March 2017

A Novel Periplasmic Protein involved in the Mannan Chain Elongation Step of Lipomannan and Lipoarabinomannan Biosynthesis in Mycobacterium smegmatis

Stephanie A. Ha
University of Massachusetts Amherst

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A NOVEL PERIPLASMIC PROTEIN INVOLVED IN THE MANNAN CHAIN ELONGATION STEP OF LIPOMANNAN AND LIPOARABINOMANNAN BIOSYNTHESIS IN MYCOBACTERIUM SMEGMATIS

A Thesis Presented
By
STEPHANIE ALLEN HA

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment Of the requirements for the degree of
MASTER OF SCIENCE

February 2017

Department of Microbiology
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Approved as to style and content by:

Yasu S. Morita, Chair

Michele M. Klingbeil, Member

M. Sloan Siegrist, Member

Steven J. Sandler, Department Head
Department of Microbiology
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ABSTRACT

A NOVEL PERIPLASMIC PROTEIN INVOLVED IN THE ELONGATION STEP OF LIPOMANNAN AND LIPOARABINOMANNAN BIOSYNTHESIS IN MYCOBACTERIUM SMEGMATIS

FEBRUARY 2017

STEPHANIE ALLEN HA, B.S. UNIVERSITY OF CONNECTICUT
M.S. UNIVERSITY OF MASSACHUSETTS AMHERST
Directed by: Dr. Yasu S. Morita

Mycobacteria are atypical bacteria possessing unusual cell envelopes comprised of an outer membrane, covalently linked to an arabinogalactan-peptidoglycan structure via waxy mycolic acids, in addition to the conventional inner membrane. This thick and highly impermeable cell envelope is a major deterrent to antibiotic treatment of clinically relevant mycobacterial pathogens, including Mycobacterium tuberculosis (Mtb), which infects a third of the world’s population and kills millions each year. Thus, the regulation of mycobacterial cell envelope biosynthesis is of great interest for the development of more effective therapeutics for treating Mtb infections. Using the model organism Mycobacterium smegmatis (M. smegmatis), we identified a novel protein, Spe2, with an unknown role in the biosynthesis of the cell envelope glycolipids lipomannan (LM) and lipoarabinomannan (LAM). Based on the observation that Δspe2 mutants produce truncated LM/LAM, I speculated Spe2 might enhance the elongation of these products. Here, I use biochemical assays to show Spe2 is localized to the periplasm where it can directly interact with the
LM/LAM biosynthetic pathway. I further utilize a genetic approach to demonstrate that Spe2 acts at the stage in which the mannosyltransferase MptA mediates periplasmic LM elongation. Moreover, native polyacrylamide gel electrophoresis (PAGE) and co-immunoprecipitation techniques failed to reveal Spe2 protein binding partners. Together, these data suggest Spe2 is a periplasmic protein involved in regulating LM/LAM biosynthesis, perhaps through direct interactions with LM intermediates.
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CHAPTER 1

BACKGROUND AND SIGNIFICANCE

1.1 Mycobacteria and Disease

The genus *Mycobacterium* consists of a diversity of sturdy microorganisms, of which many are important clinical and veterinary pathogens (1, 2). Major mycobacterial species of clinical relevance are outlined in Table 1. *Mycobacterium tuberculosis* (*Mtb*) is perhaps the most prominent of these organisms, although *Mycobacterium leprae* has historically been problematic for humans as well (2, 3). These pathogenic species of mycobacteria are generally slow growers; *Mtb*, for example, has a generation time of up to 24 hours (4). This slow growth makes culturing for diagnostics and research particularly difficult. As a result, *Mycobacterium smegmatis* (*Msm*) is a fast-growing saprophytic commensal commonly used as a model organism in place of these slow growers. Additionally, cloning in mycobacteria has greatly expanded due to the creation of strains engineered for efficient transformation, including the mc2155 strain of *Msm* that is now referred to as “wild-type” *Msm* (5). These *Msm* strains can be used to study mycobacterial physiology and other cellular processes to gain a solid foundation prior to studying these concepts in *Mtb*.

1.2 Tuberculosis

Tuberculosis (TB) is the outcome of human infection with *Mtb*, an obligate aerobic bacterium characterized by slow-growth, fastidiousness, and an unusual
acid-fast cell wall (6). *Mtb* infects a third of the world’s population—killing millions annually (7). In fact, despite improvements in controlling TB, *Mtb* killed 1.4 million individuals in 2015 alone (7). Most *Mtb* infections are considered latent, in which the bacteria are suppressed by the immune system. However, active infection, in which *Mtb* is not contained, occurs in individuals with weakened immune systems and represents roughly 5-15% of TB cases (7). Moreover, pulmonary TB is concurrent with the human immunodeficiency virus (HIV) epidemic, meaning those infected with HIV are prone to *Mtb* infection. In 2015 alone, the World Health Organization (WHO) has reported that 0.4 million HIV-positive individuals died of *Mtb* infection (7).

### Table 1. Characteristics of Clinically Important Mycobacteria*

<table>
<thead>
<tr>
<th>Organism</th>
<th>Reservoir</th>
<th>Main Disease Caused in Humans</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Mycobacterium tuberculosis</em></td>
<td>Human</td>
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</tr>
<tr>
<td><em>Mycobacterium leprae</em></td>
<td>Human</td>
<td>Leprosy</td>
</tr>
<tr>
<td><em>Mycobacterium ulcerans</em></td>
<td>Environment</td>
<td>Skin ulcerations</td>
</tr>
<tr>
<td><em>Mycobacterium bovis</em></td>
<td>Animals</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td><em>Mycobacterium marinum</em></td>
<td>Water, Fish</td>
<td>Skin granuloma</td>
</tr>
<tr>
<td><em>Mycobacterium avium</em></td>
<td>Environment</td>
<td>Tuberculosis-like</td>
</tr>
</tbody>
</table>

*Adapted from *Schaechter's Mechanisms of Microbial Disease* (p.259) by N. Engleberg, T. Dermody, and V. DiRita (Eds), 2013, Baltimore, MD: Lippincott Williams & Wilkins.*
Drug resistance in *Mtb* has been on the rise, with multi-drug resistant TB (MDR-TB) and extremely drug resistant TB (XDR-TB) steadily emerging (8, 9). This complicates the already lengthy and toxic treatment regimen of first-line and second-line antibiotics (10). Thus, there is a great need for the development of new treatments to combat TB infection. Recently, mutations in cell envelope glycolipid biosynthetic pathways have been shown to increase mycobacterial antibiotic sensitivity while simultaneously diminishing cell wall integrity (11). These studies implicate the mycobacterial cell envelope as a promising drug target for novel therapeutic strategies in treating TB infection.

1.2.1 Pathogenesis

1.2.1.1 Encounter

Mycobacteria are naturally abundant in the environment. However, in the case of *Mtb*, humans are the natural reservoir (4, 12). Aerosolized droplets produced from the coughs of an infected individual are the mechanism of spread from person to person. The infectious dose (ID50) of *Mtb* is estimated to be a range within 1-200 organisms required to establish an infection (13, 14).

1.2.1.2 Invasion

Within the alveoli of the lungs, *Mtb* primarily infects resident alveolar macrophages, although other immune cells such as dendritic cells and alveolar epithelial type II pneumocytes are potential first contacts as well (15). *Mtb* utilizes a number of strategies to enhance its uptake into these macrophages, including the use of
complement factors, surfactants, and antibody-mediated opsonization (16). Primarily, non-opsonic intake via contact with mannose receptors on macrophages, which can be upregulated by the presence of the glycoprotein surfactant protein A in the alveolar environment, increases Mtb binding and promotes phagocytosis (15, 16). Another route of entry into macrophages is through recognition of pathogen associated molecular patterns (PAMPs)—better known as microbial associated molecular patterns (MAMPs)—by Toll-like receptor 2 (TLR2). TLR2 activation, which in turn enhances CD14 affinity to Mtb, further increases the pathogen's uptake. Opsonic methods of internalization, such as complement receptor 3 (CR3), or FC-γ receptors (FCγRs) which also promotes host production of pro-inflammatory mediators, are utilized by Mtb (16). The fact that Mtb is capable of using various routes of entry highlights the pathogen's ability to readily compensate with alternative mechanisms.

