Critical Roles for Lipomannan and Lipoarabinomannan in Cell Wall Integrity of Mycobacteria and Pathogenesis of Tuberculosis

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ABSTRACT  Lipomannan (LM) and lipoarabinomannan (LAM) are mycobacterial glycolipids containing a long mannose polymer. While they are implicated in immune modulations, the significance of LM and LAM as structural components of the mycobacterial cell wall remains unknown. We have previously reported that a branch-forming mannosyltransferase plays a critical role in controlling the sizes of LM and LAM and that deletion or overexpression of this enzyme results in gross changes in LM/LAM structures. Here, we show that such changes in LM/LAM structures have a significant impact on the cell wall integrity of mycobacteria. In Mycobacterium smegmatis, structural defects in LM and LAM resulted in loss of acid-fast staining, increased sensitivity to β-lactam antibiotics, and faster killing by THP-1 macrophages. Furthermore, equivalent Mycobacterium tuberculosis mutants became more sensitive to β-lactams, and one mutant showed attenuated virulence in mice. Our results revealed previously unknown structural roles for LM and LAM and further demonstrated that they are important for the pathogenesis of tuberculosis.

IMPORTANCE  Tuberculosis (TB) is a global burden, affecting millions of people worldwide. Mycobacterium tuberculosis is a causative agent of TB, and understanding the biology of M. tuberculosis is essential for tackling this devastating disease. The cell wall of M. tuberculosis is highly impermeable and plays a protective role in establishing infection. Among the cell wall components, LM and LAM are major glycolipids found in all Mycobacterium species, show various immunomodulatory activities, and have been thought to play roles in TB pathogenesis. However, the roles of LM and LAM as integral parts of the cell wall structure have not been elucidated. Here we show that LM and LAM play critical roles in the integrity of mycobacterial cell wall and the pathogenesis of TB. These findings will now allow us to seek the possibility that the LM/LAM biosynthetic pathway is a chemotherapeutic target.

Mycobacterium tuberculosis, an etiologic agent of tuberculosis (TB), infects around one-third of the world population and kills millions of people annually (1). A critical contributor to the ability of M. tuberculosis to evade the host immune system is its hydrophobic and complex cell wall, which is composed of peptidoglycans, arabinogalactans, mycolic acids, and glycolipids layered on top of the plasma membrane (2). Lipomannan (LM), lipoarabinomannan (LAM), and phosphatidylinositol (PI) mannosides (PIMs) are mannosic-containing glycolipids that are important constituents of the cell envelope. These molecules have a PI membrane anchor and are embedded in the plasma membrane or the outer membrane by their lipid moieties (3). PIMs are highly heterogeneous in structure and carry up to four fatty acids and six mannoses (4, 5). The predominant conformations of PIM are triacylated species termed AcPIM2 and AcPIM6, which carry two and six mannoses, respectively. LM and LAM carry a much longer chain of α-1,6-linked mannoses, and the α-1,6-mannan backbone is further modified by multiple α-1,2-monomannose branches (Fig. 1) (6). In Mycobacterium smegmatis, a nonpathogenic model organism, LM/LAM mannan carries 21 to 34 mannose residues (7), and in the case of LAM, the mannan backbone is further modified with arabinan(s), which consists of α-1,5-linked arabinose backbones with α-1,3 branch points.

PIMs, LM, and LAM are synthesized by sequential additions of mannoses and arabinoses to PI, one of the major phospholipids in mycobacterial plasma membranes (5, 8–10) (Fig. 1). While the initial steps of PIM/LM/LAM biosynthesis overlap, the biosynthetic pathway of LM and LAM diverges from that of AcPIM6 at...
For the LM/LAM pathway, the elongating while they do not have any apparent defect in LM/LAM biosynthesis (12). For the LM/LAM pathway, the elongating through the intermediate AcPIM4 (11). PimE (MSMEG_5136) is an α-1,2-mannosyltransferase that commits AcPIM4 to the AcPIM6 pathway, and pimE deletion mutants cannot produce AcPIM6, while they do not have any apparent defect in LM/LAM biosynthesis (12). For the LM/LAM pathway, the elongating α-1,6-mannosyltransferase (MSMEG_4241) and branch-forming α-1,2-mannosyltransferase (MSMEG_4247) mediate mannan synthesis in M. smegmatis (7, 13). An MSMEG_4241 deletion mutant accumulates an LM intermediate carrying 5 to 20 mannose residues, suggesting that there is at least one other unidentified α-1,6-mannosyltransferase involved in the initial stage of the mannan elongation. Deletion of MSMEG_4247 resulted in ablation of branch-forming α-1,2-mannosyltransferase activities, leading to accumulation of branchless LAM and the complete absence of LM (13, 14). Interestingly, we previously reported that the overexpression of MSMEG_4247 resulted in the production of smaller LM and LAM carrying dwarfed mannan and arabinan (14). Because MSMEG_4247 is the branch-forming mansyltransferase, we initially expected a greater frequency of mono- and trimannosyl LM/LAM biosynthetic pathway and phenotypes of the mutants. See the text for details.

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mannosyltransferase involved in AcPIM6 synthesis, is critical for the maintenance of plasma membrane structure, and a ΔpimE mutant developed plasma membrane invaginations (12) (Fig. 2C, arrows). In contrast, LM/LAM mutants did not show any morphological plasma membrane aberrations, and occasional membrane invaginations in the LM/LAM mutants were no more frequent than they were in wild-type cells (~20%) (Fig. 2C). These data suggested that structural changes in LM and LAM do not have significant impact on the plasma membrane morphology. We then examined the sensitivity of these mutants to chemical compounds such as malachite green, sodium dodecyl sulfate (SDS), and crystal violet. These lipophilic compounds are toxic to mycobacteria and have been used to test the permeability of the plasma membrane (28). Consistent with the morphological abnormalities of the ΔpimE plasma membrane, the ΔpimE mutant became markedly sensitive to these compounds (Fig. 2D). In contrast, LM/LAM mutants showed little change in sensitivity, suggesting that structural changes in LM and LAM do not affect the permeability of these compounds.

