not sediment significantly. To

confirm that the *M. tuberculosis* proteins in the top fractions correspond to cytoplasmic proteins,

we performed immunoblotting against the cytoplasmic enzyme Ino1, an inositol-3-phosphate

synthetase (24), and detected this protein predominantly in fractions 1 - 2 (**Fig. 1B**). Next, we

determined the localization of the mannosyltransferase MptC, a polytopic membrane protein

found in the PM-CW in *M. smegmatis*, by immunoblotting. MptC was enriched in the denser

region of the gradient, spanning from fractions 8 - 11 (**Fig. 1B**). These fractions correspond to

the density of 1.127-1.154 g/ml, which is comparable to 1.131-1.159 g/ml for *M. smegmatis* PM-

CW (19), suggesting that MptC is a PM-CW protein in *M. tuberculosis* as well.

To examine if the IMD exists in *M. tuberculosis* in addition to the PM-CW fraction, we examined

the compositional profiles of plasma membrane phospholipids. Lipids were extracted from each

fraction and visualized by chemical staining. As shown in **Fig. 1C**, membrane phospholipids

cardiolipin (CL), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and PIMs were

detected in the PM-CW fractions as well as in another density region spanning from fractions 3

to 6. These data suggest that there is an additional membrane fraction separate from the PM-

CW. One known reaction that takes place in *M. smegmatis* IMD is the biosynthesis of

polyprenol-phosphate-mannose (PPM). We have previously shown that the PPM biosynthetic

activity is enriched in the IMD and the enzyme Ppm1 was found exclusively in the IMD in *M.*

smegmatis (16, 17). To determine the localization of this biosynthetic reaction in *M.*

tuberculosis, we performed a cell-free radiolabeling assay using GDP-[^{3}H]mannose as a

mannose donor. As shown previously (25–27), *M. smegmatis* produces heptapentenyl and

decapentenyl PPM species (**Fig. S1**). In contrast, *M. tuberculosis* does not produce

heptapentenyl polyprenols, and decapentenyl PPM is the major product (27–29). Although the

enzyme activity is not as robust as that of *M. smegmatis* enzyme, we detected the synthesis of

the expected decapentenyl PPM from *M. tuberculosis* cell lysate (**Fig. S1**), and was enriched in

fractions 3 - 5 of a sucrose density gradient (**Fig. 1D**), suggesting the subcellular localization of

PPM synthetase in these membrane fractions.

To compare the morphology of the membrane present in fractions 3 - 5 with that of the PM-CW,

we precipitated these membrane fractions by differential centrifugation and visualized by

negative staining electron microscopy (EM) (**Fig. 1E**). As in *M. smegmatis* (17), we observed

small vesicle-like structures in fractions 3 - 5, while the PM-CW fraction revealed larger

fragments of cells. Together, these data support that fractions 3 - 5 correspond to the IMD in *M.*

tuberculosis and its overall characteristics are similar to those of the *M. smegmatis* IMD.

Identification of IMD-associated proteins in *M. tuberculosis*

To uncover the IMD proteome, we purified the membranes in both IMD and PM-CW fractions by

differential centrifugation and analyzed the trypsin digests of total proteins by liquid

chromatography-mass spectrometry (LC-MS) in biological triplicates. The comparative

proteomes of the two membranes were distinct with a total of 64 or 298 proteins enriched more

than 2-fold (p < 0.05) in the PM-CW or IMD, respectively (**Fig. 2A**, **Dataset S1**). PM-CW was

associated with enzymes with activities that are related to the typical functions of plasma

membrane (**Fig. 2B**). For example, we identified Esx5 type VII secretion system components

EccB5-E5 (Rv1782, Rv1783, Rv1795), trehalose monomycolate transporter MmpL3 (Rv0206c),

a subunit of the phosphate specific transport system, PstS1 (Rv0934), and NADH-quinone

oxidoreductase subunits NuoF and NuoG (Rv3150, Rv3151). The IMD, on the other hand, was

more restricted to enzymes involved in lipid metabolism (**Fig 2B**). For instance, we found

enzymes involved in the synthesis of polyprenol-linked galactan precursor for arabinogalactan

synthesis enriched in the IMD: the GlcNAc-diphospho-decaprenol L-rhamnosyltransferase

WbbL1 (Rv3265c), the Rha-GlcNAc-diphospho-decaprenol β1,4/1,5-

galactofuranosyltransferase GlfT1 (Rv3782) and the Gal*f*-Gal*f*-Rha-GlcNAc-diphospho-

decaprenol β1,5/1,6-galactofuranosyltransferase GlfT2 (Rv3808c). The PPM synthase Ppm1

