Membrane compartmentalization of mycobacterial peptidoglycan synthesis

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Abstract: Cell wall peptidoglycan, a mesh of polysaccharides crosslinked by short peptides, encases the bacterial cell and protects it from turgor pressure lysis. Peptidoglycan synthesis is an effective antibiotic target. Assembly of the biopolymer occurs in close association with the plasma membrane, but higher order organization of the process has not been described. In mycobacteria, intracellular membrane domains comprise biochemically and spatially distinct regions within the conventional plasma membrane. We find that lipid-linked peptidoglycan precursors are made in these domains and then trafficked to the conventional plasma membrane for insertion into the

cell wall. Disorganization of the membrane rapidly delocalizes and then halts peptidoglycan assembly. Our data show that membrane compartmentalization is an essential feature of mycobacterial cell wall biogenesis.

Main Text: Many antibiotics target peptidoglycan synthesis, a well-conserved pathway that spans the cytoplasm, plasma membrane and periplasm. The polyprenol-linked, disaccharide-pentapeptide monomer lipid II is completed by the glycosyltransferase MurG in the inner leaflet of the plasma membrane (Fig. 1A). Lipid II is then flipped to the outer leaflet by MurJ and integrated into the cell wall by membrane-bound transglycosylases and transpeptidases from the penicillin-binding protein (PBP) and shape, elongation, division, and sporulation (SEDS) families (*1-4*).

The plasma membrane is a heterogeneous mixture of lipids and proteins. Mycobacteria, for example, have **i**ntracellular **m**embrane **d**omains (IMD, formerly called the PMf (*5*) for **p**lasma **m**embrane **f**ree of cell wall) that are separable from the conventional plasma membrane (designated the PM-CW, for **p**lasma **m**embrane tightly associated with **c**ell **w**all) by sucrose density gradient fractionation. The proteome and lipidome of IMD are distinct from PM-CW (*5, 6*). While PM-CW-resident proteins localize along the perimeter of live mycobacteria, IMD-resident proteins localize along sidewall but are enriched adjacent to sites of polar cell elongation (*6, 7*).

Our proteomics analysis indicated that MurG is present in the IMD while sequentially-acting PBPs preferentially associate with the PM-CW (*6*). We also observed *in situ* that the subpolar enrichment of MurG-RFP resembles that of the validated IMD marker mCherry-GlfT2 or GlfT2-GFP (*6, 8*) but that nascent cell wall at

the mycobacterial poles primarily abuts rather than colocalizes with mCherry-GlfT2 (*7*). These observations suggest that lipid II synthesis is biochemically and spatially segregated from the subsequent steps of cell wall assembly (Fig. 1A).

We expressed a functional MurG-Dendra2 fusion in *Mycobacterium smegmatis* (Fig. S1) and assayed its distribution in membrane fractions that had been separated by density gradient (Fig. 1B). The fusion to MurG, a peripheral membrane protein, was enriched in both the cytoplasmic and IMD membrane fractions (Fig. 1C; Fig. S2), recapitulating the association predicted for the native protein (*6*). In intact cells, polar enrichment of MurG-Dendra2 was coincident with that of mCherry-GlfT2 (Fig. 1D). The spatial relationship between the proteins was similar to that of MurG-RFP and GlfT2-GFP (*8*), suggesting that it is independent of the fluorescent protein tag.

The association of MurG with the IMD, but not with the PM-CW, implied that the membrane domain is the site of lipid II synthesis. We refined an *in vitro* D-amino acid exchange assay to detect lipid-linked peptidoglycan precursors from *M. smegmatis* membrane fractions (Fig. 2A; *9, 10*). In wildtype cells, we detected biotinylated molecules in both the IMD and PM-CW (Fig. 2B; Fig. S2). We hypothesized that the labeled species comprised precursors in the inner leaflet of the plasma membrane as well as lipid II that had been flipped to the outer leaflet. We, and others, have shown that depletion of MurJ results in an accumulation of biotinylated precursors (*10, 11*; Fig. S3). By performing the D-amino acid exchange reaction on membrane fractions, we found that precursors accumulate in the IMD (Fig. 2B; Fig. S2). These results suggest that lipid II is made in the IMD and transferred to the PM-CW in a MurJ-dependent manner.