1.2.1.3 Establishment of infection

Once Mtb is phagocytosed, the bacterium resides in a phagosome which later fuses with a lysosome to create a highly acidic and degradative environment. However, Mtb possesses clever strategies to impede phagosome maturation by interfering with the Ca²⁺, calmodulin, phosphoinositide 3-kinase (PI3K) signaling cascade. This prevents both ATPase incorporation into the phagosome, rendering it unable to create an acidic environment, and the delivery of lysosomal hydrolases to degrade Mtb (16, 17). In addition to blocking phagosome maturation, Mtb also is capable of impeding apoptosis and interfering with antigen presentation. For instance, the
serine hydrolase Hip1 is known to inhibit the expression of major histocompatibility complex II (MHC II) which is used by macrophages and dendritic cells to present antigens (16). *Mtb* also forces the infected macrophage to sequester MHC II through the reduction of the host protease cathepsin S, which results in the simultaneous reduced export of mature MHC II and increased export of immature MHC II (16). As a result, these strategies directly affect antigen-presenting cells and ultimately slow down the progression of the adaptive immune response for increased *Mtb* survival.

1.2.1.4 Role of PIMs/LM/LAM in TB Pathogenesis

The phosphatidyl-inositol (PI) based glycolipids including PI-mannosides (PIMs), lipomannan (LM), and lipoarabinomannnan (LAM) are abundant on mycobacterial cell envelopes. These glycolipids are important immunomodulatory molecules that aid in *Mtb* invasion and survival in the host on multiple fronts. Mannose-capped LAM, or ManLAM, binds to mannose receptors on the macrophage, enhancing *Mtb* internalization (16). However, structural differences in ManLAM, such as more or less branching, between species and strains have been noted to alter the affinity of *Mtb* attachment to macrophages (18). ManLAM additionally binds to Dectin-2 and dendritic cell specific intercellular adhesion molecule 3 grabbing non-integrin (DC-SIGN) in dendritic cells to elicit TNF-α, IL-6, and IL-10 secretion (19). DC-SIGN and mannose receptor mediated binding appears to be contingent on recognition of the mannose cap of ManLAM, as PI-capped LAM (PILAM) is not recognized by these receptors (20–22). ManLAM also blocks PI3K recruitment to the phagosome, which inhibits phagosome maturation and enables *Mtb* survival as described above (16).
Furthermore, PIMs, LM, LAM are TLR2 agonists that induce pro-inflammatory responses, such as the production of pro-inflammatory cytokines IL-10, TNFα, that ultimately cause host damage (16, 17, 23). The acylation of PIMs also appears to be an important factor for TLR2 activation (24). Interestingly, \textit{Mtb phoP} mutants lacking the transcriptional regulator PhoP possess a greater proportion of monoacylated ManLAM and decreased multi-acylated ManLAM (25). Interference of lipid raft signaling, also key to phagosome maturation, is also thought to occur, since TLR2 activation also leads to increased \textit{Mtb} binding to CD14 via LAM (26). Both LM and LAM have been shown to incorporate into lipid rafts, which can be blocked by the addition of PIMs (27). Additionally, PIMs, LM, and ManLAM can induce early stage granuloma formation, presumably through TLR2 activation—although this has not been demonstrated (23).

It is important to note that during studies of LAM-induced immunological response in the host, the cell line used and method of preparation of LAM both affect the maturation and cytokine profiles, and appears to be the cause of confusing cytokine profiles published throughout the literature (18). Despite this, it is clear that clinically isolated \textit{Mtb} strains induce greater TNF production in human macrophages compared to the virulent laboratory strain H37Rv, while H37Rv causes increased TNF production compared to attenuated H37Ra (18).
1.3 Mycobacterial Cell Structure: Atypical Bacteria with Unusual Cell Walls

1.3.1 Overview of Arrangement and Components

Mycobacteria possess an unusual cell envelope which contains an inner membrane and an arabinogalactan-peptidoglycan (AG-PG) layer, in addition to a mycomembrane (Figure 1). The mycomembrane consists of an outer membrane along with its associated glycolipids, free lipids, and waxy mycolic acids, which are covalently linked to the AG-PG structure (6, 28). This mycolyl arabinogalactan-peptidoglycan (mAGP) complex is considered the mycobacterial cell wall core—an insoluble structure critical to cell viability (6).

Furthermore, the presence of phospholipids such as phosphatidylinositol (PI), which is typically found in eukaryotic cells, is rather unusual in bacteria. The PI-based glycolipids include PI mannosides (PIMs), lipomannan (LM), and lipoarabinomannan (LAM), which will be discussed further below.

1.3.2 Biosynthesis of PIMs/LM/LAM

PIMs, LM, and LAM originate from the same initial pathway (Figure 2). PI on the inner membrane is acylated and has sequential mannose additions in a few major steps. First, the essential enzyme, MSMEG_2935 or PimA, adds the first mannose by transferring the residue from the mannose donor GDP-Man, resulting in the precursor PIM (29–31). This structure can next be modified into AcPIM2 when the acyltransferase phosphatidyl-myoinositol mannosides acyltransferase A, or PatA, transfers a fatty acid to the PIM (31, 32), followed by MSMEG_4253, or PimB'.
mediated mannose addition (31). The reverse reaction (formation of PIM2 followed by PatA acylation) is also thought to occur, but with lower preference (31). From AcPIM2, sequential mannoses are further added, with products AcPIM3 through AcPIM6 receiving mannoses from the mannose donor polyprenol-phosphate mannose (PPM) rather than GDP-Man (33–35).

The intermediate AcPIM4 is considered a branching point, in which it becomes the substrate for either PimE (MSMEG_5149), which transfers the fifth mannose to produce the final PIM product AcPIM6 (34), or the first (unidentified in Msm) mannosyltransferase of the LM/LAM biosynthetic pathway which begins the elongation of a mannose backbone via α1,6 linkages (11, 36–38). The mannosyltransferase MSMEG_4241, or MptA, further elongates the LM intermediate through α1,6 linkages to form mature LM, which possesses up to 34 mannose residues (11, 36). Simultaneously, the mannosyltransferase MSMEG_4247, or MptC, branches this backbone by addition of mannoses via α1,2 linkages (38). The expression levels of MptA and MptC seem to regulate the length of the mannan chain, as the overexpression of MptC results in shorter LM and LAM whereas the overproduction of MptA causes longer LM and LAM to be formed (38). Another potential mechanism for the regulation of LM and LAM production is through the lipoprotein LpqW (MSMEG_5130), which has been suggested to help channel AcPIM4 towards LM/LAM biosynthesis (39–42).

While LM is also a major end product, LAM is thought to be produced from LM through the addition of arabinose residues by the chain extending
arabinofuranosyltransferase EmbC (43, 44). EmbC (Mtb: Rv3793, Msm: MSMEG_6387) is essential in Mtb, but not in Msm, which supports the importance of LAM in Mtb pathogenesis (44). AftC (MSMEG_2785) and AftD (MSMEG_0359), in which the latter is interestingly essential only in Msm, are α1,3 arabinofuranosyltransferases with roles in both LAM and arabinogalactan biosynthesis (45–47). Mtb and other slow growing pathogenic mycobacteria seem to have LAM that is additionally capped with terminal mannose residues (ManLAM) (48) whereas Msm LAM is capped with a PI moiety (PILAM) (49). These differences implicate the contribution of ManLAM in pathogenesis.

Interestingly, Ppm1 (MSMEG_3859) and Ppm2 (MSMEG_3830) are essential enzymes that form the PPM synthase found in the plasma membrane tightly associated with cell wall (PM-CW), whereas PimB’ and AcPIM2 can be found in the plasma membrane free of cell wall (PMf, now termed inner membrane domain (IMD)), suggesting that mycobacteria spatially regulate the PIMs pathway (35, 50, 51). Additionally, the LM/LAM biosynthetic enzymes are all polytopic membrane proteins, suggesting LM and LAM are first made in the periplasm prior to transport to the outer membrane by an unknown mechanism.

1.4 Spe2: A Novel Cell Envelope Protein Involved in LM/LAM Biosynthesis

Previously, a forward genetic screen of pimE suppressor mutants identified a novel gene, which we termed spe2, involved in LM/LAM biosynthesis (Rahlwes et al. in preparation) identified by whole genome sequencing of three of these suppressor mutants (S1, S10, and S22) (Figure 3). Each mutant had specific point mutations,
with the exception of S22 which had a transposon insertion, in conserved regions of this gene (Figure 3). Further characterization of these isolates showed that these were suppressor mutants which still lacked AcPIM6 (Figure 4a), indicating that \textit{pimE} was not restored. However, the LM/LAM profiles of the mutants had similar shifts in size compared to that of WT (Figure 4b). Upon complementation with Spe2, the LM/LAM profile of each mutant was restored to the WT profile (Figure 4b). To ensure that the mutations in \textit{spe2} were indeed causing these changes in LM/LAM, a \textit{Δspe2} Msm strain was generated and characterized. This \textit{Δspe2} mutant had similar smaller LM/LAM as seen in each of the suppressor mutants, which was also restored by complementation with Spe2-HA under the control of either a hsp60 promoter or the endogenous promoter (Figure 5). These data suggest that Spe2 is somehow implicated in LM/LAM biosynthesis.