(Rv2051c) and acyl-CoA dehydrogenases such as FadE24 (Rv3139), FadE23 (Rv3140), and

FadE10 (Rv0873) were also found in the IMD. From the total of 298 proteins identified in the

IMD through the comparative proteomics, 218 had *M. smegmatis* homologues. Of these, 101

have also been identified previously as enriched in the IMD in a comparative proteomics of *M.*

smegmatis IMD and PM-CW (**Dataset S1**) (16). These data are consistent with the idea that the

M. tuberculosis IMD is a metabolically active membrane domain with multiple functions shared

with that of *M. smegmatis* (see below for a more refined IMD proteome).

Validation of IMD-associated proteins

Among the IMD-associated proteins was the dihydroorotate dehydrogenase PyrD, which is

involved in pyrimidine biosynthesis and is dependent on a lipidic electron carrier, menaquinone,

for the redox reaction (30, 31). Because it is an IMD-associated protein in *M. smegmatis* (16),

we chose this protein for the validation of the *M. tuberculosis* IMD proteome. We cloned the

gene with a hemagglutinin (HA) epitope tag into an expression vector, integrated into the

bacteriophage L5 integration site of *M. tuberculosis* genome, and confirmed its expected

molecular weight of 42.1 kDa (**Fig. 3A**). We then fractionated the cell lysate by sucrose density

gradient (**Fig. 3B**), and confirmed the IMD localization of PyrD-HA in contrast to the PM-CW

localization of MptC (**Fig. 3C**). These data indicate that PyrD associates with the IMD in *M.*

tuberculosis validating the comparative proteomics analysis.

Next, we took advantage of the PyrD-HA-expressing strain and purified the IMD from the

gradient fractions by vesicle immunoprecipitation using anti-HA agarose beads as performed

previously in *M. smegmatis* (16). We used the IMD fraction from the parental strain as a

negative control for immunoprecipitation. With a cutoff of 5-fold enrichment between the

experimental and negative control samples, 215 proteins were identified in the IMD of *M.*

tuberculosis (**Dataset S1**). Among them, 108 proteins were found in the IMD proteome of the

initial comparative proteomics (**Fig. 3D**). Out of these 215 immunoprecipitated proteins, 148 had

M. smegmatis homologues, with 73 localizing in the IMD of both species (**Fig. 3D**). Sixty seven

IMD-associated proteins were unique to *M. tuberculosis*, and notable proteins among them were

enzymes involved in the biosynthesis of phthiocerol dimycocerosate (PDIM) and phenolic

glycolipids (PGL; see below for further analysis). These data indicate that the IMD is an

evolutionarily conserved domain in mycobacteria with species-specific features.

Visualization of IMD proteins in *M. tuberculosis*

To visualize the IMD in *M. tuberculosis*, we constructed a mutant, in which a gene encoding

2xHA-tagRFP tag was knocked into the 3' end of the endogenous *pyrD* gene locus. The in-

frame insertion was designed to produce a PyrD-2xHA-tagRFP fusion protein from the

endogenous locus using the native promoter. This mutant was grown to log phase, and cell

lysate was fractionated by sucrose density gradient. We detected the localization of this fusion

protein to the IMD by western blotting using anti-HA antibody (**Fig. 4**). When we examined this

strain by live fluorescence microscopy, we could not detect the fluorescence (not shown),

possibly due to low abundance of the protein expressed from the endogenous promoter.

As an alternative approach, we next used an expression vector that integrates at the

mycobacteriophage L5 *attB* site and expressed an IMD-associated protein as fluorescent

protein fusions using a strong promoter. We chose two proteins: GlfT2, a galactosyltransferase

involved in galactan biosynthesis, and Psd, a phosphatidylserine decarboxylase involved in PE

biosynthesis (see **Fig. 2A**). These enzymes are also enriched in the IMD of *M. smegmatis* (16,

17). We fractionated the lysates of cells expressing either GlfT2-mNeonGreen-HA or Psd-

mNeonGreen-HA. Both GlfT2-mNeonGreen-HA and Psd-mNeonGreen-HA showed expected

molecular weights (100.9 and 53.5 kDa, respectively), and were enriched in the IMD fractions

(**Fig. 5A-B and S2A-B**) although we noted that there was a weak signal of Psd-mNeonGreen-

HA in the PM-CW.