Acid-fastness is a hallmark of mycobacteria and has been attributed to the waxy nature of the cell wall outer membrane. We wondered if the acid-fastness is affected in our mutants. While the parental strain of *M. smegmatis* showed a typical red color following carbol-fuchsin staining, both Δ4247 and Δ4247 + Phsp604247 strains were negative for this staining (Fig. 2E). These data suggested that the cell wall integrity of the LM/LAM mutants is compromised significantly despite the fact that these mutants are resistant to the abovementioned chemical compounds.

Catalytic activity of MSMEG_4247 is critical for changes in cell wall integrity. The expression of MSMEG_4247 driven by the Hsp60 promoter is significantly greater than the endogenous level (14). Therefore, we wished to exclude the possibility that the altered cell wall properties of Δ4247 + Phsp604247 are a nonspecific effect of protein overexpression. We have previously demonstrated that the smaller LM/LAM phenotype of Δ4247 + Phsp604247 can be reproduced when MSMEG_4247 is overexpressed in a wild-type background, and 15-fold overexpression is sufficient to induce this effect. We also demon-
strated that catalytically inactive MSMEG_4247 D45A mutant
protein cannot induce the same effect in the same wild-type
background (14). We took advantage of these observations and
compared wild-type cells overexpressing catalytically active
and inactive forms of the enzyme [WT
Phsp60 4247 and
WT
Phsp60 4247(D45A)]. We preferred the wild-type back-
ground because deletion mutants might have additional muta-
tions that could complicate the interpretation of our data. We
first confirmed high expression levels of MSMEG_4247 by
Western blotting (Fig. 3A). Consistent with our previous pub-
lication (see Fig. 2 [WT
Phsp60 4247 background] or Fig. 3 [wild-type
background] in reference 14), LM and LAM became smaller
only when the wild-type cells were transfected with catalytically
active MSMEG_4247 (WT
Phsp60 4247) (Fig. 3A). We then
examined if other components of the cell wall and plasma
membrane are affected by overexpression of MSMEG_4247.
We found that PIMs, phospholipids, trehalose dimycolate,
and glycopeptidolipids are all present at levels comparable be-
tween the two strains (Fig. 3B to D). In addition, α and α’ sub-
novations of mycolic acids released from the peptidoglycan-
arabinogalactan-mycolic acid core were methylated, and mycolic acid methyl ester was separated and visualized by chronic acid staining. α and α’ indicate methyl ester derivatives of
α-mycolic acid and α’-mycolic acid, respectively. (F) Killing of M. smegmatis by THP-1 cells. M. smegmatis WT+Vec (solid squares), WT+Phsp604247 (open circles), and WT+Phsp604247(D45A) (solid triangles with dashed line) were incubated with activated THP-1 cells, and survival of M. smegmatis cells was
monitored by counting recovered CFU. Experiments were performed in triplicate, and standard deviations are shown.

FIG 3 Catalytic activity of MSMEG_4247 is critical for the phenotype of the MSMEG_4247 overexpression mutant. (A) (Top) Western blot assay using anti-MSMEG_4247 antibody. (Bottom) ProQ Emerald staining showing LM/LAM profiles. (B) Extracted lipids were separated by high-performance thin-layer chromatography (HPTLC) using chloroform-methanol-13 M ammonia-1 M ammonium acetate-water (180:140:9:9:23) as a solvent system and stained for glycolipids using orcinol. (C) Extracted lipids were separated on an HPTLC plate using chloroform-methanol-13 M ammonia-1 M ammonium acetate-water (180:140:9:9:23) as a solvent system, and separated lipids were stained for phospholipids using molybdenum blue staining reagent. CL, cardiolipin; PE, phosphatidylethanolamine. (D) Extracted lipids were separated on an HPTLC plate using chloroform-methanol (9:1) as a solvent system, and separated lipids were visualized by orcinol staining. GPLs, glycopeptidolipids; TDM, trehalose dimycolate. (E) Mycolic acids released from the peptidoglycan-arabinogalactan core were methylated, and mycolic acid methyl ester was separated and visualized by chronic acid staining. α and α’ indicate methyl ester derivatives of
α-mycolic acid and α’-mycolic acid, respectively. (F) Killing of M. smegmatis by THP-1 cells. M. smegmatis WT+Vec (solid squares), WT+Phsp604247 (open circles), and WT+Phsp604247(D45A) (solid triangles with dashed line) were incubated with activated THP-1 cells, and survival of M. smegmatis cells was
monitored by counting recovered CFU. Experiments were performed in triplicate, and standard deviations are shown.
envelope perturbation has been suggested to be a major intracellular stress encountered by *M. tuberculosis* during infection of human THP-1 monocyte cells (29), so we considered the possibility that these LM/LAM mutants might be more sensitive to killing by THP-1 cells. Because *M. smegmatis* is non-pathogenic, the wild-type strains are susceptible to killing by activated THP-1 cells, and at least 99% of cells are killed within 42 h (Fig. 3F). When we compared our mutants, WT + Phsp60 Δ4247 appeared to be phagocytosed more readily as indicated by a CFU number slightly higher than that of the wild type at time zero. Furthermore, WT + Phsp60 Δ2181 was killed at a higher rate than was WT + Phsp60 Δ4247 (D45A) (Fig. 3F), suggesting that LM and LAM play a protective role against the bactericidal activity of THP-1 cells.