To better understand the function of Spe2, simple bioinformatic analyses were conducted. The transmembrane prediction using Hidden Markov Models (TMHMM) predict Spe2 to be a β-sheet rich protein with one transmembrane domain (52). A recent study by Song et al. 2008 predicted the Spe2 homolog in \textit{Mtb} (Rv0817c) to be an outer membrane protein (53). However, it should be noted that the presence of signal peptide sequences in a protein can be mistaken as a transmembrane domain in many predictive algorithms, making outer membrane protein prediction difficult. Indeed, Spe2 possesses a signal sequence, suggesting Spe2 is an extra-cytoplasmic protein (54). This signal sequence accounts for the predicted transmembrane domain and may indicate that Spe2 is an exported
protein. This speculation is further supported by sucrose density gradient fractionation data that demonstrate Spe2-HA localizes to the plasma membrane tightly associated to the cell wall (PM-CW) (Figure 6).

Based on these preliminary data, I hypothesized that Spe2 might be influencing LM/LAM biosynthesis by enhancing the activities of the elongation mannosyltransferase directly involved in this pathway. Since LM/LAM biosynthesis occurs in the periplasm, Spe2 might be a periplasmic regulator that directly interacts with these elongases. Furthermore, understanding the role of Spe2 in the regulation of PIMs/LM/LAM biosynthesis may lead to novel strategies in the development of TB therapeutics.
CHAPTER 2
MATERIALS AND METHODS

2.1 Bacterial Strains and Culture Conditions

*M. smegmatis* mc²155 and derived mutants (see Table 2) were grown at 30°C in Middlebrook 7H9 (BD) supplemented with 10% DC (11 mM glucose and 14.5 mM NaCl), and 0.05% Tween-80, or at 37°C on Middlebrook 7H10 agar supplemented with 10% DC unless otherwise indicated. Antibiotics were added to the media at the following concentrations: 100μg/ml hygromycin (Wako), 20μg/ml streptomycin (Fisher Scientific), and 20 μg/ml kanamycin (MP Biologicals).

Table 2. Strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genus</th>
<th>Species</th>
<th>Description</th>
<th>Reference</th>
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<tr>
<td>mc²155</td>
<td><em>Mycobacterium</em> smegmatis</td>
<td>WT</td>
<td></td>
<td>Snapper et al. 1990</td>
</tr>
<tr>
<td>SUMA 178</td>
<td><em>Mycobacterium</em> smegmatis</td>
<td>Δspe2</td>
<td></td>
<td>Rahlwes et al. (in preparation)</td>
</tr>
<tr>
<td>SUMA 188</td>
<td><em>Mycobacterium</em> smegmatis</td>
<td>Δspe2::Spe2-HA (P₇₀)</td>
<td></td>
<td>Rahlwes et al. (in preparation)</td>
</tr>
<tr>
<td>SUMA 228</td>
<td><em>Mycobacterium</em> smegmatis</td>
<td>Δspe2::Spe2-HA (Pnative)</td>
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<td>Rahlwes et al. (in preparation)</td>
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<tr>
<td>SUMA 228</td>
<td><em>Mycobacterium</em> smegmatis</td>
<td>Δspe2::T7-Spe2-HA (P₇₀)</td>
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<td>Rahlwes et al. (in preparation)</td>
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<tr>
<td>SAB 571</td>
<td><em>Mycobacterium</em> smegmatis</td>
<td>Transfected with pYAB133 and pYAB 288</td>
<td></td>
<td>Fukuda et al. 2013</td>
</tr>
<tr>
<td>SUMA 227</td>
<td><em>Mycobacterium</em> smegmatis</td>
<td>Transfected with pYAB133 and pYAB 288</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td><em>Escherichia</em> coli</td>
<td>XL-10 Gold chemically competent cells</td>
<td></td>
<td>Hanahan et al. 1991</td>
</tr>
</tbody>
</table>
2.2 Establishment of Recombinant *Mycobacterium smegmatis* Strains

Plasmids (see Table 3) were transformed into chemically competent *Escherichia coli* (*E. coli*) during heat shock at 42°C for 45 seconds followed by incubation at 37°C for 1 hour to allow for antibiotic expression prior to plating. Plasmids were purified and transfected into electrocompetent mycobacteria.

Table 3. Plasmids.

<table>
<thead>
<tr>
<th>Plasmid Name</th>
<th>Description</th>
<th>Selection</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pYAB133 (pTEK-4S0X)</td>
<td>revTet repressor expression vector (episomal)</td>
<td>Kan</td>
<td>Guo et al. 2007</td>
</tr>
<tr>
<td>pYAB288</td>
<td>Tet-off 4241 (MptA) expression vector (integrated)</td>
<td>Hyg</td>
<td>Guo et al. 2007</td>
</tr>
<tr>
<td>pYAB156</td>
<td>Integration vector with multiple coding sequences downstream of hsp60 promoter</td>
<td>Kan</td>
<td>Gift from William R. Jacobs Jr.</td>
</tr>
</tbody>
</table>

2.3 Preparation of Cell Lysates, SDS-PAGE, and Western Blot Analysis

Mycobacteria were grown at 30°C until log phase (OD$_{600}$ 0.5-1), at which 50 OD units (50/OD$_{600}$) were harvested by centrifugation at 3220 x g for 10 minutes at 4°C (Eppendorf 5810R). Cells were washed twice with 50 mM Hepes-NaOH (pH 7.4) (MP Biologicals). Pellets were resuspended at 200 mg wet pellet weight/ml in lysis buffer containing 25 mM Hepes-NaOH (pH 7.4), 15% glycerol, 2 mM EGTA, and protease inhibitor (Pierce). 0.5 ml of this cell suspension was transferred to a 2 ml screw cap tube containing 400 mg glass beads (Sigma) and lysed at 4°C using a BeadBug Microtube Homogenizer (Benchmark Scientific) at 4,000 rpm for 5 cycles.
each consisting of 30 seconds of homogenization followed by 1 minute incubations on ice. Cellular debris was removed by centrifugation at 100 x g for 5 minutes at 4°C (Eppendorf 5418R). The supernatant was centrifuged at 4°C for 10 minutes at 800 x g, followed by a 2,200 x g centrifugation for the same duration. The resulting protein fraction was processed for SDS-PAGE by mixing with 4X reducing loading buffer (Laemmli buffer) and run on 12 or 15% gels as indicated. Proteins were blotted onto a polyvinylidene difluoride membrane (PVDF) (BioRad). The membrane was blocked for at least 1 hour in 5% milk in phosphate buffered saline containing 0.1% Tween-20 (PBST). To visualize the following epitopes, the following primary and secondary antibodies were used as indicated in Table 4. Bound probes were visualized by chemiluminescence and captured using ImageQuant LAS 4000 mini (GE Healthcare).

Table 4. Antibodies.

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Manufacturer /Reference</th>
<th>Dilution</th>
<th>Secondary Antibody</th>
<th>Manufacturer /Reference</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA</td>
<td>Sigma</td>
<td>1:1000 (1h)</td>
<td>Anti-mouse HRP conjugate IgG</td>
<td>Amersham</td>
<td>1:1000 (1h)</td>
</tr>
<tr>
<td>FLAG</td>
<td>Sigma</td>
<td>1:5000 (O/N)</td>
<td>Anti-mouse HRP conjugate IgG</td>
<td>Amersham</td>
<td>1:10,000 (1h)</td>
</tr>
<tr>
<td>MptA</td>
<td>Sena et al. 2010</td>
<td>1:2000 (1h)</td>
<td>Anti-rabbit HRP conjugate IgG</td>
<td>Amersham</td>
<td>1:1000 (1h)</td>
</tr>
<tr>
<td>MptC</td>
<td>Sena et al. 2010</td>
<td>1:2000 (1h)</td>
<td>Anti-rabbit HRP conjugate IgG</td>
<td>Amersham</td>
<td>1:1000 (1h)</td>
</tr>
<tr>
<td>Mpa</td>
<td>Darwin et al. 2004</td>
<td>1:2000 (1h)</td>
<td>Anti-rabbit HRP conjugate IgG</td>
<td>Amersham</td>
<td>1:2000 (1h)</td>
</tr>
</tbody>
</table>
2.4 Immunoprecipitation

HA7 agarose beads (Sigma) were washed with HES (25 mM Hepes-NaOH (pH 7.4), 2 mM EGTA, 150 mM NaCl) or HEST (25 mM Hepes-NaOH (pH 7.4), 2 mM EGTA, 150 mM NaCl, 1% Triton-X100) for a total of five times. Bead beating cell lysate was added to these pre-washed beads, and incubated with HES or HEST buffer at 4°C overnight under gentle rotation at 5 rpm. Beads were washed with HES or HEST buffer five times prior to elution with HA peptide (AnaSpec Inc) at 30°C. Anti-HA elutions were run on 12% reducing SDS-PAGE and visualized via western blot as described above.