We then analyzed the localization of these proteins in live cells by fluorescence microscopy. In a

subset of cells, GlfT2-mNeonGreen-HA showed intense fluorescence near the poles of the cells

with less intense patches of fluorescence along the sidewall membrane (**Fig. 5C**). This

fluorescence pattern is consistent with the IMD fluorescence patterns observed in *M.*

smegmatis. We used structured illumination microscopy (SIM) to acquire higher-resolution

images. As shown in **Fig. 5D**, intense foci near the poles of the cells were evident and the

sidewall patches appeared consistent with the idea that the protein is a membrane-associated

protein. Even though the polar enrichment of GlfT2-mNeonGreen-HA was evident in some cells,

we often found many cells where the intensities of polar and sidewall patches were similar,

making the polar enrichment not as obvious as in the case of *M. smegmatis*. We considered the

possibility that the polar IMD enrichment may correlate with a specific cell cycle stage. To

visualize cells with or without an active septum synthesis, we labeled the cells with 3-[[(7-

hydroxy-2-oxo-2H-1-benzopyran-3-yl)carbonyl]amino]-D-alanine (HADA), a fluorescent D-amino

acid analog (**Fig. 5E**), and separated the cell population based on the presence of a septum.

We then determined the average fluorescence intensity profiles of GlfT2-mNeonGreen-HA

along the cell length as we have previously done in *M. smegmatis* (32). We observed that the

enrichment of GlfT2-mNeonGreen-HA was slightly proximal to the polar HADA labeling, and

was evident regardless of whether there is an active septum synthesis or not (**Fig. 5F**).

Comparable fluorescence patterns were also observed for Psd-mNeonGreen-HA (**Fig. S2C**).

Together, these data show that the IMD is a membrane domain that is frequently enriched in the

subpolar region of the cell.

Maturation of PGL takes place in the IMD

The biosynthesis pathways of PDIM and PGL are partially overlapping, utilizing the same

enzymes for several steps (**Fig. 6A**). The pathway can be divided into synthesis, assembly, and

maturation stages. In the synthesis stage, mycocerosates and (phenol)phthiocerols are

synthesized by Mas and PpsA-E, respectively. In the assembly stage, PapA5 esterifies two

molecules of mycocerosates to the hydroxyl groups of phthiocerol or phenolphthiocerol,

producing PDIM or a PGL precursor, phenolphthiocerol dimycocerosate (PPDIM), respectively.

In the maturation stage, PPDIM is further decorated at the hydroxyl group of the phenol residue

by several carbohydrate residues and these carbohydrates are modified by methylation to

become a mature PGL.

We found several enzymes in these pathways enriched in the IMD (**Fig. 6A**). Among them, we

generated merodiploid strains producing HA epitope-tagged PpsB, PpsE, and fPKR (Rv2951c).

PpsB and PpsE are polyketide synthases involved in the synthesis of (phenol)phthiocerols while

fPKR is a $F_{420}H_2$-dependent phthiodiolone ketoreductase (33–35). PpsE and fPKR were

enriched in the IMD based on the comparative analysis of IMD / PM-CW proteomes as well as

the proteome of immunoprecipitated IMD. PpsB was not found in the comparative proteome, but

identified in the immunoprecipitated IMD. We analyzed lysates of the merodiploid strains by

SDS-PAGE and anti-HA immunoblot, but were unable to detect any of these epitope-tagged

proteins (not shown). Next, we generated merodiploid strains producing mNeonGreen-HA-

tagged enzymes involved in the final maturation steps of PGL synthesis, namely a rhamnosyl *O*-

methyltransferase (Rv2959c), a fucosyltransferase (Rv2957), and a fucosyl *O*-methyltransferase

(Rv2955c). All three enzymes were enriched in the immunoprecipitated IMD. Rv2955c and

Rv2959c were also enriched in the IMD based on the IMD / PM-CW comparison. When

mNeonGreen-HA-tagged proteins were analyzed by immunoblot, we found significant amounts

of protein bands that were smaller than the expected molecular weight (not shown). We suspect

that those fluorescent protein-tagged fusions may be prone to protein degradation. To

circumvent the problem, we generated merodiploid strains producing fusion proteins tagged with

a short HA epitope alone. Among them, we detected a protein band that migrated near the

expected molecular weight for Rv2955c and Rv2957 whereas we could not detect Rv2959c at a

significant level. We fractionated a cell lysate from the strain producing either Rv2955c-HA or

Rv2957-HA, and determined the subcellular localization. Both proteins were localized to the

IMD although Rv2955c was distributing more broadly to a lighter density region as well (**Fig. 6B**

and C). Thus, our biochemical analysis validates that the final maturation steps of PGL

synthesis takes place in the IMD. Taken together with proteomic identifications, out data support

the idea that the majority of enzymatic reactions to synthesize PDIM/PGL take place in the IMD.