PBPs and SEDS proteins incorporate lipid II into peptidoglycan. Given the trafficking of precursors from the IMD to the PM-CW (Fig. 2B) and the association of cell wall fragments specifically with the PM-CW (*5*), we hypothesized that extracellular, peptidoglycan-acting enzymes function in the PM-CW. While our proteomics did not detect SEDS proteins, our PM-CW dataset was enriched for all of the known mycobacterial PBPs (*6*). Fluorescent derivatives of β-lactam antibiotics such as Bocillin-FL bind covalently to PBPs and can be used to image transpeptidase-active enzymes in both polyacrylamide gels and intact cells. We incubated membrane fractions from wildtype *M. smegmatis* with Bocillin-FL and identified fluorescent proteins in the PM-CW but not the IMD (Fig. 2B; Fig. S2). As expected for PBPs, the signal from these bands was diminished by pre-treatment with the β-lactam ampicillin (Fig. S4). We focused on characterizing PonA1, a bifunctional transglycosylase/transpeptidase that is essential for *M. smegmatis* growth (*12, 13*). Depletion of PonA1 (*12*) resulted in the loss of the highest molecular fluorescent band (Fig. S4), confirming that this protein is present and active specifically in the PM-CW (Fig. 2B). We next expressed a functional PonA1-mRFP fusion in *Mycobacterium smegmatis* (*13*) and found that it was more evenly distributed around the cell perimeter than MurG-Dendra2, and in a manner similar to the functional PM-CW marker PimE-GFP (Fig. 2C, Fig. S5; *6*). Together, our data show that MurG and PonA1 occupy spatially distinct compartments along the pathway of peptidoglycan synthesis.

Based on our biochemical data, we hypothesized that lipid II incorporation into the cell wall is laterally segregated from its synthesis. We previously showed that alkynyl and azido D-amino acid dipeptides (*14*) incorporate into lipid-linked

peptidoglycan precursors in *M. smegmatis* (*10*) and that metabolic labeling with alkynyl dipeptide (alkDADA or EDA-DA) is most intense in regions adjacent to the IMD marker mCherry-GlfT2 (*7*). We labeled MurG-Dendra2-expressing *M. smegmatis* with alkDADA for ~1% of generation time and detected the presence of the alkyne by copper-catalyzed azide-alkyne cycloaddition (CuAAC; Fig 2D; *10*). To tune our detection for extracellular alkynes present in lipid II and newly-polymerized cell wall, we selected picolyl azide-Cy3 as our label because the localized charge on the sulfonated cyanine dye confers poor membrane permeability (*15*). Using this optimized protocol, we observed nascent peptidoglycan deposition at the polar tip, whereas MurG-Dendra2 was more posterior (Fig. 2D). Our data support a model in which lipid II synthesis is laterally and biochemically partitioned from the ensuing steps of peptidoglycan assembly.

In the Gram-positive bacterium *Bacillus subtilis*, MurG associates with **r**egions of **i**ncreased **f**luidity (RIFs) in the plasma membrane that are marked by the accumulation of certain lipophilic fluorescent dyes (*16*). Benzyl alcohol has been used to disperse plasma membrane domains in plant, animal and bacterial cells, including *B. subtilis* RIFs (*16-18*). Mycobacteria are also Gram-positive but have a second 'myco' membrane that is covalently attached to the cell wall. We found that benzyl alcohol did not alter labeling by NalkTMM or OalkTMM (Fig. S6), which respectively incorporate into the noncovalent and covalent mycolates of the mycomembrane (*19*). It did, however, alter the distribution of FM4-64 (Fig. S7), a dye used previously to label the plasma membrane (*20*), and altered glycolipid abundance in the IMD (Fig. S8). MurG-Dendra2 was also notably less enriched in the IMD following benzyl alcohol treatment (Fig. 1C)

and, in live cells, at the cell poles (Fig. 3A). By contrast benzyl alcohol produced subtle changes in the distribution of active PBPs (Fig. 2B), although PonA1 shifted toward the poles in live cells (Fig. 3A). Disruption of plasma membrane organization by benzyl alcohol was accompanied by delocalization of cell wall assembly within 5 min (Fig. S9) as well as an overall reduction in synthesis (Fig. 3B, Figs. S9) and halt in cell elongation (Fig. S6). The phenotypes were reversible: we recovered viable, peptidoglycan-synthesizing cells following benzyl alcohol washout (Figs. S10). These data suggest that membrane partitioning is an essential feature of both peptidoglycan synthesis and cell growth in *M. smegmatis*.

We find that benzyl alcohol also inhibits cell wall assembly in *B. subtilis* (Fig. 3C), demonstrating that plasma membrane architecture may optimize peptidoglycan biogenesis in bacterial phyla with divergent cell envelope structures and modes of growth. Moreover, our previous work suggests that the biosynthetic pathways for phosphatidylethanolamine, a major phospholipid of mycobacterial plasma membrane, phosphatidylinositol mannoside, a cell envelope glycolipid, and menaquinone, the primary lipid electron carrier of the mycobacterial respiratory chain, are partitioned across the IMD and PM-CW (*5, 6, 21*). In the absence of a standard set of membrane-bound organelles, plasma membrane compartmentalization may be a general bacterial strategy for organizing pathways with lipid-linked intermediates and enzymes.