**Tetracycline-inducible suppression of MSMEG_4241 expression.** Continuous overexpression of MSMEG_4247 might select for an adaptive mutation that compensates for the decreased fitness due to aberrant LM/LAM structures. To minimize such a possibility of secondary mutation and examine the direct impact of LM and LAM on cell wall integrity, we created a tetracycline-inducible strain to suppress the expression of MSMEG_4241. As MSMEG_4241 mediates the elongation of mannan backbone, tetracycline-inducible suppression of its expression allowed us to examine the direct consequences of structural alterations of LM and LAM.

We designed a construct so that the reverse tetracycline represor (revTetR)-controlled promoter controls the expression of MSMEG_4241 (Fig. 4A). Targeted integration of the construct was confirmed by Southern blotting (Fig. 4A). The revTetR expression vector was then introduced and maintained episomally. In the resultant MSMEG_4241 Tet-off cells, MSMEG_4241 expression was reduced to undetectable levels as early as 23 h after addition of anhydrotetracycline (atac), a tetracycline analog (Fig. 4B). Both mature LM and LAM disappeared upon induction, and LM-like intermediates accumulated (Fig. 4C). Matrix-assisted laser desorption–ionization time of flight mass spectrometry (MALDI-TOF-MS) showed that these LM-like intermediates carry 9 to 18 mannoses (Fig. 4D), consistent with the intermediate species previously observed in an MSMEG_4241 deletion mutant (7). Taken together, we have established an MSMEG_4241 Tet-off mutant that becomes unable to produce mature LM and LAM upon atc addition.

We examined the effect of MSMEG_4241 Tet-off induction on growth and found that cells grew at almost the same rate, regardless of induction (Fig. 4E). We also found no difference in the plasma membrane integrity at an ultrastructural level (Fig. 4F). Furthermore, Tet-off induction did not change the sensitivity of cells to malachite green, SDS, or crystal violet (Fig. 4G). However, consistent with the phenotypes of Δ4247 and Δ2181 + Phsp60 Δ4247, MSMEG_4241 Tet-off cells lost their acid-fastness upon induction (Fig. 4H). These data suggest that the structural changes of LM and LAM have a direct and immediate impact on cell wall integrity.

**Changes in LM/LAM structures affect the pathogenesis of *M. tuberculosis*.** We next wanted to examine if the roles that LM and LAM play in *M. smegmatis* can be extended to pathogenic species. We have previously created *M. tuberculosis* mutants that either lack or overexpress Rv2181 (Δ2181 or Δ2181 + Phsp60 Δ2181, respectively) and shown that their LM/LAM profiles were aberrant in a manner similar to that of their *M. smegmatis* counterparts (Fig. 5A). Using a polyclonal antibody against Rv2181, we confirmed that Δ2181 and Δ2181 + Vec lack Rv2181 expression (Fig. 5A). We also confirmed that the Δ2181 + Phsp60 Δ2181 strain overexpresses Rv2181 protein. The doubling time of these mutants was not significantly different from that of the wild type (Table 2). Interestingly, we also found no significant differences in acid-fastness between wild-type, Δ2181 + Vec, and Δ2181 + Phsp60 Δ2181 strains (data not shown), possibly suggesting more dominant roles of the core mycolic acid-arabinogalactan-peptidoglycan layer for acid-fastness in *M. tuberculosis*. Nevertheless, when we tested the sensitivity of the mutants to antibiotics, we found that both Δ2181 + Vec and Δ2181 + Phsp60 Δ2181 were more sensitive to various antibiotics (Table 3). Interestingly, *M. tuberculosis* mutants became sensitive to wider varieties of β-lactams than did *M. smegmatis* mutants (see Discussion).

This increased sensitivity to antibiotics indicated that the changes in LM/LAM structures have a significant impact on the integrity of the *M. tuberculosis* cell wall. Because the *M. smegmatis* mutant was more sensitive to macrophage killing (Fig. 3G), we

### TABLE 1 Antibiotic sensitivities of *M. smegmatis* mutants

| Antibiotic class | Antibiotic name | Wild type + empty vector | WT + Phsp60 Δ4247 | WT + Phsp60 Δ4247(D45A) |
|----------------|----------------|--------------------------|------------------|--------------------------|
| Glycopeptide   | Vancomycin     | 0.27 ± 0.00              | 0.11 ± 0.01      | 0.21 ± 0.01              |
| Penam          | Ampicillin     | >400                     | >400             | >400                     |
|               | Carbenicillin  | >400                     | >400             | >400                     |
|               | Benzylpenicillin | >200                  | >200             | >200                     |
| Carbapenem     | Meropenem      | 2.6 ± 0.3                | 0.58 ± 0.03      | 1.9 ± 0.1                |
| Cepham         | Cephalothin    | >200                     | >200             | >200                     |
|               | Cefamandole    | >200                     | >200             | >200                     |
|               | Cefotaxime     | >800                     | 4.7 ± 8.8        | >800                     |
|               | Cefepime       | >200                     | 1.2 ± 0.3        | >200                     |
| Monobactam     | Aztreonam      | >200                     | >200             | >200                     |

* a Vancomycin, meropenem, cephalothin, and cefepime values are given as means ± standard deviations from triplicate data. Other antibiotics were tested in duplicate. IC50, 50% inhibitory concentration.