DISCUSSION

The IMD is proposed to provide a membrane surface for the cell envelope biosynthesis and

growth of *M. smegmatis*, a non-pathogenic model *Mycobacterium* (16, 17). Here, we

demonstrated that a similar membrane organization exists in *M. tuberculosis*. Furthermore, our

data demonstrate that the biosynthesis of PGL, a virulence factor produced by a subset of

pathogenic mycobacteria (36–40), takes place in the IMD. We suggest that the IMD is an

evolutionary conserved subcellular organization in mycobacteria.

A number of metabolic reactions in the IMD are conserved between the two species. First,

GlfT2, a galactofuranosyltransferase involved in arabinogalactan synthesis, was found in the

IMD of both species. Two reactions preceding the GlfT2-driven galactose chain elongation are

mediated by the rhamnosyltransferase WbbL1 (Rv3265c) and the galactofuranosyltransferase

GlfT1 (Rv3782). Both WbbL1 and GlfT1 are enriched in the IMD proteome of *M. smegmatis* as

well as *M. tuberculosis*, indicating that galactan precursor biosynthesis is a conserved metabolic

function of the IMD. Second, polyprenol-phosphate-mannose biosynthesis is IMD-associated in

M. smegmatis and the enzyme Ppm1 is enriched in this membrane domain (16, 17). In *M.*

tuberculosis, we used radiolabeled GDP-mannose to show the enzyme activity of Ppm1 in the

IMD. Furthermore, we detected Ppm1 (Rv2051c) in the *M. tuberculosis* comparative IMD

proteome although we could not detect this protein from the immunoprecipitated IMD. In *M.*

smegmatis, the PPM synthase Ppm1 and the integral membrane protein Ppm2 are produced

separately, while they are produced as a single fused polypeptide in *M. tuberculosis* (41). The

larger and more hydrophobic nature of *M. tuberculosis* Ppm1 may have contributed to the

inefficient detection of this protein by proteomics. Third, PyrD, a menaquinone-dependent

dihydroorotate dehydrogenase involved in pyrimidine biosynthesis, is an IMD-associated protein

in *M. smegmatis* (16, 42), and our current study revealed it as an IMD protein in *M. tuberculosis*

as well. These pathways represent some of the well-characterized evolutionarily conserved

pathways enriched in the IMD. In total, our data showed that 69-73% of the IMD-associated *M.*

tuberculosis proteins had *M. smegmatis* orthologs, which was comparable to the two-species

overlap found in the whole genome, where 63-69% of *M. tuberculosis* protein-coding genes

have *M. smegmatis* orthologs (43, 44).

It is notable that the IMD is often where final maturation steps of lipid biosynthesis take place.

For example, the final step of PE biosynthesis is decarboxylation of phosphatidylserine

mediated by the IMD-associated enzyme Psd (17, 42). We confirmed in this study that Psd

(Rv0437c), which was expressed as a mNeonGreen-HA fusion protein, is an IMD-associated

protein by both subcellular fractionation and fluorescence microscopy. Similarly, during the

synthesis of an electron carrier menaquinone, demethylmenaquinone produced by the PM-CW

enzyme MenA is modified by the IMD-associated demethylmenaquinone methyltransferase

MenG and menaquinone reductase MenJ to become a mature form of menaquinone (19). We

identified both MenG (Rv0558) and MenJ (Rv0561c) in the proteome of immunoprecipitated *M.*

tuberculosis IMD, suggesting a conserved compartmentalization of these final two steps of

menaquinone biosynthesis in the pathogenic species. Finally, peptidoglycan precursor lipid I

matures into lipid II by a GlcNAc transfer reaction mediated by MurG, and MurG is a previously

established IMD protein in *M. smegmatis* (32). In *M. tuberculosis*, MurG (Rv2153c) showed four-

fold enrichment in the IMD compared to the PM-CW, although we could not detect MurG in the

immunoprecipitated IMD proteome. Observations of conserved IMD-associated lipid maturation

steps in multiple lipid biosynthetic pathways indicate the role of the IMD in lipid modifications.

