LmeA is required for mycobacterial lipomannan elongation

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Keywords: bacterial genetics, carbohydrate metabolism, cell envelope, glycolipid, glycosyltransferase, lipomannan, Mycobacterium smegmatis, PimE

ABSTRACT

The integrity of the distinguishing, multilaminate cell envelope surrounding mycobacteria is critical to its survival and pathogenesis. The prevalence of phosphatidylinositol mannosides in the cell envelope suggests an important role in the mycobacterial life cycle. Indeed, deletion of the pimE gene (ΔpimE) encoding the first committed step in phosphatidylinositol hexamannoside biosynthesis in Mycobacterium smegmatis results in the formation of smaller colonies than wildtype colonies on Middlebrook 7H10 agar. To further investigate potential contributors to cell-envelope mannan biosynthesis while taking advantage of this colony morphology defect, we isolated spontaneous suppressor mutants of ΔpimE that reverted to wildtype colony size. Of 22 suppressor mutants, 6 accumulated significantly shorter lipomannan or lipoarabinomannan. Genome sequencing of these mutants revealed mutations in genes involved in the lipomannan/lipoarabinomannan biosynthesis, such as those encoding the arabinosyltransferase EmbC and the mannosyltransferase MptA. Furthermore, we identified three mutants carrying a mutation in a previously uncharacterized gene, MSMEG_5785, that we designated lmeA. Complementation of these suppressor mutants with lmeA restored the original ΔpimE phenotypes, and deletion of lmeA in wildtype M. smegmatis resulted in smaller lipomannan as observed in the suppressor mutants. LmeA carries a predicted N-terminal signal peptide, and density gradient fractionation and detergent extractability experiments indicated that LmeA localizes to the cell envelope. Using a lipid ELISA assay, we found that LmeA binds to plasma membrane phospholipids such as phosphatidylethanolamine and phosphatidylglycerol. LmeA is widespread throughout the Corynebacteriales; therefore, we concluded that LmeA is an evolutionarily conserved cell-envelope protein critical for controlling the mannan chain length of lipomannan/lipoarabinomannan.

The rise in multi-drug resistant Mycobacterium tuberculosis (Mt) is a global concern. One avenue to search for novel drug targets is through the biosynthetic pathway of the mycobacterial cell envelope components (1). Several enzymes in biosynthetic pathways of the key cell envelope glycolipids, such as phosphatidylinositol mannosides (PIMs), lipomannan (LM), and lipoarabinomannan (LAM), are shown or predicted to be essential in Mt (2, 3). Moreover, alterations in the LM/LAM structures make both Mt and nonpathogenic Mycobacterium smegmatis (Msme) susceptible to β-lactam antibiotics, which otherwise cannot cross...
the mycobacterial cell wall efficiently (4). These observations suggest that PIMs/LM/LAM play important structural roles within the cell envelope to maintain the permeability barrier. Furthermore, PIMs/LM/LAM are critical for virulence, with recognized host factor interactions (5-7).

The biosynthesis of PIMs/LM/LAM begins with phosphatidylinositol (PI) and is mediated by sequential actions of two mannosyltransferases: PimA and PimB, followed by the acylation of the mannose residue by an acyltransferase, PatA, to produce AcPIM2 (Fig. 1A) (8-11). Sequential addition of mannoses to AcPIM2 is mediated by an undetermined mannosyltransferase(s), resulting in the production of AcPIM4 (12). The AcPIM4 intermediate serves as a branch point for PIMs/LM/LAM biosynthesis, feeding into either LM/LAM formation or AcPIM6 production (Fig. 1A). The α1–2 mannosyltransferase PimE transfers a mannose residue from polyisoprenyl-phosphate-mannose (PPM) to AcPIM4, committing the pathway to the production of AcPIM6 (13). LM/LAM biosynthesis is mediated by α1–6 mannosyltransferase MptA (MSMEG_4241) and the α1–2 mannosyltransferase MptC (MSMEG_4247) (14-18). MptA is a PPM-dependent polymerase of the α1–6 mannose backbone, and the deletion of mptA results in the accumulation of an immature LM intermediate that carries 5-20 mannose residues instead of 21-34 mannose residues found in mature LM/LAM (14). MptC is another PPM-dependent mannosyltransferase, involved in mono-mannose side chain addition. While the functional roles of mono-mannose side chains remain obscure, we have previously suggested that mannan chain biosynthesis requires a balance between the enzymatic activities of MptA and MptC (18).

LpqW is a possible regulator of LM/LAM biosynthesis at the AcPIM4 branching point (19-21). Mutants lacking lpqW show defective LM/LAM biosynthesis and colony size smaller than wildtype (WT) (19). These phenotypic defects were spontaneously resolved in suppressor mutants of ΔlpqW, and mutations in pimE identified in these mutants suggest that alterations in AcPIM6 biosynthesis can phenotypically compensate for defects in LM/LAM. These studies highlight the complex interplay of PIMs/LM/LAM to create a functional cell envelope. In the current study, our initial observation that ΔpimE shows smaller colony morphology when grown on Middlebrook 7H10 agar led us to isolate spontaneous suppressors of ΔpimE, which restored the small colony morphology to that of WT. In some of these suppressor mutants, we found structural alterations in LM/LAM, which were caused by mutations in a previously uncharacterized protein, MSMEG_5785. Characterization of MSMEG_5785 reveals this protein as a cell envelope-associated phospholipid binding protein, involved in the mannan elongation of LM/LAM.