Role of Mycobacterial Mannan in Pathogenesis
considered the possibility that these *M. tuberculosis* mutants may show defects in infection of mice. To test this hypothesis, we intra-tracheally injected C57BL/6 mice with wild-type, Δ2181+Vec, and Δ2181+Phsp602181 strains and monitored bacterial growth and survival of mice. We determined the level of infection by lung CFU. We found that bacterial colonization was efficient in mice infected with either mutant strain, although we observed slightly lower lung CFU from mice infected with the Δ2181+Phsp602181 mutant, especially at 12 weeks postinfection (Fig. 5B). More strikingly, Δ2181+Phsp602181 was unable to kill the mice under the condition where mice infected with wild-type *M. tuberculosis* started to die after 6 weeks (Fig. 5C), suggesting that its virulence was significantly compromised. Because LM and LAM have been implicated in immunomodulatory activities, we monitored local inflammation and cytokine responses in the infected lungs during the course of infection. There were no clear changes in either the...
severity of inflammation (Fig. 5D) or the levels of cytokine production (Fig. 5E) between mice infected with wild-type *M. tuberculosis* and those infected with mutants. We also tested but did not detect significant production of other cytokines such as interleukin-12 p70 (IL-12p70), IL-4, IL-17, IL-2, and granulocyte-macrophage colony-stimulating factor (GM-CSF). Although we cannot eliminate the possibility that cytokines that we did not test play roles in responding to the exposure to LM and LAM, these data are consistent with the possibility that LM and LAM play a role in establishing *M. tuberculosis* infection without having dominant roles in inflammatory responses.

**DISCUSSION**

In this study, we examined the roles for LM and LAM in the integrity of the mycobacterial cell envelope. Structural changes in LM and LAM significantly compromised the cell wall integrity of *M. smegmatis*, as demonstrated by the loss of acid-fastness, increased sensitivity to antibiotics, and faster killing by macrophages. Similarly, defects in LM/LAM structures in *M. tuberculosis* led to increased antibiotic sensitivity and attenuated infectivity. Thus, our data demonstrate that LM and LAM are critical for maintaining cell wall integrity. In addition, while previous studies demonstrated that the deletion of *Rv2181* is not lethal in *M. tuberculosis* (14, 15), *Rv2174* is predicted to be an essential gene (21). Furthermore, *embC*, which encodes an arabinosyltransferase for LAM biosynthesis, cannot be deleted in *M. tuberculosis* (26). Taken together with these previous observations, the LM/LAM biosynthetic pathway can be considered a candidate for drug targets.

We found that changes in LM/LAM structures have a significant impact on the cell envelope integrity of both *M. smegmatis* and *M. tuberculosis*. However, there were some differences between the two species. For example, *M. smegmatis* lost the acid-fast property of the cell wall upon deletion or overexpression of *MSMEG_4247*, as well as tetracycline-induced downregulation of *MSMEG_4247*, as well as tetracycline-induced downregulation of *MSMEG_4247* (Fig. 2E and 4H). In contrast, equivalent *M. tuberculosis* mutants did not show defects in the acid-fastness (not shown). These data indicate that LM and LAM do not have a significant impact.

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**FIG 5** Phenotypes of *M. tuberculosis* mutants. (A) (Top) Western blot assay using anti-Rv2181 antibody. (Bottom) ProQ Emerald staining showing LM/LAM profiles. (B) Growth of *M. tuberculosis* mutants in mouse lung. Experiments were performed in triplicate, and the data represent the mean CFU ± standard deviation from three independent experiments on three mice for each condition. The 12-week time point for mice infected with wild-type *M. tuberculosis* was not calculated because of an insufficient number of surviving mice. Asterisk, *P* < 0.05. (C) Survival of mice after infection with *M. tuberculosis* mutants. Asterisk, *P* < 0.05. (D) Histological analysis of lung tissues infected with *M. tuberculosis* mutants for 8 weeks. In all cases, inflammatory responses such as infiltration of neutrophils and lymphocytes as well as proliferation of macrophages are evident. Bars, 1 mm. (E) Pulmonary cytokine levels during infection (*n* = 3, ±standard deviation). Solid circles, wild type; open triangles, Δ2181 + Vec; solid squares, Δ2181 + Phsp60. IFN-γ, gamma interferon; TNF-α, tumor necrosis factor alpha. The data in panels B to E are representative of 2 independent experiments.
on the acid-fastness of M. tuberculosis, which could be due to the compositional differences in the M. tuberculosis outer membrane. While M. tuberculosis mutants maintain the acid-fastness of the cell wall, these mutants became sensitive to a wider variety of β-lactams than did M. smegmatis mutants. Slow penetration of antibiotics through the cell wall has been suggested as a contributor to the intrinsic drug resistance of M. tuberculosis (30). Therefore, in M. tuberculosis, LM and LAM might play more important roles in restricting the physical pores that allow penetration of β-lactams. Alternatively, the longer doubling time of M. tuberculosis may allow higher accumulation levels of β-lactams, making wider varieties of β-lactams effective against the slow-growing pathogen. Other contributing factors, such as β-lactamase and efflux pumps, may also explain the species difference. In particular, the intrinsic resistance of M. tuberculosis to β-lactams is well established (31), and blatC, the gene encoding β-lactamase, is known to play a dominant role (32). It is possible that β-lactamase and/or efflux pumps have different substrate specificities in M. smegmatis and M. tuberculosis. Further studies are needed to clarify which of these parameters play dominant roles in the differential antibiotic sensitivities.