For use of Protein A magnetic beads (Fisher), 36 µl of bead-beating lysate was incubated with ~5 µg of primary antibody with bead-beating lysis buffer with protease inhibitor (described previously) in a total volume of 500 µl for 1 hour at room temperature with gentle mixing prior to addition to Protein A magnetic beads pre-washed in tris-buffered saline (TBS) containing 0.05% Tween-20 and 0.5 M NaCl (TBS wash buffer). Sample was then incubated with the beads for 1 hour at room temperature with gentle mixing. The magnetic beads were separated from the flow through with a magnetic stand, then washed with the TBS wash buffer. Beads were resuspended in 15 µl 4X reducing loading buffer (laemmli buffer) and boiled for 10 minutes. Beads were separated from eluate on a magnetic stand and eluate was immediately analyzed by SDS-PAGE.
2.5 **Proteinase K Accessibility Assays**

Cultures were grown to log phase in 7H9 then harvested for 50 OD units. Cells were washed in 1 ml phosphate buffered saline (PBS) then resuspended in 5 ml PBS containing 40% sucrose. 1.5 ml aliquots were removed. To each aliquot, 5 mM DTT (MP Biologicals) and 1 mg/ml Proteinase K (Fisher) was added. Proteinase K was not added to untreated controls. Samples were then incubated for 1 or 2 hours at 37°C shaking. To stop Proteinase K activity, 2 mM PMSF (Sigma) was added directly to the samples. Samples were then washed twice in PBS containing protease inhibitor. Proteins were either recovered by incubation at 37°C with 1% SDS for 30 minutes followed by boiling for 10 minutes, or resuspended at 200 mg wet pellet/ml PBS containing protease inhibitor and subjected to bead-beating lysis as described above.

For lysozyme and glycine weakening of the cell wall, cultures were grown in 7H9 until log phase, then ~43 OD units was harvested. Cells were washed in 1ml PBS, then resuspended in either 500µl of PBS or PBS containing 50 µg/ml lysozyme and 1.2% glycine. Samples were incubated at 37°C shaking for 3.5 hours. 0.1 mg/ml or 1 mg/ml of Proteinase K was added directly to the samples and incubated at 37°C shaking for 3 hours. Untreated controls did not receive Proteinase K. 2 mM PMSF was added to the samples, which were subsequently homogenized by bead-beating lysis.
2.6 Lipid/LM/LAM Extraction and Analysis

WT cultures were grown to log-phase. Knockdowns of SAB571 and SUMA 227 were induced with 40 ng/ml anhydrotetracycline (atc) at the time of inoculation and timepoints were taken at log (18-24 hours), stationary (44-48 hours), and late stationary (68-72 hours) phase. Lipids were sequentially extracted twice with chloroform-methanol (2:1, vol/vol) and once with chloroform-methanol-water (1:2:0.8, vol/vol/vol). The resulting aqueous phase (PIMs and other lipids) was subject to drying under a nitrogen stream followed by 1-butanol-water (2:1, vol/vol) partitioning, while the pellet (LM/LAM) was subject to phenol-water (1:1, vol/vol) extraction at 55°C for 2 hours. Phospholipids and PIMs were analyzed by HPTLC (Millipore) in chloroform-methanol-13M NH₃-water (180:140:9:9:23) and visualized by orcinol (PIMs) or molybdenum blue (phospholipids). LM/LAM was run on 15% or 20% (where specified) reducing SDS-PAGE and visualized with ProQ Emerald 488 Glycoprotein Gel and Blot Stain Kit (Molecular Probes).

2.7 MALDI-TOF MS Analysis

LM/LAM hot phenol extracts were further purified via octyl-Sepharose column chromatography (GE Healthcare) and lyophilized. 0.5 µl of the octyl-Sepharose purified LM/LAM was mixed with 0.5 µl of matrix solution (20 mg/ml sinapinic acid (SA), 30% acetonitrile, 1% trifluoroacetic acid (TFA) in water). Samples were analyzed on a Bruker Microflex MALDI-TOF instrument (Bruker Daltonics) using linear mode and positive ion detection.
CHAPTER 3
RESULTS

3.1 Subcellular Localization of Spe2

Based previous observations that LM and LAM are both smaller in a Δspe2 M. smegmatis (Msm) mutant, we speculate Spe2 may regulate LM/LAM biosynthesis. To test this hypothesis, the subcellular localization of Spe2 first needed to be determined. Since LM/LAM biosynthesis takes place in the periplasmic space, Spe2 must be a periplasmic protein or an inner membrane protein with a periplasmic face in order to interact with components of this pathway.

In order to identify Spe2 as a periplasmic associated protein, I must confirm that Spe2 is not a cytoplasmic protein. Approaches including immunoprecipitation (IP) and other protease accessibility assays were utilized to address this question regarding the localization of Spe2.

Immunoprecipitation of Spe2-HA from Whole Cell Lysate

To distinguish between cytoplasmic or periplasmic localization of Spe2, I performed anti-HA IP of whole cell lysates from a C-terminal HA-tagged Spe2 strain of Msm and a C-terminal HA-tagged GlnA1 (MSMEG_4290) Msm strain. GlnA1, an established cytoplasmic protein (50), was successfully pulled down with anti-HA agarose beads (Figure 7b). Conversely, Spe2-HA was not pulled down under the same conditions (Figure 7b). These results indicate Spe2 might not be a cytoplasmic protein. I then considered the possibility that, if Spe2 is a periplasmic protein, the
HA epitope would not be accessible by the beads due to the inner membrane acting as a barrier. With this in mind, anti-HA IP of Spe2-HA was performed in the presence of the mild, nonionic detergent TritonX-100 to help disrupt the inner membrane (Figure 7a). Under this condition, Spe2-HA was successfully precipitated from cell lysates (Figure 7b). The inaccessibility of the HA tag on Spe2 in the absence of detergent implies that the tag is blocked by the inner membrane—a barrier that is absolved with the addition of mild detergent. Thus, these IP results coincide with the idea that Spe2 is a periplasmic protein.

3.1.1 Immunoprecipitation from Whole, Intact Cells

To further distinguish the localization of Spe2, IP was performed on whole, intact cells to assess potential surface exposure. Periplasmic and cytoplasmic proteins should not be accessible to anti-HA beads in whole cells, even with the addition of detergent, due to the intact mAGP core. Indeed, using the same IP procedure as described above with whole, intact cells, Spe2-HA was not pulled down even with the addition of 1% TritonX-100 (Figure 8a). However, a proper outer membrane protein control is necessary to validate such a technique. Thus, I generated a strain of Msm expressing FLAG-tagged Mtb12 (MSMEG_3903), one of a number of predicted Msm outer membrane proteins as described by He and De Buck et al. 2010 (55). In order to use the same IP system to pull down both FLAG and HA tagged proteins, I utilized protein A magnetic beads pre-incubated with the respective antibodies to IP outer membrane proteins from intact cells.
van der Woude et al. 2013 describe a method by which *Mycobacterium marinum* (*M. marinum*) outer membrane proteins can be selectively extracted from fractionated cell lysates with the non-ionic detergent *n*-octyl-β-D-glucopyranoside (OBG) (56). A similar detergent of the same family has also been described in the literature for selective extraction of the *Msm* outer membrane porin MspA (57). Thus, I reasoned that outer membrane proteins on whole, intact cells could be more accessible to antibodies with the presence of mild detergent—particularly OBG. In a pilot experiment, incubation with 1% OBG for one hour at room temperature was the optimal condition for pulling down Mtb12-FLAG from whole, intact cells (Figure 8b, right panel; Supplemental Figure 3). Unfortunately, while Mtb12-FLAG was successfully immunoprecipitated from whole intact cells the first three of five total experimental replicates, the last two attempts were unsuccessful. As expected, neither Spe2-HA nor GlnA1-HA was pulled down from intact cells in the presence of OBG (Figure 8b, left panels). The lack of recovery of Spe2-HA from intact cell IP suggests Spe2-HA is not an outer membrane protein. However, the unsuccessful attempt to pull down Mtb12-FLAG in the same experiment confounds this result.

### 3.1.2 Protease Accessibility in Intact Cells

Protease accessibility assays are frequently used in the literature to determine surface exposure of proteins. This procedure utilizes Proteinase K, a non-specific protease that cleaves peptide bonds, to degrade proteins on the surface of intact cells. During short Proteinase K treatment, surface exposed proteins are degraded while cytoplasmic and inner membrane proteins remain intact. Additional
published measures such as the use of phosphate buffered saline (PBS) containing 40% sucrose and the presence of dithiothreitol (DTT) were included in order to maximize efficiency of Proteinase K treatment (55, 58). Proteins were solubilized with 30 minutes of 1% SDS incubation and subsequently boiled for western blot analysis.