RESULTS
Isolation of ΔpimE suppressor mutants. When the ΔpimE MsmeG mutant was grown on Middlebrook 7H10 agar plates for 4 days at 37°C, we noticed that the colony size of the mutant was significantly smaller than WT (WT, 4.17 ± 0.91 mm; ΔpimE, 1.31 ± 0.45 mm; average ± standard deviation, n = 50). Interestingly, we noticed occasional appearances of spontaneous large colony mutants after sub-culturing (Fig. 1B). We isolated a total of 22 spontaneous large colony revertants. Because ΔpimE was generated by replacing the central 213 bp of the pimE gene with kanamycin-resistant gene (13), it seemed unlikely that the pimE gene was restored. Indeed, all 22 isolates remained kanamycin-resistant (not shown) and failed to produce AcPIM6 (Figs. 1C and S1). These results indicated that these revertants are suppressor mutants rather than same site revertants of the pimE gene.

Suppressor mutants show changes in LM/LAM structure. As described in the Introduction, the growth defect observed in ΔlpqW was restored by suppressor mutations in pimE (19). Inspired by these studies, we examined if our suppressor mutants of ΔpimE show structural changes in LM/LAM. Various changes in both LM/LAM size and abundance were noted in several of the suppressor mutants (Figs. 1D and S2). For example, the suppressor mutant S4 shows smaller LAM while LM appears comparable to that of the WT. Another prominent example is found in S1, S10, S20, S21, and S22, where both LM and LAM appeared smaller. We also found mutants with changes in the amount of LM/LAM (e.g., S5, S9, S12, S18, and S19). While there are a number of suppressor mutants that show no apparent changes
in LM/LAM, these initial observations suggested that one way for the ΔpimE mutant to revert back to the WT colony morphology is to alter the abundance or structures of LM/LAM.

**Whole genome sequencing reveals mutations in genes involved in LM/LAM biosynthesis.** We used whole genome sequencing of the parental ΔpimE mutant and the subset of suppressor mutations with apparent structural changes in LM/LAM to identify genetic alterations consistent with altered LM/LAM biosynthesis (Dataset S1). Table 1 summarizes the mutations found in the sequenced mutant strains. In the mutant S4, where smaller LAM was observed, we found a point mutation in *embC*, an arabinosyltransferase involved in LAM biosynthesis, being consistent with the LAM-specific defect. For other mutants accumulating smaller LM and LAM, such as S1, S10, S20, S21, and S22, we suspected that there might be a mutation in *mptA* gene, which encodes α1–6 mannosyltransferase involved in LM/LAM mannan elongation. We therefore PCR-amplified *mptA* gene and determined the gene sequence by the Sanger method. As suspected, we identified a mutation in the *mptA* gene amplified from S20 and S21 (Table 1). These two mutants have the same 24 bp deletion, suggesting that they originated from the same parental mutant. In contrast, we found no mutation in the *mptA* gene from S1, S10, and S22. Therefore, we subjected S1 and S10 to whole genome sequencing, and found missense mutations (G170D and V181G, respectively) in the gene MSMEG_5785, which has no previously assigned function (Fig. 2). We asked whether the third suppressor mutant (S22) had mutations in MSMEG_5785 by PCR amplification (see Table S1 for primer sequences). Interestingly, S22 had an insertion of a 2,276 bp transposon (TnpR) after the first 14 bp of the MSMEG_5785 gene (Fig. 2). The TnpR insertion into the near 5’ end of the gene suggested that the phenotypic changes in LM/LAM are likely due to the loss of the gene function. Given these mutant characteristics and additional features described below, we termed MSMEG_5785 as lipomannan elongation factor A (*lmeA*). To confirm loss of *LmeA* function, we introduced a P<sub>hsp60</sub>-*lmeA-HA* expression vector into each suppressor mutant. No changes in PIMs were detected in the complemented suppressor mutants (Fig. 3A). Importantly, mature LM and LAM were restored in each mutant when complemented with the P<sub>hsp60</sub>-*lmeA-HA* vector (Fig. 3B). Additionally, the complemented strains restored the ΔpimE small colony morphology (Fig. 3C). Taken together, three out of the twenty-two suppressor mutants of ΔpimE carried a loss-of-function mutation in the novel gene *lmeA*.

**LmeA is critical for LM/LAM mannan chain length maturation.** The LM/LAM phenotype of the suppressor mutants suggested a potential role of LmeA in LM/LAM biosynthesis. However, the phenotype could be dependent on the ΔpimE background. To test a direct role of LmeA in LM/LAM biosynthesis, we generated a strain carrying a markerless deletion of *lmeA* (ΔlmeA) in the WT background (Fig. S3A). The deletion of *lmeA* was confirmed by PCR (Fig. S3B). The ΔlmeA mutant showed the production of smaller LM and more disperse LAM, which corroborates the phenotype of the suppressor mutants (Fig. 4A). Importantly, there were no apparent changes in the biosynthesis of PIMs or other phospholipids (Figs. 4B and S4A). Moreover, the ΔlmeA mutant colony morphology was similar to WT (Fig. 4C). By complementing with either P<sub>hsp60</sub>-*lmeA-HA* or P<sub>native</sub>-*lmeA-HA*, mature LM/LAM production was restored (Fig. 4A). We also complemented the deletion mutant with *lmeA* carrying the point mutations found in the suppressor mutants S1 and S10. Neither of the *lmeA* mutants was able to restore mature LM/LAM (Fig. S4B), further supporting our notion that the mutations are loss-of-function. Interestingly, the expression levels of the mutated LmeA proteins were lower than that of WT protein and were undetectable by western blot (Fig. S4C), indicating that the mutant LmeA may be unstable and susceptible to protein degradation. These results indicate that LmeA is involved in LM/LAM biosynthesis and the *lmeA* mutant phenotype is independent of the *pimE* deletion.