We found that both deletion and overexpression of branch-forming α-1,2-mannosyltransferase (MSMEG_4247 or Rv2181) compromised cell wall integrity in M. smegmatis and M. tuberculosis. While the deletion has a significant impact on cell wall integrity in both species, the effect of overexpression on cell wall integrity was greater, as measured by higher sensitivity to antibiotics. These effects of overexpression are unlikely to be due to the toxic effect of protein overexpression, because overexpression of catalytically inactive enzyme did not affect cell wall integrity in M. smegmatis (Fig. 3; Table 1). Furthermore, tetracycline-induced downregulation of MSMEG_4241 expression, which caused the disappearance of mature LM and LAM, led to similar phenotypes. These data suggest that LM and LAM with shorter mannose backbones have greater impacts on cell wall permeability. In Gram-positive bacteria, polymers known as lipoteichoic acids and wall teichoic acids are thought to play important roles in the maintenance of cell wall integrity (33, 34), and one proposed function is to strengthen the cell wall permeability barrier by filling in the pores and cavities present in the peptidoglycan mesh (35). Our data suggest that LM and LAM may have a similar function and that LM and LAM with shorter mannose backbones are ineffective at fulfilling such functions. A recent study indicated that clinical isolates of M. tuberculosis produce a truncated LAM with reduced arabinan and mannose sizes (36). It would be interesting to examine if these phenotypes were produced as a consequence of overexpression of Rv2181 relative to Rv2174 and if these clinical isolates show differences in cell wall permeability.

TABLE 2  Doubling time of M. tuberculosis mutants

| M. tuberculosis strain | Doubling time (h) |
|------------------------|------------------|
| Wild type              | 25.4 ± 1.1       |
| Δ2181                  | 25.0 ± 0.3       |
| Δ2181 + empty vector   | 28.5 ± 1.6       |
| Δ2181 + Rv2181          | 24.6 ± 0.9       |

LAM arabinans from pathogenic species are modified by oligomannose capping, while those from nonpathogenic species are either modified by inositol phosphate or unmodified. Despite a number of studies indicating that oligomannose capping is involved in immune modulation (10, 25), mutant Mycobacterium marinum and Mycobacterium bovis BCG lacking the mannose cap did not show any defects in infection of zebrafish and mouse models, respectively (37). Therefore, the true functions of the oligomannose cap remain to be determined. In M. tuberculosis, Rv2181 is not only involved in the addition of monomannose side chains to the mannann backbone but is also responsible for adding terminal α-1,2-mannoses in the mannose cap structure (15). Therefore, we expect that our Rv2181 deletion mutant will lack the terminal mannose modifications in addition to the monomannose side chains of the mannann backbone. In the current study, the Rv2181 deletion mutant infected mice effectively, and its growth in the lung was comparable to that of the wild type. These data are consistent with the previous observations that mannose cap structure does not have a dominant role during host infection.

In contrast to the Rv2181 deletion mutant, there was a defect in the ability of the Rv2181 overexpression mutant to establish infection. The Rv2181 overexpression mutant showed slightly less effective establishment of infection in the lung and failed to kill mice as effectively as did wild-type M. tuberculosis. We noticed that the lung CFU started to decline after 8 weeks in mice infected with the Rv2181 overexpression strain. Although it is beyond the scope of the current study, these data might indicate the role of an acquired immune response. While we cannot exclude the possibility that Rv2181 overexpression affected the mannose cap structure of LAM, this cannot explain the defective cell wall integrity of the equivalent mutant in M. smegmatis. Indeed, the MSMEG_4247 overexpression mutant showed increased sensitivity to various antibiotics and macrophage killing, comparable to those of the M. tuberculosis mutants. A more likely possibility is that shortening of the mannann backbone has a significant impact on the integrity of the M. tuberculosis cell wall and affects the ability of the pathogen to establish infection in mice. Based on our analysis of the MSMEG_4241 Tet-off strain, we predict that inhibition of the orthologous Rv2174 would have similar changes in LM/LAM structures and a similar impact on cell wall integrity in M. tuberculosis. We therefore suggest that key enzymes such as Rv2174 in the LM/LAM biosynthetic pathway could be potential targets for TB chemotherapy.

MATERIALS AND METHODS

Mycobacterial strains and culture conditions. M. smegmatis mc2155 (38) and derived mutants (14) were grown at 30°C in Middlebrook 7H9 broth (BD, Franklin Lakes, NJ) supplemented with 0.2% (wt/vol) glucose, 0.2% (vol/vol) glycerol, 15 mM NaCl, and 0.05% (vol/vol) Tween 80. Viability was determined by counting CFU on Middlebrook 7H10 agar plates supplemented with 0.2% (wt/vol) glucose, 0.2% (vol/vol) glycerol, and 15 mM NaCl. M. tuberculosis H37Rv and derived mutants were grown at 37°C in Middlebrook 7H9 broth supplemented with Middlebrook albumin-dextrose-catalase (ADC) enrichment (BD) and 0.05% (vol/vol) Tween 80 or Middlebrook 7H10 agar supplemented with Middlebrook oleic acid-albumin-dextrose-catalase (OADC) enrichment (BD).

Electron microscopy. Conventional transmission electron microscopy was performed (39). The bacteria were fixed in 2.5% glutaraldehyde and 0.05% ruthenium red in 0.1 M HEPES (pH 7.4) for 2 h on ice and then washed, dehydrated, and embedded in epoxy resin. Ultrathin sections were obtained with an F66/UC6 ultramicrotome (Leica Microsystems, Tokyo, Japan), counterstained with lead citrate for 10 min, and then observed with a JEM-1011 electron microscope (JEOL, Tokyo, Japan).