Within two hours of 1 mg/ml Proteinase K treatment, the outer membrane control Mtb12-FLAG was degraded in intact cells (Figure 9a). This disappearance of Mtb12-FLAG is only seen when I carefully titrated the protein loading onto the SDS-PAGE gel, emphasizing the significant impact of over-expression of Mtb12-FLAG on Proteinase K digestion. Neither Spe2-HA nor GlnA1-HA were recovered, even in the untreated samples (Figure 9b). Proteinase K activity was confirmed during treatment of lysate, in which all three proteins were degraded upon addition of 1 mg/ml Proteinase K (Figure 9a and b). Therefore, I concluded the lack of recovery of Spe2-HA and GlnA1-HA from both untreated and treated samples was due to 1% SDS as a poor method for solubilizing internal proteins. In order to address this issue, the experiment was scaled up so the samples could be homogenized through bead-beating lysis, which should improve protein recovery. Using this method, Spe2-HA and GlnA1-HA were both recovered from untreated samples, proving the validity of this experimental method (Figure 9c). Spe2-HA was not degraded after two hours of Proteinase K treatment in intact cells (Figure 9c). Similarly, the cytoplasmic protein GlnA1-HA was not degraded in intact cells under the same
conditions (Figure 9c). Mtb12-FLAG, however, does not appear to be degraded in this experiment (Figure 9c).

3.1.3  **Protease Accessibility of Spe2 in Lysozyme and Glycine Treated Cells**

One final alternative strategy for determining the localization of Spe2 is the use of Proteinase K to degrade proteins protected by the thick mycobacterial cell wall. Lysozyme and glycine treatment has been used in the literature to weaken mycobacterial cell walls for applications such as spheroplast preparation and genomic DNA extraction (59–61). Here, I applied this concept to make the cell wall leaky, thus enabling Proteinase K accessibility to proteins normally protected by the cell wall. I predicted that short treatment with Proteinase K would result in the degradation of solely periplasmic proteins and that longer treatment would result in the degradation of cytoplasmic proteins in addition to periplasmic proteins.

Pre-treatment of Spe2-HA expressing cells with lysozyme and glycine at 37°C followed by treatment with or without Proteinase K resulted in the retention of the cytoplasmic proteasome-associated ATPase Mpa (MSMEG_3902) (62) and the loss of Spe2-HA with 1mg/ml Proteinase K treatment within the same *Msm* strain (Figure 10). Interestingly, cells that were not treated with lysozyme and glycine had similar trends in retention and degradation of Mpa and Spe2-HA compared with lysozyme and glycine pre-treated cells, suggesting that lysozyme and glycine pre-treatment was not required for Proteinase K access to internal proteins during this experiment (Figure 10). Considering that Spe2-HA is under the control of a heat shock promoter, incubations at 37°C would mean Spe2-HA was overproduced by
these cells. Such levels of Spe2-HA might explain why Spe2-HA requires 1mg/ml of Proteinase K for complete degradation. Overall, these results reinforce that Spe2 is not a cytoplasmic protein, as Spe2-HA was not retained in the same manner as the cytoplasmic control Mpa.

Together, the results from these independent experiments provide substantial evidence pointing to Spe2 localization to the periplasm, the site of LM/LAM biosynthesis, and not the cytoplasm. Thus, the data reinforce our hypothesis that Spe2 regulates the mannosyltransferases in this pathway.

3.2 Genetic Interaction of Spe2 with Elongation Mannosyltransferases

Preliminary data has shown that the deletion of Spe2 results in the truncation of both LM and LAM, suggesting Spe2 might be a positive regulator of either one or both of the elongation mannosyltransferases in LM/LAM biosynthesis: unknown mannosyltransferase and MptA. Genetic manipulation was utilized to better understand the relationship between Spe2 and the enzymes involved in this biosynthetic pathway.

3.2.1 Comparative LM/LAM Analysis of MptA Knockdown in a Δspe2 Strain

We have previously generated a strain of Msm that has a tetracycline (tet)-off inducible knockdown system for conditionally shutting off MptA expression (11), herein referred to as WT/MptA KD. In this system, a reverse tet repressor (revTetR) responsive promoter (tetO) was introduced in front of the endogenous MptA (MSMEG_4241) gene by homologous recombination. An episomal copy of the
revTetR was then introduced. Upon the addition of anhydrotetracycline (atc), atc binds to revTetR, which then binds to tetO. This in turn blocks transcription of \textit{mptA}, thereby diminishing further MptA production.

To examine the role of Spe2 in the LM/LAM biosynthetic pathway (Figure 11), the knockdown system described above was introduced into a \textit{Δspe2 Msm} strain, herein referred to as \textit{Δspe2/MptA KD} (Figure 12a). Western blotting confirmed that with the addition of atc, MptA was knocked down by the time the cells reached logarithmic phase (Figure 12b).

I then visualized LM and LAM extracts as previously described (11). Without the addition of atc, LM and LAM appeared similar to WT and \textit{Δspe2} (see Figure 5) in the WT/MptA KD and \textit{Δspe2/MptA KD} strains, respectively (Figure 13). Conversely, with the addition of atc, both WT/MptA KD and \textit{Δspe2/MptA KD} strains showed smaller LM and little to no LAM (Figure 13). Interestingly, both knockdown mutants show an accumulation of LM over time (Supplemental Figure 4).

### 3.2.2 MALDI-TOF Analysis of LM Products in the \textit{Δspe2/MptA Tet-Off} Strain

In order to determine whether these intermediate LM species are similar in size and structure, matrix assisted laser desorption – time of flight (MALDI-TOF) mass spectrometry of octyl-sepharose purified LM/LAM extracts was performed at the UMass Amherst Mass Spectrometry Center. Indeed, the mass spectrum of LM from the \textit{Δspe2/MptA KD} strain was identical to that of the WT/MptA strain (Figure 14). Despite the presence of sinapinic acid (SA) adducts originating from the matrix,
these spectra match theoretical atomic masses in addition to previously published spectra (11) once the SA adducts are subtracted from the recorded mass. Analysis of butanol lipid extracts via HPTLC and subsequent chemical staining established that no other changes in PIMs (Figure 15a) nor PI (Figure 15b) occur with the knockdown of MptA. Together, these data indicate that the absence of Spe2 does not interfere with PI, PIMs, nor LM/LAM biosynthesis upstream of MptA, thereby suggesting Spe2 acts either at or downstream of MptA activity.

3.3 Protein-Protein Interactions of Spe2

From the previous sections, I can draw the conclusion that Spe2 is likely a periplasmic protein that acts downstream of the unknown elongase in LM biosynthesis. MptA is responsible for elongating LM to its mature length, and thus, Spe2 might interact with MptA directly, indirectly, or through other targets to enhance its activity. In order to elucidate potential protein-protein interactions with Spe2, native PAGE and co-immunoprecipitation techniques were implemented.

3.3.1 Native PAGE Analysis of Spe2-HA

Native PAGE is a technique that avoids the use of the traditional reducing agents, such as SDS and DTT. Such omission of these agents enables the visualization of protein complexes and/or proteins in their native conformations that would have otherwise been disrupted. Accordingly, lysate from the Spe2-HA expressing *Msm* strain was run on a 12% native gel under reducing and non-reducing conditions (Figure 16a). Varying percentages of TritonX-100 was added to promote mobilization of native proteins during native PAGE. Under reducing conditions and
regardless of the percentage of TritonX-100 added, Spe2-HA appears as a single band at just below 37 kDa (Figure 16a). However, under non-reducing conditions, Spe2-HA is seen as both the ~37 kDa band in addition to smears above this molecular weight, suggesting the presence of protein-protein interactions involving Spe2-HA (Figure 16a). Interestingly, the addition of 1% TritonX-100 diminishes this smear. Next, I sought to examine the IP of Spe2-HA under native conditions under the premise that IP is more likely to enrich true Spe2-protein interactions. Anti-HA IP eluates were run on native PAGE alongside lysate with either 0.5 or 1% TritonX-100. Surprisingly, no higher molecular weight bands were seen in the eluates either under reducing or non-reducing conditions (Figure 16b). However, the higher molecular weight bands, particularly the ~75 kDa band, was reproduced in lysate under non-reducing conditions as seen in Figure 16a (Figure 16b), implicating that these are not likely to be random artifacts.