In addition to these shared features, *M. tuberculosis* IMD revealed roles that are specific to

slow-growing pathogens and not found in *M. smegmatis*. In particular, we found multiple

enzymes associated with the PDIM/PGL biosynthesis (Fig. 6A). These lipids are not produced

by *M. smegmatis*, and genes encoding the biosynthetic enzymes are absent in its genome. We

found PpsA-E (Rv2931-2935), polyketide synthases that produce phthiodiolone and

phenolphthiodiolone, enriched in the IMD proteomes. They are large proteins, ranging 159 - 231

kDa, and therefore, we were initially concerned if the IMD enrichment of PpsA and PpsE over

the PM-CW might have been a consequence of these large proteins sedimenting further in a

density gradient than other average-sized proteins due to their large size. However, all five

enzymes were identified in the proteome of immunoprecipitated IMD but not from the mock

immunoprecipitation of a wildtype cell lysate. Therefore, our data suggest that these enzymes

are truly associated with the IMD. Unfortunately, our attempts to express epitope-tagged PpsB

and PpsE were unsuccessful, and validation of the subcellular localization of these enzymes

must await future studies. PapA5 transfers two mycocerosates to either a phthiodiolone or a

phenolphthiodiolone, producing phthiodiolone dimycocerosate or phenolphthiodiolone

dimycocerosate, respectively. These lipids are further modified by sequential reactions of

$F_{420}H_2$-dependent phthiodiolone ketoreductase (fPKR, Rv2951c) and

phthiotriol/phenolphthiotriol dimycocerosates methyltransferase (PMT, Rv2952) to become a

mature PDIM or phenolphthiocerol dimycocerosate (PPDIM), a precursor of PGL (34, 35, 45,

46). Comparative proteomic analysis showed that both fPKR and PMT are enriched in the IMD,

and fPKR was also detected in the immunoprecipitated IMD. We attempted to express fPKR

with C-terminal mNeonGreen-HA or HA tag, but could not detect the protein production by

immunoblotting. fPKR has a predicted molecular weight of 41.3 kDa, and has no predicted

transmembrane domains. Therefore, it is likely that fPKR peripherally associates with the IMD,

but further validations are needed.

Finally, we attempted to produce epitope-tagged glycosyltransferases and methyltransferases

that are involved in the maturation of PPDIM to PGL. Two methyltransferases (Rv2955c and

Rv2959c) and a fucosyltransferase (Rv2957) were found in the IMD proteomes. While we could

not detect stable production of epitope-tagged Rv2959c, we were able to produce Rv2955c and

Rv2957 with C-terminal HA tag. Both proteins were found enriched in the IMD and absent in the

PM-CW, confirming the proteomic analysis. Rv2955c was more widely distributed into lighter

density regions (Fractions 2-3), which is a feature observed for some *M. smegmatis* proteins as

well (*e.g.* MurG, ThiD) (32, 42). The biochemically validated IMD association of two enzymes

involved in the final maturation step of PGL synthesis, combined with other enzymes found in

the IMD proteome, indicate that the PDIM/PGL biosynthesis takes place in the IMD.

In sum, we conclude that the IMD is a conserved membrane domain of mycobacteria enriched

in lipid biosynthetic reactions.

MATERIALS AND METHODS

Cell cultures

M. tuberculosis mc²6230 ΔRD1/panCD strain was used throughout this study. It was grown in

Middlebrook 7H9 broth or Middlebrook 7H10 agar supplemented with OADC (final

concentrations: 190 µM oleic acid, 0.5% (w/v) bovine serum albumin, 11.1 mM dextrose, 13.9

mM NaCl, 0.0004% (w/v) catalase), 0.05% Tween 80, and 50 µg/ml pantothenic acid. When

required, the medium was supplemented with 100 µg/ml hygromycin B (Wako), 20 µg/ml

kanamycin sulfate (MP Biochemicals), 50 µg/ml streptomycin, or 5% sucrose.

Density gradient fractionation and protein analysis

Log phase cells (OD_{600} = 0.5-1.0) were lysed and the lysate was fractionated by sucrose density

gradient as described previously (16). The protein concentration for each fraction was

determined by bicinchoninic acid (BCA) assay (Pierce). Sucrose density of each fraction was

determined by a refractometer (ATAGO). SDS-PAGE and western blotting of the sucrose

gradient fractionation were performed using equal volume of each fraction as described before

(16). Anti-MptC antibody was previously raised (47) and anti-HA antibody was purchased from

Sigma-Aldrich.

Cell-free radiolabeling assay

To determine PPM biosynthesis activities, sucrose density fractions were incubated with GDP-

[2-^{3}H]mannose (American Radiolabeled Chemicals, 20 Ci/mmol) as described before (17),

except that the time of incubation was extended to 1 hour. The lipids were extracted, purified,

and resolved on high-performance thin layer chromatography (HPTLC) silica gel using

chloroform/methanol/13 M ammonia/1 M ammonium acetate/water (180:140:9:9:23, v/v/v/v/v)

as a solvent, and visualized by fluorography using En^{3}Hance.