Permeability of chemical compounds. The membrane permeability test was performed as described previously (40). Briefly, stationary-phase
TABLE 3 Antibiotic sensitivities of M. tuberculosis mutants

| Antibiotic class | Antibiotic name | Wild type | Δ2181 + Phop60|2181 | Δ2181 + empty vector |
|-----------------|-----------------|-----------|---------------|------|---------------------|
| Glycopeptide    | Vancomycin      | 4.1 ± 0.6 | 2.8 ± 0.1     | 7.8 ± 0.8 |
| Penam           | Ampicillin      | >800      | 133 ± 6       | 247 ± 31 |
|                 | Carbenicillin   | >400      | 42.8 ± 0.9    | 62.4 ± 1.8 |
|                 | Benzylpenicillin| 156 ± 4   | 10.2 ± 0.2    | 19.0 ± 2.5 |
| Carbapenem      | Meropenem       | 3.3 ± 0.2 | 1.9 ± 0.1     | 2.6 ± 0.0 |
| Cepham          | Cephalothin     | >100      | 10.7 ± 0.1    | 17.3 ± 0.6 |
|                 | Cefamandole     | 143 ± 11  | 9.0 ± 0.8     | 19.2 ± 0.2 |
|                 | Cefotaxime      | 15.3 ± 1.6| 4.6 ± 0.3     | 6.8 ± 0.2 |
|                 | Cefepine        | 5.4 ± 0.2 | 3.2 ± 0.1     | 5.2 ± 0.2 |
| Monobactam      | Aztreonam       | >200      | >200          | >200   |

* All data are given as means ± standard deviations from triplicate data except for aztreonam, which was tested in duplicate.
Southern blot analysis. Genomic DNA was extracted as described previously (45). A 10-μg aliquot of digested DNA was resolved by electrophoresis on an 0.8% agarose gel in 1× Tris-acetate-EDTA (TAE) buffer and blotted onto a Hybond N+ nylon membrane (Amersham Biosciences). Probe hybridization and signal detection were performed as previously described (12). The probe was prepared by PCR amplification of a DNA fragment from M. smegmatis genomic DNA using the primer pair 5’ CATCATACGGTCGCCGTTGATC 3’ and 5’ AAGGTCGTTAGGCT TACGG 3’ (Fig. 4A).

MALDI-TOF-MS analysis. For MALDI-TOF-MS analysis, 1.0 μg of purified LM/LAM sample was mixed with 1.0 μl of the matrix solution, which consisted of 10 μM/mL 2,5-dihydroxybenzoic acid and 0.1% trifluoroacetic acid in water-acetonitrile (1:1, vol/vol). Samples were analyzed on a Bruker Ultraflex MALDI-TOF/TOF instrument (Bruker Daltonics, Billerica, MA) using reflector mode and in negative mode detection.

Mouse infection. Animal experimentation was carried out in accordance with the guidelines for animal care approved by the National Institute of Infectious Diseases, Japan. C57BL/6 mice (female, 6 weeks old; SLG, Shizuoka, Japan) were maintained under specific-pathogen-free conditions in a biosafety level 3 facility. Mice (12 per group) were infected via intratracheal injection of 5.0 × 10^4 CFU of M. tuberculosis H37Rv suspended in 50 μl PBS. Survival curves were analyzed statistically using a log rank test. At the indicated number of weeks postinfection, lung homogenates were diluted 10^3- to 10^6-fold in PBS and spread on 1% Ogawa egg medium (Kyokuto Pharmaceutical Industrial, Tokyo, Japan) for colony counting (CFU ± standard deviation, n = 3). The CFU values were analyzed statistically using the Mann-Whitney U test.

Analysis of infected mouse lungs. Lung tissues from infected mice were fixed in 10% formalin-PBS for histological analysis. The paraffin-embedded sections were stained with hematoxylin and eosin. The cytokine levels in lung homogenates were determined by FlowCytomx (Bio-Science, San Diego, CA) using a FACScalibur flow cytometer (BD, Franklin Lakes, NJ), according to the manufacturer’s instructions.