**LmeA is a cell envelope protein.** LmeA is a conserved protein of unknown functions widely present in the order *Corynebacteriales* (Fig. S5A), but are apparently absent in other bacteria and other domains of life. Its ortholog in *Mtb*, Rv0817c, is 60% identical to *Msme* LmeA at the amino acid level, and the missense mutations found in the suppressor mutants S1 and S10 are both conserved in *Mtb* LmeA (Fig. S5B). LmeA has a conserved
hydrophobic region near the amino terminus, and SignalP 3.0, an algorithm effective for the prediction of mycobacterial signal peptides (22), predicted both Rv0817c and MSMEG_5785 to carry a signal peptide with high probabilities (0.998-1.000). Indeed, \( Mtb \) LmeA is a periplasmic protein identified by a recent secretome analysis (23). Furthermore, \( Mtb \) LmeA is found in the proteome of \( Mtb \) cell lysate but not in that of cell filtrate (24), implying cell envelope association. In elucidating the accessibility of \( Msmeq \) LmeA-HA, we noticed that the protein could not be immunoprecipitated from crude cell lysate using anti-HA antibody (Fig. 5A). This is in contrast to GlnA-HA, a cytoplasmic protein readily immunoprecipitated using anti-HA antibody. When we added Triton X-100, a mild detergent, to the cell lysate, we were able to pull down the LmeA-HA protein (Fig. 5A). These data also implied that LmeA-HA is a cell envelope protein and the HA epitope is not exposed for the antibody recognition when detergent is not present. To confirm further, we performed sucrose density gradient sedimentation to fractionate the cell envelope, intracellular membrane domain (IMD), and cytoplasmic fractions (25, 26). Markers, MptC, PimB’, and Mpa, respectively, showed separation of these three subcellular fractions, and demonstrated that LmeA-HA co-localizes to the cell envelope fraction (Fig. 5B). These data provide further evidence that LmeA is an extracytoplasmic cell envelope-associated protein.

**LmeA acts on a biosynthetic step genetically hypostatic to MptA-mediated LM mannan elongation.** Small intermediates of LM have been previously identified in the knockdown or knockout of MptA, the mannosyltransferase that elongates the mannan chain during LM/LAM biosynthesis (14, 27). This immature LM intermediate is known to carry 5-20 mannoses. While LmeA has no homology to known mannosyltransferases, the small LM produced in the \( \Delta lmeA \) mutant suggested that LmeA plays a role in the elongation of LM. At least two mannosyltransferases are thought to be involved in the elongation step: an unknown \( \alpha 1-6 \) mannosyltransferase(s) that extends \( \alpha 1-6 \) mannosyl chain on AcPIM4 to produce the LM intermediates seen in \( mptA \) deletion or knockdown strains, and MptA, the \( \alpha 1-6 \) mannosyltransferase that extends the chain generated by the unknown polymerase(s) (18). We hypothesize that LmeA may facilitate either the unknown polymerase or MptA. In order to determine the genetic interaction of LmeA with either polymerase, we introduced a tet-off \( mptA \) knockdown system (27) into the \( \Delta lmeA \) mutant, and suppressed the expression of \( mptA \) by anhydrotetracycline (atc). As shown in Fig. 6A, the small LM accumulating in \( \Delta lmeA \) (-atc, lane 3) became even smaller on SDS-PAGE upon suppression of the \( mptA \) expression (+atc, lane 4). The small LM accumulating in \( \Delta lmeA \) strain under \( mptA \) knockdown condition was apparently no different from the small LM accumulating in the \( mptA \) knockdown under the WT background (compare lanes 2 and 4). To confirm further, we analyzed the size distribution of LM by MALDI-TOF using a previously established method (27). We observed that the \( \Delta lmeA \) LM carried 10-22 mannose residues, being larger than the LM produced under \( mptA \) knockdown, which carried 11-17 mannoses (Fig. 6B). Nevertheless, \( \Delta lmeA \) LM was still significantly smaller than WT LM (21-34 mannose residues). Therefore, these analyses by MALDI-TOF were consistent with the SDS-PAGE migration patterns. Importantly, the LM intermediates accumulating in the \( mptA \) knockdown strains were identical regardless of \( lmeA \) deletion. Taken together, these data suggest that MptA is epistatic to LmeA.

The hypostatic nature of LmeA suggested that it might facilitate the function of MptA or other downstream enzymes. We therefore wondered if the over-expression of LmeA-HA could restore the small colony morphology in other suppressor mutants such as S4 and S21, which have mutations in \( embC \) (S4) and \( mptA \) (S21), respectively. However, the additional expression of LmeA had no obvious impact on the colony size (Table S2), indicating that LmeA does not have dominant effects on these other mutants.