Lipid analysis

Lipids were extracted from each gradient fraction as described (16) and analyzed by HPTLC as

described above. Phospholipids and PIMs were detected by molybdenum blue and orcinol

staining, respectively.

Negative staining EM

Samples of pooled sucrose gradient fractions of IMD and PM-CW were pelleted at 100,000x *g*

for 60 min on TLA100.2 rotor (Beckman), and washed with HES buffer (25mM HEPES-HCl, pH

7.4, 2mM EGTA, 150mM NaCl) twice. The samples were prepared for EM observation as

described (16).

Proteome preparation and analysis

Pooled IMD and PM-CW fractions were pelleted and washed, as described above, in biological

triplicates. The protein samples were then separated on a 12% SDS-PAGE gel for a short

distance and the gel slice containing the entire protein bands was subjected to in-gel trypsin

digestion. The peptides were analyzed by nano-LC ESI MS on an Orbitrap mass spectrometer

(Thermo Scientific Q Exactive) as described (16). The data were analyzed and annotated using

TubercuList (Release 27, https://mycobrowser.epfl.ch/). Proteins with a minimum of two-fold

enrichment ($p < 0.05$) in either the IMD or the PM-CW were considered to be part of the

respective proteome. Functional annotation was performed using DAVID Bioinformatics

Resources as described (16).

The immunoprecipitated IMD proteome was analyzed using IMD fractions from cells expressing

PyrD-HA, using wild-type as a negative control. Immunoprecipitation using HA-agarose beads

(Pierce), proteome analysis and functional annotation analysis were as described (16).

Construction of plasmids

pMUM036 – To create an expression vector for PyrD (Rv2139), which is C-terminally tagged

with an HA epitope, the *pyrD* gene was amplified with primers A161/A162 from *M. tuberculosis*

genomic DNA (**Table S1**). pMUM012 (16) was digested with EcoRV and ScaI and the vector

backbone was blunt-end-ligated with the PCR product.

pMUM145 – To create a knock-in strain, in which an epitope-tagged PyrD is expressed from the

endogenous locus, the upstream and downstream of the *pyrD* gene were amplified using

primers A536/A537 and A538/A539, respectively (**Table S1**). The PCR products were digested

with Van91I. The two fragments were then ligated into Van91I-digested pCOM1 as previously

described (16) to create an intermediate vector, pMUM133. Using pMV261-CO-TagRFP as a

template, tagRFP was amplified and HA tag was added using primers A557/A558. The PCR

product and pMUM133 were then digested with VspI and BspT1 and ligated to create a knock-in

vector to insert a gene encoding 2xHA-tagRFP at the 3' end of the *pyrD* gene.

pMUM215 – To create expression vector for C-terminally mNeonGreen-HA-tagged Psd

(Rv0437c), *psd* was cloned by PCR using primers A630/A631 (**Table S1**). In a separate

experiment, a gene encoding mNeonGreen was amplified from pMUM044, an unpublished

plasmid which carries the same *mNeonGreen* gene as our published plasmid pMUM072 (16),

using primers A483/A484. The PCR product was blunt-end-ligated with ScaI-digested

pMUM098 (19) to create pMUM111. The PCR product carrying *psd* and pMUM111 were

digested with NdeI and BspTI and ligated to create the final expression vector.

pMUM222 – To create an expression vector for GlfT2 (Rv3808c) with C-terminal mNeonGreen-

HA epitope tag, the *glfT2* gene was amplified with primers A628/A629 (**Table S1**). The PCR

product carrying *glfT2* and pMUM111 were digested with NdeI and BspTI and ligated to create

the final expression vector.

pMUM257 – We first created an expression vector for Rv2955c with C-terminal mNeonGreen-

HA epitope tag. The *rv2955c* gene was amplified with primers A849/850 (**Table S1**). Both the

PCR product carrying *rv2955c* and pMUM235, which is a derivative of pMUM111, were digested

with NdeI and BspTI and ligated to create the Rv2955c-mNeonGreen-HA expression vector

(pMUM245). To remove mNeonGreen, we digested pMUM245 with PacI and AflII, blunt-ended

the digested plasmid using T4 DNA polymerase, and ligated, resulting in pMUM257, the

Rv2955c-HA expression vector.

pMUM258 – As in pMUM257, we first created an expression vector for Rv2957 with C-terminal

mNeonGreen-HA epitope tag. The *rv2957* gene was amplified with primers A851/852 (**Table

S1**). The Rv2957-HA expression vector, pMUM246, and the Rv2957-HA expression vector,

pMUM258, were created following the identical procedure described for pMUM257.