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REFERENCES

1. World Health Organization. 2011. Global tuberculosis control: WHO report 2011. World Health Organization, Geneva, Switzerland.
2. Brennan PJ. 2003. Structure, function, and biogenesis of the cell wall of Mycobacterium tuberculosis. Tuberculosis (Edinb.) 83:91–97.
3. Pitarque S, Larrouy-Maunus G, Payré B, Jackson M, Puzo G, Nigou J. 2008. The immunomodulatory lipopolysaccharides, lipoolarabinomannan and lipo- mann, are exposed at the mycobacterial cell surface. Tuberculosis (Edinb.) 88:560–565.
4. Miller M, Quiñones M, Puzo G. 2003. Acylation state of the phosphatidylmannose membrane lipopolysaccharides from Mycobacterium bovis bacillus Calmette Guerin and Mycobacterium tuberculosis H37Rv and its implication in Toll-like receptor response. J. Biol. Chem. 278:29880–29889.
5. Morita YS, Fukuda T, Sena CB, Yamayo-Botte Y, McConville MJ, Kinoshita T. 2011. Inositol lipid metabolism in mycobacteria: biosynthesis and regulatory mechanisms. Biochim. Biophys. Acta 1810:630–641.
6. Chatterjee D, Hunter SW, McNeil M, Brennan PJ. 1992. Lipoolarabinomannan. Multiglycosylated form of the mycobacterial mannosylphosphatidylinositol. J. Biol. Chem. 267:6228–6233.
7. Kaur D, McNeil MR, Kho KH, Chatterjee D, Crick DC, Jackson M, Brennan PJ. 2007. New insights into the biosynthesis of mycobacterial lipomannan arising from deletion of a conserved gene. J. Biol. Chem. 282:22453–227140.
8. Guerin ME, Koudalová J, Alzari PM, Brennan PJ, Jackson M. 2010. Molecular basis of phosphatidyl-myo-inositol mannoside biosynthesis and regulation in mycobacteria. J. Biol. Chem. 285:33577–33583.
9. Kaur D, Guerin ME, Skovierová H, Brennan PJ, Jackson M. 2009. Biogenesis of the cell wall and other glycoconjugates of Mycobacterium tuberculosis. Adv. Appl. Microbiol. 69:23–78.
10. Mishra AK, Driessen NN, Appelmelk BJ, Besra GS. 2011. Lipoolarabinomannan and related glycoconjugates: structure, biogenesis and role in Mycobacterium tuberculosis physiology and host-pathogen interaction. FEBS Microbiol. Rev. 35:1126–1157.
11. Kovacevic S, Anderson D, Morita YS, Patterson J, Haits RE, McMillan BN, Coppell R, McConville MJ, Billman-Jacobe H. 2006. Identification of a novel protein with a role in lipoolarabinomannan biosynthesis in mycobacteria. J. Biol. Chem. 281:9011–9017.
12. Morita YS, Sena CB, Waller RF, Kurokawa K, Sernee MF, Nakatani F, Haits RE, Billman-Jacobe H, McConville MJ, Maeda Y, Kinoshita T. 2006. Pipm is a polypropen-phosphate-mannose-dependent mannosyltransferase that transfers the fifth mannose of phosphatidylinositol mannoside in mycobacteria. J. Biol. Chem. 281:25143–25155.
13. Kaur D, Berg S, Dinadayala P, Gicquel B, Chatterjee D, McNeil M, Brennan PJ, Besra GS. 2011. Lipoarabinomannan biosynthesis. Proc. Natl. Acad. Sci. U. S. A. 103:13664–13669.
14. Sena CB, Fukuda T, Miyaniagi K, Matsumoto S, Kobayashi K, Murakami Y, Maeda Y, Kinoshita T, Morita YS. 2010. Controlled expression of branch-forming mannosyltransferase is critical for mycobacterial lipoolarabinomannan biosynthesis. J. Biol. Chem. 285:13326–13336.
15. Kaur D, Obregón-Henao A, Pham H, Chatterjee D, Brennan PJ, Jackson M. 2008. Lipoarabinomannan of Mycobacterium: mannos capping by a multifunctional terminal mannosyltransferase. Proc. Natl. Acad. Sci. U. S. A. 105:17973–17977.
16. Mishra AK, Alderwick LJ, Rittmann D, Tattiri RV, Ngou J, Gillerón M, Eggeling L, Besra GS. 2007. Identification of an alpha(1→6) mannopyranosyltransferase (MptA), involved in Corynebacterium glutamicum lipomannan biosynthesis, and identification of its ortholog in Mycobacterium tuberculosis. Mol. Microbiol. 65:1503–1517.
17. Chatterjee D, Khoo KH, McNeil MR, Dell A, Morris HR, Brennan PJ. 1993. Structural definition of the non-reducing termini of mannosylated LAM from Mycobacterium tuberculosis through selective enzymatic degradation and fast atom bombardment-mass spectrometry. Glycobiology 3:497–506.
18. Chatterjee D, Lowell K, Rivoire B, McNeil M, Brennan PJ. 1992. Lipoarabinomannan of Mycobacterium tuberculosis. Capping with mannosyl residues in some strains. J. Biol. Chem. 267:6234–6239.
19. Venisse A, Berjeaud JM, Chaourd P, Gillerón M, Pujo G. 1993. Structural features of lipoolarabinomannan from Mycobacterium bovis BCG. Determination of molecular mass by laser desorption mass spectrometry. J. Biol. Chem. 268:12401–12411.
20. Dinadayala P, Kaur D, Berg S, Amin AG, Vissa VS, Chatterjee D, Brennan PJ, Crick DC. 2006. Genetic basis for the synthesis of the immunomodulatory mannosic caps of lipoolarabinomannan in Mycobacterium tuberculosis. J. Biol. Chem. 281:20027–20035.
21. Sattawi CM, Boyd DH, Rubin EJ. 2003. Genes required for mycobacterial growth defined by high density mutagenesis. Mol. Microbiol. 48:77–84.
22. Briken V, Porcelli SA, Besra GS, Kremer L. 2004. Mycobacterial lipoolarabinomannan and related lipoglycans: from biosynthesis to modulation of the immune response. Mol. Microbiol. 53:391–403.
23. Gillerón M, Jackson M, Ngou J, Pujo G. 2008. Structure, biosynthesis, and activities of the phosphatidy-l-myo-inositol-based lipoglycans, p 75–105. In Dufè M, Reyart JM (ed), The mycobacterial cell envelope. ASM Press, Washington, DC.
24. Schlesinger LS, Azad AK, Torrelles JB, Roberts E, Verge I, Deretic V. 2008. Determinants of phagocytosis, phagosome biosynthesis and autophagy for Mycobacterium tuberculosis, p 1–22. In Kaufmann SHE, Britton WJ (ed), Handbook of tuberculosis: immunology and cell biology. Wiley-VCH Verlag, Weinheim, Germany.
25. Torrelles JB, Schlesinger LS. 2010. Diversity in Mycobacterium tuberculosis mannosylated cell wall determinants impacts adaptation to the host. Tuberculosis (Edinb). 90:84–93.

26. Goude R, Amin AG, Chatterjee D, Parish T. 2008. The critical role of embC in Mycobacterium tuberculosis. J. Bacteriol. 190:4335–4341.