**LmeA binds to phospholipids.** While LmeA is widely conserved in Corynebacteriales, standard BLAST analyses did not reveal any homologs with a protein of known function. Using RaptorX Structure Prediction algorithm (28), we were able to predict the \( \beta \)-rich structure of LmeA with a P-value of 4.90 x 10\(^{-4} \) using several templates including the bactericidal/permeability-increasing protein (BPI). BPI is a lipid-binding protein produced by lymphocytes and specifically binds to the lipid
portion of bacterial lipopolysaccharides (29). Given this homology, we were prompted to test the possibility that LmeA binds the plasma membrane phospholipids or LM intermediates directly to exert its function in the LM biosynthesis. We created an *Escherichia coli* strain transformed with an IPTG-inducible His-LmeA expression vector and prepared a cell lysate of *E. coli* heterologously expressing His-LmeA. We tested the *E. coli* lysate by anti-His western blotting and found a single band with the expected molecular weight of 29 kDa (Fig. 7A). We modified an established LAM-binding assay (30) and designed a lipid ELISA assay to test if His-LmeA binds to any lipids. Commercially available lipids were first separated by TLC and stained by cupric acetate to evaluate their purities (Fig. S6A-C). We also purified the small LM intermediates from the *mptA*-knockdown strain. We found that the binding of His-LmeA to the LM intermediates was minimal (Figs. 7B and S6D). In contrast, we observed more robust binding of His-LmeA to phospholipid species such as PI and phosphatidylethanolamine (PE), (Fig. 7B), which are major structural components of *Msmeg* plasma membrane (31, 32). The binding of His-LmeA to PI and PE was dose-dependent and saturated at ~2 µM (Fig. S6E-F). Although our current assay system has limitations that make the accurate determination of Kd values difficult, we determined the apparent Kd values from the available data shown in Fig. S6 to compare the relative binding affinities of LmeA to different lipid species. The Kd values for PI and PE were 0.242 µM and 0.487 µM, respectively, showing that the binding affinity of LmeA to PI was comparable but slightly higher than that to PE. Because LmeA bound both PI and PE effectively, we used phosphatidic acid (PA), a “headless” phospholipid, to examine if the phospholipid head group is relevant to the binding (Fig. 7B and S6G). The binding of LmeA to PA was comparable to PI with the Kd value of 0.297 µM, indicating that the phospholipid head groups do not affect the binding affinity. We also tested triacylglycerol (TAG), but LmeA did not bind to this glycerolipid (Figs. 7B and S6H), indicating that the phosphate residue is critical for the binding. Because mannan polymerization for LM biosynthesis utilizes PPM as the mannose donor, we tested if LmeA binds to poly-prenol-phosphate with a comparable affinity to PA. However, binding of LmeA to geranylgeranyl monophosphate (GGP) was minimal with an apparent Kd value of 8.65 µM (Fig. S6I). Finally, we tested if LmeA binding to PE could be inhibited by the addition of soluble mannose 1-phosphate or GDP-mannose. However, up to 10 mM of these compounds had no effects on the binding of LmeA to PE, implying that mannose-containing molecules are not involved in the substrate recognition of LmeA (Fig. S7). Taken together, we conclude that the binding of LmeA is specific to glycerophospholipids.

**DISCUSSION**

In this study, we revealed that the previously uncharacterized protein LmeA encoded by *MSMEG_5785* is involved in LM/LAM biosynthesis through a forward genetic screen of the Δ*pimE* mutant. We propose that LmeA is a cell envelope-associated phospholipid-binding protein that facilitates the maturation of mannan chain length. Several pieces of evidence suggest that LmeA is directly involved in mannan elongation of LM/LAM biosynthesis. First, the deletion of the *lmeA* gene resulted in the production of LM/LAM with shorter chain length. Second, LmeA is an extra-cytoplasmic cell envelope-associated protein. Third, LmeA is genetically hypostatic to MptA. Together, these observations are consistent with the idea that LmeA regulates the mannan chain polymerization by controlling the catalytic activity or processivity of MptA (Fig. 8). However, the precise function of LmeA remains unknown. One possibility is that LmeA controls the availability of the mannose donor substrate PPM or poly-prenol-phosphate-based carbohydrate donors in general. Such a function of LmeA could rescue other suppressor mutants such as S4 and S21 by increasing the general availability of poly-prenol-phosphate-based carbohydrate donors. However, we did not observe such effects of LmeA on these other suppressor mutants. Furthermore, lipid binding assays suggested that LmeA preferentially binds glycerophospholipids over geranylgeranyl-phosphate and that the binding of LmeA was not competitively inhibited by GDP-mannose or mannose 1-phosphate. Therefore, we suggest that LmeA is not involved in the metabolism and trafficking of poly-prenol-phosphate-based substrates.
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Based on the fact that LmeA is extra-cytoplasmic and binds to phospholipids such as PI and PE, we propose that LmeA is a protein peripherally bound to the plasma membrane. Nikaido and colleagues have taken the approach of reverse micelle extraction to show that major phospholipid species such as PI and PE are found predominantly in the plasma membrane and relatively depleted in the mycobacterial or corynebacterial outer membrane (33, 34). Therefore, the ability of LmeA to bind to phospholipid species suggests that LmeA is a periplasmic plasma membrane-associated protein (Fig. 8). In *Mycobacterium marinum*, the LmeA ortholog (MMAR_4866) was relatively resistant to a differential detergent extraction, which was effective in selectively extracting known outer membrane proteins (35), further supporting the periplasmic location of LmeA. Nevertheless, *Mtb* homolog of LmeA (Rv0817c) is predicted to be an outer membrane β-barrel (36). These contradictory observations highlight that further studies are needed to determine functional and structural features of LmeA. Although our attempts have so far been unsuccessful, an important next step is the purification of LmeA in its active form.