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Supplementary Materials:

Materials and Methods

Figures S1-S10

Tables S1

Fig. 1. MurG is enriched in the IMD. (A) Membrane-bound steps of peptidoglycan synthesis with hypothesized partitioning into the IMD and PM-CW. NAM, N-acetylmuramic acid; NAG, N-acetylglucosamine; circles, amino acids: light green, L-ala; red, D-glu; deep blue, diaminopimelic acid; yellow, D-ala. (B) Plasma membrane fractionation. Bacteria are lysed by nitrogen cavitation and cell lysate is sedimented on a sucrose density gradient. (C) Lysates from MurG-Dendra2-expressing *M. smegmatis* were fractionated as in (B) and separated by SDS-PAGE. Protein detected by in-gel fluorescence. Incubation of bacteria with 100 mM benzyl alcohol (BA) for 1 hour decreased the enrichment of MurG-Dendra2 in the IMD. (D) *M. smegmatis* coexpressing MurG-Dendra2 and mCherry-GlfT2 was imaged by structured illumination

228 microscopy (SIM-E, left). Fluorescence distribution of the fusion proteins from 59 cells
229 was quantitated from parallel conventional fluorescence microscopy (right). Signal was
230 normalized to the length and total fluorescence intensity of the cell. Cells were oriented
231 such that the brighter pole is on the right hand side of the graph. a.u., arbitrary units,
232 scalebar 5 μm.
233
234 **Fig. 2.** Lipid II is synthesized in the IMD and trafficked to the PM-CW. (A) Detection of
235 lipid-linked peptidoglycan precursors from organic extracts of *M. smegmatis* membrane
236 fractions. Terminal D-alanines (yellow) of endogenous precursors are exchanged for
237 biotin-D-lysine (BDL; pink) via purified *S. aureus* PBP4. Biotinylated species are
238 detected by blotting with streptavidin-HRP. (B) Detection of peptidoglycan precursors
239 and PonA1 activity from density gradient fractions. Precursors are in both the IMD and
240 PM-CW in wildtype *M. smegmatis* but accumulate in the IMD upon MurJ depletion (*10*).
241 PonA1 binds Bocillin-FL in the PM-CW before or after 1 hour of 100 mM benzyl alcohol
242 treatment. Wildtype *M. smegmatis* membrane fractions (50 μg/mL of total protein each)
243 were incubated with 40 μM Bocillin-FL and separated by SDS-PAGE. Active PBPs
244 detected by in-gel fluorescence in PM-CW. PonA1 was identified in Fig. S3. (C) SIM-E
245 of *M. smegmatis* coexpressing PonA1-mRFP and PimE-GFP, scalebar 5 μm. (D) Top,
246 metabolic labeling of mycobacterial cell wall synthesis (*10*). Bottom, *M. smegmatis*
247 expressing MurG-Dendra2 were incubated with alkDADA for 2 min (~1% generation).
248 Surface-exposed alkynes on fixed cells were detected by CuAAC with picolyl azide-Cy3
249 (*10*). Cells imaged by SIM-E, scalebar 5 μm.
250

Fig. 3. Perturbation of plasma membrane organization disrupts peptidoglycan biogenesis. (A) *M. smegmatis* coexpressing PonA1-mRFP and MurG-Dendra2 were imaged +/- benzyl alcohol by SIM-E (left). Fluorescence distribution of the fusion proteins from 42<n<56 cells was quantitated from parallel conventional fluorescence microscopy (right). Signal was normalized as Fig. 1D, scalebar 5 μm. (B) Wildtype *M. smegmatis* +/- benzyl alcohol were incubated with both azido D-amino acid dipeptide and mycomembrane probe OalkTMM (*10, 19*) for 15 min and fixed in 2% formaldehyde. Alkynes and azides were detected by sequential CuAAC reactions with picolyl azide TAMRA and alkyne carboxyrhodamine 110 (Click Chemistry Tools) labels with a wash step between, scalebar 5 μm. (C) *B. subtilis* were exposed to indicated antibiotics or benzyl alcohol for 10 min then incubated with alkDADA for an additional 5 min. Cells were fixed and alkynes were detected by CuAAC with picolyl azide CR 110. MFI, median fluorescence intensity obtained by flow cytometry. Experiments performed three times in triplicate. Error bars, +/- standard deviation of biological replicates. ***, $p < 0.0005$; ****, $p < 0.00005$, Tukey multiple comparison test.