27. Torrelles JB, DesJardin LE, MacNeil J, Kaufman TM, Kurzbach B, Knaup R, McCarthy TR, Gurcha SS, Besra GS, Clegg S, Schlesinger LS. 2009. Inactivation of Mycobacterium tuberculosis mannosyltransferase pimB reduces the cell wall lipoarabinomannan and lipomannan content and increases the rate of bacterial-induced human macrophage cell death. Glycobiology 19:743–755.

28. Podobnik M, Tyagi R, Matange N, Dermol U, Gupta AK, Matteo R, Seshadri K, Visveswariah SS. 2009. A mycobacterial cyclic AMP phosphodiesterase that moonlights as a modifier of cell wall permeability. J. Biol. Chem. 284:32846–32857.

29. Fontán P, Aris V, Ghanny S, Soteropoulos P, Smith I. 2008. Global transcriptional profile of Mycobacterium tuberculosis during THP-1 human macrophage infection. Infect. Immun. 76:717–725.

30. Chambers HF, Moreau D, Yajko D, Miick C, Wagner C, Hackbarth C, Flores AR, Parsons LM, Pavelka MS, Jr. 2007. Silencing matis and susceptibility to beta-lactam antibiotics. J. Bacteriol. 189:4614–4623.

31. Iland CN. 1946. The effect of penicillin on the tubercle bacillus. J. Pathol. Bacteriol. 58:495–500.

32. Flores AR, Parsons LM, Pavelka MS, Jr. 2005. Genetic analysis of the beta-lactamases of Mycobacterium tuberculosis and Mycobacterium smegmatis and susceptibility to beta-lactam antibiotics. Microbiology 151:521–532.

33. Peschel A, Vuong C, Otto M, Götz F. 2000. The d-alanine residues of Staphylococcus aureus teichoic acids alter the susceptibility to vancomycin and the activity of autolytic enzymes. Antimicrob. Agents Chemother. 44:2845–2847.

34. Sieradzki K, Tomasz A. 2003. Alterations of cell wall structure and metabolism accompany reduced susceptibility to vancomycin in an isogenic series of clinical isolates of Staphylococcus aureus. J. Bacteriol. 185:7103–7110.

35. Weidenmaier C, Peschel A. 2008. Teichoic acids and related cell-wall glycopolymers in Gram-positive physiology and host interactions. Nat. Rev. Microbiol. 6:276–287.

36. Torrelles JB, Knaup R, Kolareth A, Slepushkina T, Kaufman TM, Kang P, Hill PJ, Brennan PJ, Chatterjee D, Belisle JT, Musser JM, Schlesinger LS. 2008. Identification of Mycobacterium tuberculosis clinical isolates with altered phagocytosis by human macrophages due to a truncated lipoarabinomannan. J. Biol. Chem. 283:31417–31428.

37. Appelmelk BJ, den Dunnen J, Driessen NN, Ummels R, Pal M, Ngou J, Larrouy-Mamuss G, Gurcha SS, Movahedzadeh F, Geurtsen J, Brown EJ, Eysink Smeets MM, Besra GS, Willemsen PT, Lowary TL, van Kooyk Y, Maaskant JJ, Stoker NG, van der Ley P, Puzo G, Vandenbroucke-Grauls CM, Wieland CW, van der Poll T, Geijtenbeek TB, van der Sar AM, Bitter W. 2008. The mannosic cap of mycobacterial lipoarabinomannan does not dominate the Mycobacterium-host interaction. Cell. Microbiol. 10:930–944.

38. Snapper SB, Lugosi L, Jekkel A, Melton RE, Kieser T, Bloom BR, Jacobs WR, Jr. 1988. Lysogeny and transformation in mycobacteria: stable expression of foreign genes. Proc. Natl. Acad. Sci. U. S. A. 85:6987–6991.

39. Bleck CK, Merz A, Gutierrez MG, Walther P, Dubochet J, Zuber B, Griffiths G. 2010. Comparison of different methods for thin section EM analysis of Mycobacterium smegmatis. J. Microsc. 237:23–38.

40. Banai N, Kincaid EZ, Lin SY, Desmond E, Jacobs WR, Jr, Ernst JD. 2009. Lipoprotein processing is essential for resistance of Mycobacterium tuberculosis to malachite green. Antimicrob. Agents Chemother. 53:3799–3802.

41. Shoub HL. 1923. A comparison of the Ziehl-Neelsen and Schulte-Tigges methods of staining tubercle bacilli. J. Bacteriol. 1:121–126.

42. Morita YS, Patterson JH, Billman-Jacobe H, McConville MJ. 2004. Biosynthesis of mycobacterial phosphatidylinositol mannosides. Biochem. J. 378:589–597.

43. Hinchey J, Jeon BY, Alley H, Chen B, Goldberg M, Derrick S, Morris S, Jacobs WR, Jr, Porcelli SA, Lee S. 2011. Lysine auxotrophy combined with deletion of the SecA2 gene results in a safe and highly immunogenic candidate live attenuated vaccine for tuberculosis. PLoS One 6:e15857. http://dx.doi.org/10.1371/journal.pone.0015857.

44. Guo XV, Monteleone M, Klotzsche M, Kamionka A, Hillen W, Braunstein M, Ehrト Schnappinger D. 2007. Silencing Mycobacterium smegmatis with tetracycline repressors. J. Bacteriol. 189:4614–4623.

45. Jeevarajah D, Patterson JH, Taig E, Sargeant T, McConville MJ, Billman-Jacobe H. 2004. Methylation of GPLs in Mycobacterium smegmatis and Mycobacterium avium. J. Bacteriol. 186:6792–6799.