Complex interplay of PIMs/LM/LAM in the integrity of the mycobacterial cell envelope is an emerging theme. Previous studies showed that mutations in *pimE* can compensate for the growth defect of the *lpqW* mutant that produces reduced levels of LM/LAM (19, 21). Our current study showed an opposite biological response where the growth defect of Δ*pimE* was compensated by additional structural changes in LM/LAM. It appears that the balance between the levels of AcPIM6 and LM/LAM is critical for fitness, and mycobacteria regain optimal growth by genetic changes that warrant the homeostasis of the glycocalyx. A conceptually similar glycan compensation is well-known as chitin emergency response in *Saccharomyces cerevisiae* (37, 38). In this stress response, compromised cell wall integrity due to defects in surface glycans such as β-glucan, mannan, O-linked glycans, or glycosylphosphatidylinositol anchors is compensated by the upregulation of chitin synthesis. More recently, it was reported that the lack of one particular glycan structure in mammalian cells is compensated by the production of bioequivalent glycans in the Golgi apparatus (39). Such principles of glycan homeostasis may apply to the maintenance of cell surface glycocalyx in mycobacteria as well. However, completely different scenarios are also possible. For example, it has not been explored if PIMs play a role as signaling molecules to facilitate colony growth. We have previously reported that *Msmeg* as well as *Corynebacterium glutamicum* produce PI 3-phosphate (40), but nothing is known if inositol polyphosphates is released by a phospholipase. Similarly, it is completely unknown if there are phospholipases that can act on PIMs to release the glycan head groups and if such released molecules can function as signaling molecules. Interestingly, a recent report suggested that synthetic lipid-linked arabinomannan heptasaccharide can effectively inhibit biofilm formation in *Msmeg* (41), implying that comparable molecules like phospholipase-digested AcPIM6 may have similar biological activities. Finally, lipid-free D-arabino-D-mannan, D-mannan, and their phosphorylated counterparts are found in the extracellular capsules of *Mtb* and other mycobacteria, implying that there may be phospholipases that can act on larger PI-anchored glycolipids such as LM and LM (42-46). We speculate that such phospholipases may promiscuously act on PIMs as well. The Δ*pimE* suppressor mutants that we identified includes many additional mutants that do not show changes in LM/LAM structures, suggesting that there are multiple pathways to rescue the small colony morphology of Δ*pimE*. Analysis of other suppressor mutants may further reveal the potential molecular mechanisms behind the biological responses of PIMs/LM/LAM mutants.

The enzymes for the synthesis of AcPIM2, such as PimA and PimB', are essential for the viability of *Msmeg* (8, 9). AcPIM2 is a mature product, but also serves as a precursor for the synthesis of AcPIM6, LM, and LAM. Therefore, it remains unknown if AcPIM2 or any of the downstream products are essential. Interestingly, a recent study revealed that a corynebacterial membrane protein
NCgl2760 is involved in LM biosynthesis likely at a step prior to the MptA-mediated mannan elongation (47). The orthologous gene is predicted to be essential in Mtb and cannot be deleted from the endogenous locus in Msmeg (MSMEG_0317) unless an extra copy of the gene is present (47). These data imply that defects in the early stage of LM/LAM biosynthesis might be lethal to Msmeg. In contrast, mild structural defects in LM/LAM, such as the one caused by the mptA depletion, can be tolerated in Msmeg. Therefore, the non-essential nature of LmeA further supports our hypothesis that this protein acts on the downstream of MptA-mediated mannan elongation.

In contrast to Msmeg, the requirement of PIMs/LM/LAM appears to be more stringent in Mtb. EmbC, an arabinosyltransferase involved in LAM biosynthesis, has been experimentally shown to be essential for viability in Mtb (3). Genome-wide transposon mutagenesis studies further predict that many genes involved in PIMs/LM/LAM biosynthesis, such as pimA, pimB, pimE, and mptA are essential in Mtb (2). We have previously created a mutant strain of Mtb over-expressing MptC (Rv2181) (27). This mutant produces aberrant LM/LAM with truncated mannan chain, and such mild modifications were sufficient to make Mtb defective in establishing infection in mice. Indeed, the lmeA ortholog (Rv0817c) is predicted to be essential in Mtb (2). The cell envelope localization of LmeA, its predicted essentiality in Mtb, and the absence of its homologs in human make this protein a potentially attractive drug target. To this end, we are currently generating lmeA mutant in Mtb to demonstrate that lmeA is an essential gene.

EXPERIMENTAL PROCEDURES

**Mycobacterial growth conditions**

*Msmeg* mc²155 and derived mutants were grown at 37°C on Middlebrook 7H10 agar (BD) supplemented with 0.2% glucose (w/v) and 15 mM NaCl as described (48). Liquid cultures were at 30°C in Middlebrook 7H9 broth (BD) supplemented with 0.2% glycerol (v/v), 0.05% Tween 80 (v/v), 0.2% glucose (w/v) and 15 mM NaCl (48). Knockdown of mptA was induced with 40 ng/mL of atc (Acros) for 48 hours. Antibiotic concentrations used were 20 μg/mL kanamycin (MP), 50 μg/mL streptomycin (Fisher Scientific), and 100 μg/mL hygromycin (Wako).

**DNA purification, whole genome sequencing, and mutation analysis**

Genomic DNA from select suppressor mutants was purified as previously described (49). Whole-genome sequencing was performed on the Illumina MiSeq platform with 251 bp paired-end sequencing. Each genomic DNA (300 ng) was sheared to an average size of 600 bp with the Covaris S220 (Covaris). The DNA library was prepared using the KAPA Library Preparation Kit (Kapa Biosystems) and TruSeq adapters (Illumina) according to manufacturer’s instructions. Sequences were filtered and trimmed based on quality score using Quick Read Quality Control (Buffalo V. 2011. Quick read quality control. Bioconductor, Seattle. Available at www.bioconductor.org/packages/release/bioc/html/qrqc.html. Accessed October 17, 2013.), Sickle (Joshi N. 2011. Sickle — A windowed adaptive trimming tool for FASTQ files using quality. GitHub, San Francisco. Available at https://github.com/najoshi/sickle. Accessed October 17, 2013.) and Scythe (Buffalo V. 2011. Scythe—A very simple adapter trimmer. GitHub, San Francisco. Available at https://github.com/ucdavis-bioinformatics/scythe. Accessed October 17, 2013.), were aligned to the reference *Msmeg* NC_008596.1 genome using Bowtie2 (50), and variant calls were made using Samtools (51, 52). The sequence data were analyzed by the Integrative Genomics Viewer (53). Additional suppressor mutants were analyzed specifically for pimE (A115 & A118), mptA (A193 & A194), and MSMEG_5785 (A197 & A198) by amplifying the genes by PCR (see Table S1) and sequencing the amplified products by standard Sanger sequencing.