Plasmid constructs were electroporated into *Mycobacterium tuberculosis mc^{2}6230*

ΔRD1/panCD for integration and homologous recombination as previously described (16).

Fluorescence microscopy

Standard fluorescence microscopy was performed as described (42). To distinguish between

cells with or without septa, cells were labeled with the peptidoglycan probe HADA (Tocris

Bioscience, Bristol, UK). *M. tuberculosis* cells expressing GlfT2-mNeonGreen-HA were grown to

mid-log (OD$_{600}$ = 0.5) and then labeled with 500 µM HADA for 2 hour. Labeled cells were

washed twice in PBS, spotted onto a 1% agar pad slide and covered with a glass coverslip sealed to the slide with nail polish. Live cells were then imaged with a Nikon Eclipse E600 with a 100x objective lens. mNeonGreen fluorescence profiles along cell lengths were calculated with Oufti (48) and sorted based on the presence or absence of HADA-labelled septa. mNeonGreen fluorescence profiles of septated and non-septated populations were aligned by their brightest pole, normalized by cell length and then averaged using a custom Python script. For SIM, images were acquired by Nikon Eclipse Ti N-SIM E microscope equipped with a Hamamatsu Orca Flash 4.0 camera (numerical aperture,1.49) as described before (12), and reconstructed on NIS Elements imaging software.

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FIGURE LEGENDS

Figure 1. Membrane compartmentalization in *M. tuberculosis* mc²6230. **A**. Sucrose density

gradient fractionation of attenuated *M. tuberculosis* strain. The sucrose density and protein

concentration of the fractions are shown. **B**. Western blot detection of cytoplasmic marker Ino1

(40 kDa) and PM-CW marker MptC (47 kDa). **C**. Distribution of membrane phospholipids across

the gradient fractions. Phospholipids were stained by molybdenum blue staining. PIMs were

detected by orcinol staining. **D.** Cell-free PPM biosynthesis detection by GDP-[³H]-Mannose

radiolabeling. **F.** Negative staining EM of the IMD and PM-CW fractions.

Figure 2. Comparative proteome of the conventional plasma membrane (PM-CW) and the IMD.

Cell growth, lysate preparation, membrane purification and proteomic analysis were repeated

three times. **A.** The volcano plot of proteins enriched in either the PM-CW or the IMD. Proteins

that were enriched more than two-fold with $p < 0.05$ were considered significant (black dots).

Other identified proteins are shown in gray dots. Red dots correspond to significantly enriched

proteins involved in PGL/PDIM biosynthesis. Green dots correspond to other proteins analyzed

biochemically and/or by fluorescent microscopy in this study. **B.** Functional annotation of

proteins enriched in the membrane fractions using UniProt keywords, showing that the IMD is

enriched in proteins related to membrane and lipid metabolism. The enrichment represents the

proportion of the term in the IMD proteome compared to its expected proportion in the genome.

The area of the circles are proportional to the number of proteins in each category.

Figure 3. PyrD is an IMD-associated protein. **A**. Western blotting of a crude lysate prepared

from a merodiploid *M. tuberculosis* strain producing PyrD-HA. In this strain, the expression

vector was integrated at L5 *attB* site. Expected molecular weight of PyrD-HA is 42.1 kDa. **B.**

Sucrose density gradient fractionation. The density and protein concentration for each fraction

are shown. **C**. Western blot detection of PyrD-HA in the IMD fractions and the PM-CW marker

MptC (Rv2181, 47 kDa). **D.** Proteome of an immunoprecipitated IMD. IMD was purified from the

PyrD-HA-expressing strain using anti-HA antibody. Left, Venn diagram showing the overlap of

proteins found in the comparative IMD proteome (Fig. 2) and immunoprecipitated IMD

proteome. Right, Venn diagram showing the orthologs found commonly in the

immunoprecipitated IMD proteomes of *M. tuberculosis* and *M. smegmatis*.

Figure 4. PyrD-2xHA-tagRFP, expressed from the endogenous locus, showing IMD

association. **A**. Sucrose gradient density fractionation of *M. tuberculosis* strain where the

endogenous *pyrD* gene was replaced with *pyrD-2xHA-tagRFP*. Sucrose density and protein

concentration for each fraction are shown. **B**. Western blot showing the IMD localization of

PyrD-2xHA-tagRFP (68.2 kDa). MptC, PM-CW marker (47 kDa). Experiments are repeated

twice and representative results are shown.