To better understand why the eluates lose these higher molecular weight bands seen in lysate under non-reducing conditions, I considered that weak or transient interactions with Spe2 might be disrupted during the IP procedure. In light of this, NaCl was removed from IP buffers. As a reference, 150mM NaCl (the same concentration as in the IP buffers) was added to lysate, which does not originally contain NaCl. Indeed, the removal of NaCl in eluates under non-reducing conditions results in the visualization of higher molecular weight smears, in addition to reduced signal at the ~37kDa band (Figure 17a). This phenotype suggests Spe2 in its native state is potentially interacting with another protein.
3.3.2 Analysis of Potential Protein Co-Immunoprecipitation with Spe2-HA

Since the higher molecular weight bands are only seen in non-reducing conditions in native PAGE, it seemed reasonable to investigate potential binding partners. To determine whether Spe2 physically interacts with mannosyltransferases in the LM/LAM biosynthetic pathway, anti-HA IP eluates were probed for MptA, the elongase, and MptC, the branching mannosyltransferase. Interestingly, MptA did not co-immunoprecipitate (co-IP) with Spe2 in either reducing or non-reducing conditions (Figure 17b, upper panel). However, western blot analysis of anti-HA (-) NaCl eluates under both reducing and non-reducing conditions showed that multiple bands appear when probed for MptC (Figure 17b, lower panel).

Due to the presence of multiple bands in the anti-MptC western blot analysis of anti-HA eluates, I sought to confirm the specificity of both the higher molecular weight smears/bands from lysate and eluates. To do so, I probed WT lysate and anti-HA IP of WT lysate. When probed with anti-HA, both lysates from WT and the Spe2-HA expressing strain produce similar banding patterns, including the ~75kDa band seen previously (Figure 16), under a range of reducing and non-reducing conditions (Figure 18a). Similarly, when probing for MptC in WT and Spe2-HA anti-HA eluates, similar banding patterns appear when NaCl is removed from the immunoprecipitation buffers (Figure 18b, right panel). These nearly identical binding patterns in both lysates and eluates indicate that the high molecular weight smears and bands are non-specific and unlikely to be true Spe2 binding partners.
However, two bands do appear to be specific to the Spe2-HA anti-HA eluate when probing for MptC (Figure 18b, right panel), and may indicate co-immunoprecipitation with Spe2-HA.
CHAPTER 4
DISCUSSION

The mycobacterial cell wall is an extremely potent barrier against environmental stresses, including the host immunity and antibiotics (11, 63). Modulation of cell envelope components—which requires intimate knowledge of the respective biosynthetic pathways—appears to be a promising strategy for fighting mycobacterial infection.

The biosynthetic pathway of the abundant glycolipids LM and LAM is largely unraveled in terms of the biosynthetic enzymes involved. However, its regulation is still unclear. Here, I describe a novel mycobacterial protein termed Spe2, which plays a role in the regulation of LM biosynthesis.

First, I suggest Spe2 is a periplasmic protein through a number of biochemical techniques. IP of Spe2 in lysate requires the addition of mild detergent, which is not needed for the IP of cytoplasmic proteins (Figure 7). Additionally, Spe2 is not surface exposed, as IP and Proteinase K treatment of whole, intact cells did not recover (Figures 8 and 9). Moreover, Proteinase K treatment in PBS starved cells with or without lysozyme and glycine cell wall weakening of whole, intact cells was successful in degrading Spe2 but not the cytoplasmic protein Mpa within the same Msm strain (Figure 10). Unfortunately, the intended outer membrane protein control Mtb12 was problematic throughout these experiments. IP of Mtb12-FLAG from intact cells was successful during the optimization of the technique but failed...
when attempted months later alongside Spe2-HA and GlnA1-HA. While Proteinase K treatment of Mtb12-FLAG in intact cells generated the desired phenotype, but only when a small amount of sample was analyzed by SDS-PAGE (Figure 9), this shows that Proteinase K degradation of surface exposed proteins might be working, but somehow cannot completely degrade Mtb12-FLAG. A potential explanation for this phenomenon includes taking into consideration the small size of the protein, and the over-expression of Mtb12-FLAG. Mtb12 in its mature form is 13.3 kDa (64), which may be too small for Proteinase K to access in the outer membrane. Additionally, Mtb12 was discovered in Mtb as a culture filtrate protein, meaning the protein can be found abundantly in the supernatant of Mtb cultures (64). It is possible that Mtb12 is naturally produced in high abundance, and in addition to heat shock promoter-induced over-expression, can be constitutively produced by the cell. As a result, the detection of Mtb12-FLAG in western blots during Proteinase K treatments, for example, could potentially be due to cytoplasmic pools of Mtb12-FLAG, or newly replaced Mtb12-FLAG at the surface of the cell. Thus, an alternative approach could be to utilize Mtb12-FLAG as a positive control for proteins secreted out of the cell. If Mtb12-FLAG, but not Spe2-HA is found in the supernatant of cultures, one might expect Spe2 is not externally secreted, and thus, more likely to be periplasmically localized.

A study by van der Woude et al. 2013 describe a method to selectively extract outer membrane proteins from Mycobacterium marinum with OBG. In their technique, whole cells were subjected to lysis and ultracentrifugation to isolate a
cell envelope fraction. This fraction was further subjected to OBG treatment, in which the supernatants were claimed to contain the extracted outer membrane proteins. However, it is important to note the limitations of this study. The caveat of this technique is that OBG does not appear to specifically extract outer membrane proteins. Using mass spectrometry analysis of cell envelope fraction OBG extracts, the authors claim that over 90% of predicted outer membrane proteins found in the cell envelope fraction were extracted. Conversely, only 30% of predicted inner membrane proteins were extracted by OBG. However, an even greater 31-89% of proteins extracted by OBG were undefined (which the authors termed “middle”) in terms of their localization (65). OBG has also been used to permeabilize the outer membrane of Msm, and similar members of this family of detergents have been used to selectively extract the Msm outer membrane porin MspA (57). The difficulty in isolating mycobacterial outer membrane proteins has been reviewed in the literature (66). It is likely that membrane proteins may not be exclusively found in the outer membrane, and features of mycobacterial outer membrane-specific proteins may have unknown unique properties not previously seen in Gram-negative outer membranes.

Despite the lack of an efficient outer membrane protein control, the data from previous experiments such as the sucrose gradient showing that Spe2 localizes to the PM-CW (Figure 6), as well as the experiments using cytoplasmic protein controls described in this study, all support the concept of Spe2 as a periplasmic protein by suggesting Spe2 is neither an outer membrane nor cytoplasmic protein.
It must be noted that these results could be interpreted as Spe2 being an inner membrane protein. In accordance with this interpretation, Spe2 must be an inner membrane protein with a periplasmic active face in order to be able to interact with the LM/LAM biosynthetic pathway. The rationale is, as mentioned earlier, that the deletion or mutation of Spe2 results in the shift of both LM and LAM (Figures 4 and 5), which clearly indicates its association with the LM/LAM biosynthetic pathway. One approach to distinguish between periplasmic and inner membrane localization is to examine whether the signal sequence of Spe2 is cleaved. If this sequence is not cleaved, Spe2 would remain in the inner membrane post-export. However, if the sequence is cleaved, Spe2 is likely periplasmic. A strategy to determine this is to visualize the loss of an N-terminal epitope tag on Spe2-HA (i.e. T7-Spe2-HA), which would indicate the loss of the signal sequence. In either case, Spe2 must be able to interact with the components of this pathway, and its periplasmic or inner membrane localization supports such a role.

In this study, I demonstrate that Spe2 acts downstream of the unknown elongase of LM biosynthesis by showing that same-size LM intermediates accumulate in both WT/MptA KD and Δspe2/MptA KD strains (Figure 13). Since no other changes in the precursors of this pathway, such as PI and PIMs are seen (Figure 15), and the mass spectra are identical (Figure 14), the LM intermediates in WT/MptA KD and Δspe2/MptA KD must be the same intermediate. However, it is important to note that I could not—in my own hands—analyze larger species of LM or LAM, such as WT LM, by MALDI-TOF. It is possible I could not use MS to detect...
slightly larger species of LM not visible by SDS-PAGE and ProQ Emerald due to this limitation. Interestingly, polysaccharide species of lower mass are seen in both spectra (Figure 14). These also have the same distance between peaks (162 mass units) and form a high intensity curve not unlike that of LM. Based on the masses, this curve could potentially be a range of PIM species, such as AcPIM6 and AcPIM4. Comparisons of these spectra with those of butanol lipid extracts analyzed under the same conditions might confirm whether this is the case.