**Lipid extraction and analysis**

Crude lipids were extracted as described and the delipidated pellet was incubated with phenol/water (1:1) for 2 hours at 55°C to extract LM/LAM (18). PIMs were separated by high performance thin layer chromatography (TLC) silica gel 60 (EMD Merck) using chloroform/methanol/13 M ammonia/1 M ammonium acetate/water (180:140:9:9:23) as a mobile phase and visualized by orcinol staining as described (18). LM/LAM
samples were separated by SDS-PAGE (15% gel) and visualized using ProQ Emerald 488 glycan staining kit (Life Technologies). For the mass spectrometric analysis and lipid binding assay (see below), LM/LAM were purified using an octyl-Sepharose column (GE Healthcare) as before (18).

Construction of plasmids

Knockout and expression vectors were constructed as detailed below.

**pMUM57**: The upstream and downstream region of *lmeA* were amplified using A217/A218 and A219/A220 primer sets (Table S1), respectively. These two fragments were then digested with Van91I and ligated into Van91I-digested pCOM1 (26), resulting in the *MSMEG_5785* knockout construct.

**pMUM54**: *lmeA* was amplified using primers A215 and A216. The fragment was then digested with NdeI and ScaI and ligated into pMUM12 (26), which was digested with NdeI and ScaI, to generate pMUM54, an expression vector for Phsp60-*lmeA*-HA.

**pMUM107, pMUM125 and pMUM126**: Primers A470 and A472 were designed to amplify *lmeA* including 165 bp of upstream native promoter region from either WT (pMUM107), S1 (pMUM125) or S10 (pMUM126). The PCR fragment was then digested by KpnI and Xbal and ligated into pMV306 that was digested by KpnI and Xbal, resulting in pMUM107, pMUM125, and pMUM126, expression vectors for P*native*-lmeA-HA, P*native*-lmeA(G170D)-HA, and P*native*-lmeA(V181G)-HA, respectively.

**pMUM121**: Primers A552 and A553 were designed to amplify *lmeA*, excluding the sequence coding for the predicted N-terminal signal sequence. A TOPO cloning kit (Invitrogen) was used to insert the fragment into pET100, creating an IPTG inducible His-LmeA expression construct.

**MALDI-TOF MS analysis**

Octyl-Sepharose-purified LM/LAM preparation (0.5 µl) was mixed with an equal volume of matrix solution (20 mg/ml sinapinic acid (Millipore-Sigma), 30% acetonitrile (Fisher Scientific), 1% trifluoroacetic acid (Fisher Scientific) in water). Samples were analyzed on a Bruker Microflex MALDI-TOF instrument (Bruker Daltonics) using linear mode and positive ion detection. The data were analyzed using the Microflex software.

**Bead-beating cell lysis**

Cell pellets were washed in 50 mM HEPES/NaOH (pH 7.4) twice and resuspended in a lysis buffer containing 25 mM HEPES/NaOH (pH 7.4), 15% glycerol, 2 mM EGTA and a protease inhibitor mix. Four times the pellet weight of acid-washed glass beads (Millipore-Sigma) was added, and cells were lysed by a BeadBug Microtube Homogenizer (Benchmark Scientific) at 4°C with beating at 4000 rpm for 30 sec. Bead-beating was repeated for 5 times with 1 min interval on ice. Beads and cell debris were removed by centrifugation.

**SDS-PAGE and western blotting**

Protein samples (12 µl) were mixed with reducing sample loading buffer, denatured on ice for 30 min or boiled for 5 min, and separated on SDS-PAGE (12% gel). After western blot transfer, the PVDF membrane was incubated with a primary antibody at 1:2000 dilution (mouse anti-HA (Millipore-Sigma), rabbit anti-MptC (18), mouse anti-Penta-His (Qiagen), rabbit anti-PimB′ (18), or rabbit anti-Mpa (54)), followed by incubation with a horseradish peroxidase-conjugated secondary antibody, either anti-rabbit or anti-mouse IgG (GE Healthcare), at a 1:2000 dilution. Bands were visualized by chemiluminescence and recorded using ImageQuant LAS 4000mini (GE Healthcare).

**Sucrose density gradient sedimentation and fractionation**

Sucrose density gradient fractionation was performed as before (25, 26).

**Immunoprecipitation**

Anti-HA agarose beads (Millipore-Sigma) were washed with a buffer containing 25 mM HEPES-NaOH (pH 7.4), 2 mM EGTA, and 150 mM NaCl (HES) or another buffer containing 25 mM HEPES-NaOH (pH 7.4), 2 mM EGTA, 150 mM NaCl, and 1% Triton-X100 (HEST). Bead beating cell lysate (36 µl) was added to the pre-washed beads (10 µl bed volume), and incubated with HES or HEST buffer at 4°C overnight under gentle rotation at 5 rpm. Beads were washed with 1 ml of HES or HEST buffer prior to elution with 10 µl of 1 mg/ml HA peptide (AnaSpec) twice at 30°C. Eluates were separated on SDS-PAGE and visualized via western blot as described above.
E. coli cell transformation and lysis
E. coli BL21 strain was transformed with pMUM121 by heat shock. The transformed strain was grown in terrific broth to log phase and induced with 1 mM IPTG (Fisher Scientific) for 3 hours at 37°C. Untransformed E. coli was grown under the same condition. Cells were incubated with 1 mg/mL lysozyme (Fisher Scientific) for 20 min at room temperature in a buffer containing 50 mM HEPES-NaOH (pH 7.4) 200 mM NaCl, and 1x FastBreak Cell Lysis Reagent (Promega) and was lysed by sonication. The lysate was centrifuged and the supernatant was used for all binding assays or western blot analysis.