Figure 5. Polar enrichment and sidewall patches of GlfT2-mNeonGreen-HA, an IMD-associated

protein. **A.** Density gradient and protein concentration profile. **B.** Western blotting of GlfT2-

mNeonGreen-HA (100.9 kDa). MptC, PM-CW marker. **C.** Conventional fluorescence

microscopy images of log phase cells. **D.** SIM images of log phase cells. **E.** Cells expressing

GlfT2-mNeonGreen-HA, labeled with HADA for 2 hours and visualized by conventional

fluorescence microscopy. **F.** Fluorescence intensity profile of GlfT2-mNeonGreen-HA from cells

labeled with HADA, for which representative images are shown in the panel E. Cells were

categorized into two populations based on the presence or absence of a septum, as determined

by HADA labeling. Intensity profiles are aligned with the brightest pole on the left and are shown

in arbitrary units. Green and blue fluorescence intensity profiles correspond to GlfT2-

mNeonGreen-HA and HADA respectively. Experiments are repeated at least twice and

representative results are shown.

Figure 6. PDIM and PGL biosyntheses take place in the IMD. **A.** Biosynthetic pathways of

PDIM and PGL. Enzymes involved in the biosyntheses are shown in ovals, which are color-

coded to indicate the subcellular localization based on proteomic analyses. **B.** Western blotting

of Rv2955c-HA (37.8 kDa). **C.** Western blotting of Rv2957-HA (32.9 kDa). MptC, PM-CW

marker.

Supplemental Figures

Figure S1. Cell-free PPM biosynthesis detection of crude lysate by GDP-[^{3}H]-Mannose

radiolabeling, comparing *M. smegmatis* and *M. tuberculosis*.

Figure S2. IMD association of Psd-mNeonGreen-HA. **A.** Density gradient and protein

concentration profile. **B.** Western blotting of Psd-mNeonGreen-HA (53.5 kDa). MptC, PM-CW

marker. **C.** SIM images of log phase cells. Experiments are repeated twice and representative

results are shown.

Table S1. Primers to create plasmids used in this study.

Plasmid	Gene ID	Protein	Forward Primer Reverse Primer	
pMUM036	Rv2139	PyrD	A161	ATCCATATGTATCCCCTGGTGCGT
			A162	ACTGCTGGGTTGCCGACGTCTT
pMUM145	Upstream of Rv2139	PryD knock-in	A536	TTTTTTTTTCCATAGATTGGCACAAGTTGGTTTTCGCCGTG
			A537	TTTTTTTTTCCATATTTTGGATTAATGCTGGGTTGCCGACGTCTT
	TagRFP		A557	TTTTTTTTTATTAATTACCCGTACGACGTCCCGGACTACGCGTACCC GTACGACGTCCCGGACTACGCGCAATTGAGCAAGGGCGAGGAGCTG
			A558	TTTTTTTTTCTTAAGCTTGTAGAGCTCGTCCATGCC
	Downstream of Rv2139		A538	TTTTTTTTTCCATAATTTGGCTTAAGTAAAGCGCTAACGCTGCTCG
			A539	TTTTTTTTTCCATCTTTTGGATACAAGGCCAACGTCGTCC
pMUM215	mNeonGreen	Psd- mNeon Green	A483	ACTCTTAAGATGGTTAGTAAAAGAGAAGAAGA
			A484	ACTGTTAATTAACAATTGTTTGTAGAGCTCATCCATGCCA
	Rv0437c		A630	TTTTTTTTTTCATATGGTGGCCAGACGCCCCCGC
			A631	TTTTTCTTAAGCAATTGTCGACATTCGGCCAGCACGG
pMUM222	Rv3808c	GlfT2	A628	TTTTTTTTTTCATATGATGAGTGAACTCGCCGC
			A629	TTTTTCTTAAGCAATTGGCCATGCTCGGGCTCT
pMUM257	Rv2955c	Rv2955c	A849	TTTTTTTTTCATATGCAGTTCCAAGATGTGCGCC
			A850	TTTTTTTTTCTTAAGTCCCTTCCGGCTCGGAATAAAGA
pMUM258	Rv2957	Rv2957	A851	GTGGCAGAAAAGACGTCCGA
			A852	CACGTCGTAGTAGAGCGAGAACT

Figure 1

A

B

Ino1

MptC

C

CL
PE
PI

Ac₂PIM₂
AcPIM₂

Ac₂PIM₆
AcPIM₆

D

PPM

PIMs

IMD   PM-CW

E

IMD   PM-CW

Figure 2

A

Figure 3

Figure 4

Figure 5

Fig. 6

A. PDIM and PGL Biosynthesis

Figure S1

Figure S2

A

B

MptC

C

Phase   mNeonGreen

5 µm