Our hypothesis is that Spe2 acts during the elongation of LM, as Δspe2 LM/LAM has smaller LM and LAM compared to WT (Figure 5), meaning Spe2 potentially modulates MptA activity. However, the data presented in this study, do not provide enough evidence to distinguish whether Spe2 is active at the same stage as MptA or downstream of MptA. It is likely Spe2 works upstream of LAM synthesis. One interpretation of the small LM/LAM phenotype of Δspe2 is that Spe2 promotes the maturation of LM, which in turn, affects the maturation of LAM. If the mutant begins LAM biosynthesis with smaller LM (compared to WT), the resulting LAM is likely smaller as well. MptC is another mannosyltransferase involved in LM biosynthesis. This enzyme has been suggested to work simultaneously with, in addition to upstream of, MptA. Thus, MptC might also be affected by Spe2 activity. Moreover, the over-expression of MptC has been shown to shift LM/LAM size similar to that of Δspe2 (38). Therefore, it would be interesting to compare WT/MptA KD/MptC KD with Δspe2/MptA KD/MptC KD and examine the effect of the double knockdowns, as Spe2 may somehow keep MptC activity in check.
In an attempt to elucidate the function of Spe2, I explored potential protein-protein interactions involving Spe2 and other enzymes in the LM biosynthetic pathway—mainly MptA and MptC. Native PAGE has been successfully utilized in the literature to identify components within a protein complex, particularly with two-dimensional gels in which a protein complex is run on a native gel, the entire lane cut out, and subsequently run on SDS-PAGE to separate the individual components of the complex (67). Instead, I opted to examine the appearance of higher molecular weight bands or smears when Spe2-HA is run on a native gel lacking SDS. Although the presence of smears above the expected molecular weight of Spe2-HA was visible in non-reducing conditions but lacking in reducing conditions (Figures 15 and 16), these smears were also present when WT lysate or immunoprecipitation eluates were run under the same conditions (Figure 17). The seemingly promising results from the removal of NaCl—with the intent to visualize weak or transient interactions—in the immunoprecipitation buffers also increased non-specificity (Figures 16 and 17). However, when probing anti-HA eluates for MptC, two bands appeared to be specific co-immunoprecipitation with Spe2-HA, since neither of the two bands were seen in the WT lysate anti-HA IP control (Figure 18b). Although Spe2 may be interacting with MptC. I did not further examine this possibility. The results are interesting since, as mentioned earlier, the overexpression of MptC results in smaller LM and LAM similar to that of the Δspe2 mutant (38). In light of this connection, Spe2 may physically interact with MptC to negatively regulating its activity and enable proper elongation of the mannan chain by MptA.
Alternative, non-biased approaches were attempted but not fully optimized. These attempts included silver staining of anti-HA immunoprecipitation eluates (not shown) to visualize proteins that may have co-immunoprecipitated with Spe2-HA and crosslinking using two crosslinkers: dithiobis [succinimidylpropionate] (DSP) and formaldehyde (not shown) in the event that the required TritonX-100 for immunoprecipitation of Spe2-HA from lysate simultaneously disrupts interactions with the protein.

To further speculate on the function of Spe2, I must consider preliminary data from my colleague Kathryn C. Rahlwes. Kathryn is developing a Spe2 binding assay similar to an enzyme linked immunosorbent assay (ELISA) in which Spe2-HA in the form of lysate, immunoprecipitation eluates, or purified Spe2-HIS from *E. coli* is added to wells coated with lipids such as PIMs and LM from my MptA knockdowns lacking LAM. In her system, she is detecting Spe2 binding to MptA knockdown LM as well as AcPIM6. If this is indeed true, Spe2 might play a role in LM biosynthesis by regulating the length of the mannan chain. One speculation is that Spe2 is involved in steric control of the mannan chain. For instance, Spe2 might bind to intermediate LM produced by unknown elongase and enable only MptA to access the chain up a particular length. The idea of a molecular ruler which determines the maximum chain length of these glycolipids is a novel concept in this field, and thus, is particularly worth exploring further.

Mishra et al. 2008 demonstrate that MptB, the mannosyltransferase in the related organism *Corynebacterium glutamicum* ATCC 13032, is essential for LM and
LAM biosynthesis, as it mediates the initial elongation of LM (37). This group, however, notes that its Msm homolog (MSMEG_3120) is not essential, suggesting that other mannosyltransferases, including MptA, might compensate. Interestingly, Kathryn has performed BLASTP for Spe2 in this strain of *C. glutamicum* and found no homology. In light of these findings, one thought is that Spe2 might be involved in transferring LM precursor to MptA, thereby providing it a substrate pool, and that in the absence of MSMEG_3120 can compensate by transferring AcPIM4 or AcPIM6. In this case, the lack of phenotype in the MSMEG_3120 deletion (37) could be explained by the presence of Spe2, which is lacking in *C. glutamicum* ATCC 13032.

As mentioned previously, PIMs, LM, or LAM are essential in mycobacteria, which implies their significant contribution to cell wall integrity. Of note, the pimE suppressor mutants that also had mutations in spe2 that abolished its activity had restored large colony morphology as seen in WT (Figure 3). Since ΔpimE results in the accumulation of AcPIM4 (34) and Δspe2 results in smaller LM and LAM, the combination of these two suggest mycobacteria lacking these two enzymes prefer to accumulate these small glycolipids to somehow strengthen cell wall integrity. Thus, Spe2 might be a key player in the biosynthesis of these cell envelope glycolipids, which are critical for cell wall integrity as well as immunomodulation by mycobacteria. These are both excellent targets for generating novel therapeutic strategies to treat mycobacterial infections, thereby highlighting the importance of further characterization of Spe2.
In addition to the canonical plasma membrane, mycobacteria possess an outer membrane with an unusual repertoire of lipids and glycolipids. This mycomembrane is covalently linked to the arabinogalactan-peptidoglycan (AG-PG) layer via waxy mycolic acids. This mycolyl arabinogalactan peptidoglycan (mAGP) core is rather thick and confers resistance to harsh environmental stresses encountered by the cells.
Figure 2. PIMs/LM/LAM Biosynthetic Pathway in *Mycobacterium smegmatis*.

The biosynthesis of phosphatidyl-inositol (PI)-based glycolipids begin with the mannosylation of PI to form the first major product AcPIM2, which is further extended to form the intermediate AcPIM4. At this point, AcPIM4 can feed into AcPIM6 biosynthesis or LM/LAM biosynthesis. These end products are assumed to be transported into the outer membrane by unknown mechanisms. Essential enzymes are shown in yellow. EmbC, which is essential in *Mycobacterium tuberculosis*, but not in *Mycobacterium smegmatis (Msm)*, is denoted with an asterisk.
Figure 3. Sequencing of S1, S10, and S22 reveals mutations in unannotated gene spe2.

Whole genome sequencing of *pimE* suppressor mutants S1, S10, and S22 revealed point mutations in S1 and S10, in addition to a 2 kb transposon insertion in S22, within the same unannotated gene. The point mutations, shown in red, are in conserved regions of the protein. (Rahlwes et al. in preparation)
Figure 4. S1, S10, and S22 have shifts in LM and LAM that are restored by complementing with Spe2.

(a) High performance thin layer chromatography (HPTLC) of butanol lipid extracts containing PIMs. (b) 15% SDS-PAGE followed by ProQ Emerald 488 glycan staining. These pimE suppressor mutants have similar shifts (smaller) LM and LAM (b), which is restored to the WT phenotype upon complementation with Spe2 (b), the gene that all three mutants had mutations in. However, no changes in PIMs (a) were seen, indicating the specific effect of these mutations in Spe2 on LM/LAM. (Rahlwes et al. in preparation)
Figure 5. Δspe2 LM/LAM is restored upon complementation.

15% SDS-PAGE followed by ProQ Emerald 488 glycan staining. Deletion of Spe2 results in a similar shift in LM/LAM as seen in the suppressor mutants. Complementation of Spe2 with Spe2-HA under various promoters shows the restoration of WT LM/LAM, thereby implicating the relationship between Spe2 and LM/LAM biosynthesis. (Rahlwes et al. in preparation)
Figure 6. Spe2-HA localizes to the PM-CW.

Sucrose density gradient fractionation shows that Spe2-HA localizes to the plasma membrane tightly associated with cell wall (PM-CW) and not to the mycobacterial membrane domain (MMD, also known as the PMf). Expected molecular weights: Spe2-HA, 29.5 kDa; PimB', 41.4 kDa; MptC, 48.2 kDa. (Rahlwes et al. in preparation)
Figure 7. Spe2 is inaccessible to α-HA beads in cell lysate without the presence of mild detergent.

(a) Schematic of the expected results from immunoprecipitation (IP) of whole cell lysate generated from a Spe2-HA expressing Msm strain or from a GlnA1-HA expressing Msm strain using α-HA agarose beads with or without 1% TritonX-100 in the IP buffer. Spe2 is only accessible to the α-HA agarose beads due to the solubilization of the inner membrane when TritonX-100 is present (a, right). (b) α-HA western blot of the IP outlined in (a). Inputs are ¼ of the amount used during IP. Spe2-HA is only pulled down when TritonX-100 is present in the system, whereas GlnA1-HA is pulled down under both conditions. Expected molecular weights: Spe2-HA, 29.5 kDa; GlnA1-HA, 54.6 kDa.
Figure 8. Spe2-HA is not immunoprecipitated from intact cells.