Lipid binding assay
L-α-phosphatidylinositol (PI) from soybean, L-α-phosphatidylethanolamine (PE) from egg yolk, and glycerol trioleate (TAG) were purchased from Millipore-Sigma. L-α-phosphatidic acid from chicken egg was purchased from Avanti. Geranylgeranyl monophosphate was purchased from Lardan. PI was a crude preparation with many contaminants, and was further purified by preparative TLC. Purity of each lipid was examined by TLC using two solvent systems: hexane / diethyl ether / formic acid (40:10:1) for TAG or chloroform / methanol / 13 M ammonia / 1 M ammonium acetate / water (180:140:9:9:23) for PI, PE, PA and GGM. For lipid ELISA, lipids were serially diluted in isopropanol by two-fold from an initial amount of 0.4 nmol/well and the solvent was evaporated in 96-well immunoplates (Brand GmbH). Once the wells were completely dried, they were blocked at 4°C overnight with 200 µl of 5% milk in phosphate buffer saline with 0.05% Tween-20 (PBST20). A mixture of 8 µl cell lysate and 32 µl of PBST20 was added to each well and incubated at 37°C for 2 hours. After washing with PBST20, 40 µl of mouse anti-Penta-His IgG (1:2000 dilution, Qiagen) was added and incubated at 4°C overnight. After washing with PBST20, 40 µl of horseradish peroxidase-conjugated antimouse IgG (1:4000 dilution, GE Healthcare) was added and incubated at room temperature for 1 hour. After washing with PBST20, 100 µl of 3,3′,5,5′-tetramethylbenzidine (BD) was added and the colorimetric changes were read at 650 nm after 1 hour incubation at room temperature. Kd values were calculated using a nonlinear regression function of Prism 7 (GraphPad Software), assuming that LmeA has one lipid binding site. For competition assays, microtiter plates were coated with 0.05 nmol of PE (corresponding to 1.25 µM). E. coli lysate was pre-incubated with 10 mM mannose 1-phosphate (Millipore-Sigma) or GDP-Mannose (Millipore-Sigma) at room temperature for 30 min before being added to the PE-coated well.

Acknowledgements: This work was supported by a Biomedical Research Grant (RG-414805) from the American Lung Association and a Research Grant from the Pittsfield Anti-Tuberculosis Association to YSM. APTO was a summer research student supported by the UMass Amherst PREP Program. We thank Dr. Stephen Eyles (Institute for Applied Life Sciences, University of Massachusetts Amherst) for help with mass spectrometry and Dr. Heran Darwin (New York University) for the gift of anti-Mpa antibody. We also thank Julia Puffal, William Eagen, and Sarah Osman for discussion and critical reading of the manuscript.

Conflicts of interest: The authors declared no conflicts of interest with the content of this article.

Author contributions: KCR conducted most of the experiments, analyzed the results, and wrote the paper. SAH conducted a part of the experiments shown in Figs. 5 and 6. DM, JAM and SN sequenced and analyzed the whole genome of the suppressor mutants (Fig. 2 and Table 1). LRB and APTO conducted some initial experiments shown in Fig. 1. JNS contributed to the experiments shown in Fig. 7. YSM conceived the idea, designed the study and wrote the paper.

REFERENCES
LmeA is required for mycobacterial lipomannan elongation


LmeA is required for mycobacterial lipomannan elongation.


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Sci USA. 111, 4958–4963


52. Li, H. (2011) A statistical framework for SNP calling, mutation discovery, association mapping
and population genetical parameter estimation from sequencing data. *Bioinformatics.* 27, 2987–2993


### Table 1. Mutations in the suppressor mutants that showed changes in LM/LAM.

<table>
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<tr>
<th>Phenotype</th>
<th>Strain</th>
<th>Gene</th>
<th>Analysis</th>
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<td>Small LAM</td>
<td>S4</td>
<td>MSMEG_6385 (embC)</td>
<td>Whole genome</td>
<td>Missense (P352L)</td>
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<tr>
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<td>MSMEG_5785 (lmeA)</td>
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<tr>
<td>Small LM/LAM</td>
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<td>Missense (V181G)</td>
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<td>Transposon insertion</td>
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*1 The deletion included 2 bp of the 3' end of the gene, stop codon, and 19 bp of the 3' untranslated region.

### Figure Legends

**Figure 1.** Isolation of ΔpimE suppressor mutants. A) PIMs/LM/LAM biosynthetic pathway. Mannosyltransferases involved in the biosynthesis of the LM intermediate are unknown. MptA is α1–6 mannosyltransferase involved in the mannan backbone elongation while MptC is α1–2 mannosyltransferase that adds mono-mannose side chains. The mannose donors, GDP-mannose (GDP-Man) and PPM, are indicated for mannosyltransferase reactions. B) Colony morphology of WT, ΔpimE, and the suppressor mutant S1. The suppressor mutant S1 (as well as the other 21 suppressor mutants, not shown) restored WT colony morphology. Scale bar = 1 cm. C) Profile of PIMs purified from the suppressor mutants. Lipid extracts of WT, ΔpimE, and the suppressor mutants S1-S6 were separated by TLC and visualized by orcinol staining. Only a part of the TLC plate is shown. None of the suppressor mutants were able to restore the production of AcPIM6 (arrowhead). See Fig. S1 for the other suppressor mutants. D) LM/LAM profile of the suppressor mutants, showing the small LM/LAM phenotype of the mutant S1. Extracts of LM/LAM were separated on SDS-PAGE and visualized by ProQ Emerald 488 glycan staining. See Fig. S2 for the other suppressor mutants.

**Figure 2.** Three suppressor mutants (S1, S10 and S22) have distinct mutations in *MSMEG_5785*. The red lines indicate where the mutations were found. Mutated bases and amino acids were shown in red. The insertion of a 2.27 kbp transposon in S22 caused a frameshift mutation.

**Figure 3.** Complementation of the suppressor mutants S1, S10 and S22 by *lmeA-HA* restores the ΔpimE phenotype. A) AcPIM6 not restored by *lmeA-HA* complementation. PIMs were purified from the suppressor mutants S1, S10, and S22 with or without complementation, separated by TLC, and visualized by orcinol staining. Only a part of the TLC plate is shown. B) The normal sizes of LM/LAM restored upon complementation. LM/LAM purified from the suppressor mutants with or without complementation were separated by SDS-PAGE and visualized by ProQ Emerald 488 glycan staining. C) Colony size of the suppressor mutants was restored to the smaller colony morphology of ΔpimE when complemented with LmeA-HA. The box plots of colony sizes (n = 44) are shown. Bars, standard deviations; x, maximum and minimum data points.
Figure 4. Phenotypes of \textit{lmeA} deletion mutant. A) \textit{lmeA} deletion in WT background, resulting in accumulation of smaller LM and diffusely distributed LAM. LM/LAM purified from $\Delta lmeA$ and its complemented strains were separated by SDS-PAGE and visualized using ProQ Emerald 488 glycan staining. Expression vectors, P\text{native}-\textit{lmeA-HA} and P\text{hsp60}-\textit{lmeA-HA}, are driven by native and strong hsp60 promoters, respectively. P\text{native}-\textit{lmeA-HA} was more effective in restoring mature LM/LAM. B) PIMs from $\Delta lmeA$ and its complemented strains, showing that $\Delta MSMEG\_5785$ does not impact AcPIM6 biosynthesis. Only a part of the TLC plate is shown. C) Colony morphology, demonstrating similar morphologies of WT and $\Delta lmeA$ colonies. Scale bar = 1 cm.

Figure 5. LmeA is a cell envelope protein. A) Immunoprecipitation (IP) of LmeA-HA with (+) or without (-) a mild detergent. Western blot using anti-HA antibody, showing that anti-HA IP of LmeA-HA was possible only when Triton X-100 was present during the incubation. Two separate strains expressing either LmeA-HA (29.5 kDa) or a cytosolic marker, GlnA1-HA (54.6 kDa), were used. B) Sucrose gradient sedimentation of LmeA-HA expressed from the native promoter. Expected molecular weights: anti-HA, LmeA-HA (29.5 kDa); anti-MptC, cell envelope marker (48.2 kDa); anti-PimB’, intracellular membrane domain (IMD) marker (41.4 kDa); and anti-Mpa, cytosolic marker (68.0 kDa).

Figure 6. Effect of MptA depletion on $\Delta lmeA$. A) LM/LAM profile. LM/LAM were extracted after either 48-hour incubation with (+) or without (-) atc to induce \textit{mptA} knock down in $\Delta lmeA$ mutant and WT control. B) MALDI-TOF mass spectra of LM for WT, $\Delta lmeA$, \textit{mptA} knockdown (KD), and \textit{mptA} KD in $\Delta lmeA$. Mannose residues are indicated for their corresponding peaks. LM peaks are detected as sinapinic acid adducts.

Figure 7. LmeA binds to phospholipids. A) Anti-His western blot of \textit{E. coli} lysate upon 3 hour IPTG induction. T, transformed with the His-LmeA expression vector; UT, untransformed. Ponceau S staining shows protein loading. B) Binding assay using \textit{E. coli} lysate expressing His-LmeA. Microtiter plates were coated with 2.5 $\mu$M of TAG, PI, PE, PA, GGP, small LM intermediates, and a fixed amount (8 $\mu$l) of \textit{E. coli} lysate, transformed (T) with the His-LmeA expression vector or untransformed (UT), was added.

Figure 8. A model of LmeA function and localization in LM/LAM biosynthesis. LmeA potentially binds to plasma membrane phospholipids on the periplasmic side, and functions downstream of MptA. MptC is not shown.
Figure 1

A

B

C

D
Figure 2
Figure 3

A

WT ΔspinE S1 + S10 + S22 + P

AcPIM2
AcPIM4
AcPIM6

B

WT ΔspinE S1 + S10 + S22 + P

LAM
LM

C

Colony size (mm)

WT ΔspinE S1 + S10 + S22 + P

kDa

29

18

14
Figure 4

A

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B

LAM

LM

kDa

29

18

14

C

AcPIM2

AcPIM4

AcPIM6

WT

ΔlmeA
Figure 5

A

Input  Anti-HA IP
-  -  +  +

TritonX-100
- 37 kDa

LmeA

- 75 kDa

GlnA1

- 50 kDa

B

Cytosol  IMD  Cell Envelope

1  2  3  4  5  6  7  8  9  10  11  12

kDa

37  37  37  50  37  75

Anti-HA

Anti-MptC

Anti-PimB

Anti-Mpa
Figure 7

A

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B

![Graph showing A_max values for different samples](image)

- PE
- PI
- PA
- GGP
- TAG
- LM

T

UT
Figure 8
The cell envelope-associated phospholipid-binding protein LmeA is required for mannose polymerization in mycobacteria
Kathryn C. Rahlwes, Stephanie A. Ha, Daisuke Motooka, Jacob A. Mayfield, Lisa R. Baumöl, Justin N. Strickland, Ana P. Torres-Ocampo, Shota Nakamura and Yasu S. Morita

*J. Biol. Chem. published online August 29, 2017*

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