Lysate or whole cells from a Spe2-HA expressing strain was immunoprecipitated using anti-HA agarose beads in the presence or absence of 1% TritonX-100 (a). In a different immunoprecipitation (IP) system, using Protein A magnetic beads, neither GlnA1-HA (b, upper arrow) nor Spe2-HA (b, bottom arrow) were immunoprecipitated from whole, intact cells. Conversely, Mtb12-FLAG was immunoprecipitated from whole cells, suggesting that Spe2-HA is not surface exposed. The band seen at ~75 kDa is the heavy chain of α-HA antibody and the band at ~25 kDa is the light chain of the α-HA antibody, which can remain in the eluate of the magnetic bead IP system. Inputs were ¼ of the amount used during IP and appears faint due to poor western blotting detection. Expected molecular weights: Spe2-HA, 29.5 kDa; GlnA1-HA, 54.6 kDa; Mtb12-FLAG, 13.3 kDa (64).
Intact cells or lysate from cells expressing Mtb12-FLAG, Spe2-HA, or GlnA1-HA were subjected to Proteinase K treatment at 37°C. Proteins from intact cells were recovered by incubation with 1% SDS at 37°C for 30 minutes. After 1 hour of 1mg/ml Proteinase K treatment of lysate, all three proteins were completely degraded (a and b). In intact cells, Spe2-HA and GlnA1-HA were not recovered (b). Recovery of proteins from intact cells by bead-beating lysis (c) show that Mtb12-FLAG, in addition to Spe2-HA and GlnA1-HA, are maintained up to 2 hours of treatment with Proteinase K. A non-specific band at 37 kDa is occasionally seen in (b) during 1:1000 incubations with primary and secondary antibody. Input was 1.8 mg, with Mtb12-FLAG in (c) as the exception with an input of 0.9 mg. Expected molecular weights: Spe2-HA, 29.5 kDa; GlnA1-HA, 54.6 kDa; Mtb12-FLAG, 13.3 kDa.
Figure 10. Lysozyme and glycine weakening of the cell wall.

Whole cells from log phase were incubated in PBS or PBS containing 50 µg/ml of lysozyme and 1.2% glycine for 3.5 hours, followed by treatment with or without Proteinase K at the following concentrations: 0.1 mg/ml or 1 mg/ml. Spe2-HA is retained in whole cells when treated with 0-0.1 mg/ml of Proteinase K. Conversely, the addition of 1 mg/ml Proteinase K simultaneously results in the loss of Spe2-HA and the retention of the cytoplasmic protein Mpa. Expected molecular weights: Spe2-HA, 29.5 kDa; Mpa, 68 kDa.
Figure 11. Rationale for generating an inducible MptA knockdown strain of *M. smegmatis* in Δspe2 background.

Predicted sizes (number of mannoses) of LM in a Spe2 deletion strain (a and b) or MptA inducible knockdown in the Δspe2 background (c and d) of *Msm*. If Spe2 enhances the activity of unknown elongase (a or c) or MptA (b or d) in the WT background, the terminal LM product, which is slightly smaller than mature LM, accumulates when only spe2 is deleted (a and b). However, when knocking down MptA in the Δspe2 background, the final LM product that accumulates is detectably larger when Spe2 interacts with MptA (d) compared to when it interacts with the unknown elongase (c).
Using the MptA knockdown system established by Fukuda et al. 2013, the addition of anyhdrotracycline (atc) can halt MptA transcription. (a) A revTetR responsive promoter (TetO) was introduced immediately before \textit{mptA} by homologous recombination into WT or \textit{Δspe2} Msm strains. The revTetR was subsequently introduced episomally. (b) Western blot confirmation of MptA knockdown by log phase. WT lysate (input of 1.8 mg) was used as a western blot positive control. Expected molecular weights: MptA, 54.3 kDa.
Figure 13. Lipomannan (LM) of identical size accumulate in the MptA knockdowns.

MptA knockdown was induced through stationary phase (44 hours) with a final concentration of 40 ng/ml atc. LM and LAM from each strain were extracted by chloroform/methanol (2:1), followed by chloroform/methanol/water (2:1:0.8), and then hot phenol. Extracts were diluted for better visualization of distinct LM/LAM species, then run on 20% SDS-PAGE and visualized by ProQ Emerald 488 glycoprotein staining.
Figure 14. MALDI-TOF MS of LM from MptA knockdowns.

MptA knockdown LM/LAM extracts from stationary phase were further purified with octyl-sepharose and analyzed by MALDI-TOF mass spectrometry. Spectra of WT/MptA KD (upper panel) and Δspe2/MptA (lower panel), shown with sinapinic acid adducts, are identical. The number of mannoses (each addition of mannose corresponds with 162 mass unit intervals) are indicated above each relevant peak.
Butanol lipid extractions were run on HPTLC with chloroform-methanol-13M NH$_3$-water (180:140:9:9:23) as the solvent. Chemical staining and analysis of the subsequent migrations of PIMS (a) and phospholipids (b) are well-established. The staining of (a) PIMs by orcinol or (b) phospholipids by molybdenum blue confirm that no significant changes in PIMs (a) nor PI (b) occur with the knockdown of MptA.

Figure 15. No changes in LM precursors are identified in the MptA knockdowns.
Figure 16. Comparisons of Spe2-HA under reducing and non-reducing conditions on native PAGE.

Native PAGE analysis of (a) lysate or (b) anti-HA immunoprecipitation (IP) eluates originating from a Spe2-HA expressing strain of Msm. Samples were run on a 12% native gel with reducing (containing DTT and SDS) or non-reducing (contains no DTT nor SDS) loading buffers. 0, 0.1, 0.5, or 1% TritonX-100 was added to samples to promote protein migration through the gel. Expected molecular weights: Spe2-HA, 29.5 kDa.
Lysate or anti-HA IP eluates from a *Msm* strain expressing Spe2-HA were run on 12% native gels under reducing or non-reducing conditions in the presence of TritonX-100. (a) Comparison of the presence or absence of NaCl in samples. (b) Analysis of potential co-immunoprecipitation of MptA or MptC with Spe2-HA during anti-HA immunoprecipitation. The expected migration of MptA and MptC are highlighted with arrows. Expected molecular weights: Spe2-HA, 29.5 kDa; MptA, 54.3 kDa; MptC, 48.2 kDa.
Figure 18. Native PAGE with WT controls shows non-specific binding.

Native PAGE analysis of (a) lysate and (b) anti-HA IP eluates generated from WT or Spe2-HA expressing strains of *Msm*. (a) A range of reducing and non-reducing loading buffers containing different combinations of DTT or SDS additives was utilized to compare lysates. Analysis of potential MptA or MptC co-IP with Spe2-HA during anti-HA IP with or without the presence of salt (NaCl). Probing for MptC in anti-HA IP eluates (b, right panel) shows both non-specific bands in both WT and Spe2-HA lysate IP. However, two bands at 50 kDa and 100 kDa appear to be specific to the Spe2-HA lysate anti-HA IP, suggesting potential co-IP. Expected molecular weights: Spe2-HA, 29.5 kDa; MptA, 54.3 kDa; MptC, 48.2 kDa.
Supplemental Figure 1. Spe2-HA is pulled down in the presence of a number of detergents.

Spe2-HA is pulled down in the presence of TritonX-100 (HEST), TritonX-100 with NaDOC (HESD), and a combination of TritonX-100, NaDOC, and SDS (RIPA). However, Spe2-HA is not pulled down without any detergent (HES).
Supplemental Figure 2. Spe2-HA is not as effectively pulled down with OBG.

Spe2-HA is pulled down with both TritonX-100 and n-octyl-β-D-glucoside (OBG) in lysate, however the pull down is less efficient with OBG. Spe2-HA is not pulled down from intact cells with either detergent.
Supplemental Figure 3. Immunoprecipitation of Mtb12-FLAG.

Mtb12-FLAG is pulled down with protein A magnetic beads with OBG when incubated at room temperature for 0.5 or 1 hour, or at 4°C overnight (O/N). Interestingly, Mtb12-FLAG is not pulled down after 2 hours of incubation at room temperature.
Supplemental Figure 4. MptA knockdowns in WT background and Spe2 deletion background.

ProQ Emerald 488 glycan staining of LM and LAM on 20% SDS-PAGE. MptA was knocked down with the addition of anhydrotetracycline (atc) during log phase (log), stationary (stat), and late stationary (L-stat) growth in WT/MptA KD and Δspe2/MptA KD strains.


