Controlled Expression of Branch-forming Mannosyltransferase Is Critical for Mycobacterial Lipoarabinomannan Biosynthesis

Chubert B. C. Sena, Takeshi Fukuda, Kana Miyano, Sohkichi Matsumoto, Kazuo Kobayashi, Yoshihiko Murakami, Yusuke Maeda, Taroh Kinoshita, and Yasu S. Morita

From the Research Institute for Microbial Diseases and WPI-Immunology Frontier Research Center, Osaka University, Osaka 565-0871, Japan, the Department of Bacteriology, Osaka City University Graduate School of Medicine, Osaka 545-8585, Japan, and the Department of Immunology, National Institute of Infectious Diseases, Tokyo 162-8640, Japan

Lipomannan (LM) and lipoarabinomannan (LAM) are phosphatidylinositol-anchored glycans present in the mycobacterial cell wall. In Mycobacterium smegmatis, the mannan core of LM/LAM constitutes a linear chain of 20–25 α1,6-mannoses elaborated by 8–9 α1,2-monomannose side branches. At least two α1,6-mannosyltransferases mediate the linear mannos chain elongation, and one branching α1,2-mannosyltransferase (encoded by MSMEG_4247) transfers monomannose branches. An MSMEG_4247 deletion mutant accumulates branchless LM and interestingly fails to accumulate LM, suggesting an unexpected role of mannos branching for LM synthesis or maintenance. To understand the roles of MSMEG_4247-mediated branching more clearly, we analyzed the MSMEG_4247 deletion mutant in detail. Our study showed that the deletion mutant restored the synthesis of wild-type LM and LAM upon the expression of MSMEG_4247 at wild-type levels. In striking contrast, overexpression of MSMEG_4247 resulted in the accumulation of dwarfed LM/LAM, although monomannose branching was restored. The dwarfed LAM carried a mannan chain less than half the length of wild-type LAM and was elaborated by an arabinan that was about 4 times smaller. Induced overexpression of an elongating α1,6-mannosyltransferase competed with the overexpressed branching enzyme, alleviating the dwarfing effect of the branching enzyme. In wild-type cells, LM and LAM decreased in quantity in the stationary phase, and the expression levels of branching and elongating mannosyltransferases were reduced in concert, presumably to avoid producing abnormal LM/LAM. These data suggest that the coordinated expressions of branching and elongating mannosyltransferases are critical for mannan backbone elongation.

Mycobacterium tuberculosis is an etiologic agent of tuberculosis, an infectious disease that remains a global problem. Glycoconjugates from the mycobacterial cell wall are involved in pathogenesis and immune modulation. In particular, phosphatidylinositol mannosides (PIMs), lipomannan (LM), and lipoarabinomannan (LAM) form a class of glycoconjugates found in all species of mycobacteria, including pathogenic M. tuberculosis and saprophytic and experimentally tractable Mycobacterium smegmatis, and are known to have potent immunomodulatory activities (1–3). PIMs are anchored to the plasma membrane by a phosphatidylinositol (PI) and carry two or six mannoses, which are directly linked to the 2-OH and 6-OH of the inositol residue (4, 5) (Fig. 1A). Monomannose attached to the 2-OH of inositol is further modified by a fatty acid, making tricyclic PIMs the major lipid species. The 3-OH attached of inositol can be further modified by a fatty acid to become a tetra-acylated species (6). LM carries a much longer chain of mannoses. For example, in M. smegmatis, 20–25 α1,6-mannose residues were thought to form a linear chain, which is elaborated by 8–9 α1,2-mannose monomer branches (7). A more recent report estimated M. smegmatis LM to carry 21–34 mannose residues (8), highlighting even greater heterogeneity of this molecule. LAM is an arabinosylated LM, in which ~70 residues of D-arabinofuranose form arabinan(s) comprising α1,5 linear stretches linked by multiple α1,3 branch points (9, 10). It is not known if a single molecule of LAM carries a single large arabinan moiety or multiple smaller arabinans.

PIMs, LM, and LAM all contain PI as a common lipid core moiety and are thought to be biosynthesized in a sequential order. PIMs are synthesized from a PI by sequential additions of mannoses, resulting in dimannosyl or hexamannosyl PIMs (6, 11–13). PIMα and PIMβ mediate the transfers of first two mannoses in a GDP-mannose-dependent manner, and an acyltransferase, encoded by MSMEG_2934 in M. smegmatis, adds one fatty acid onto a mannose residue to produce a dimannosyl species, AcPIM2 (14–16). Then, an additional four mannose residues are transferred onto AcPIM2, producing AcPIM6.

The abbreviations used are: PIM, phosphatidylinositol mannoside; HPAEC, high performance anion exchange chromatography; HPTLC, high performance thin layer chromatography; LAM, lipoarabinomannan; LM, lipomannan; PI, phosphatidylinositol; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
Polyphenol-phosphate-mannose-dependent mannosyltransferases are thought to be involved in these later reactions (11, 13), but only the fifth mannosyltransferase, PimE, has so far been identified (17). AcPIM4 has been thought to be the branching point from which LM/LAM biosynthesis diverges from the PIM biosynthetic pathway, and a lipoprotein, LpqW, is thought to be involved in controlling the metabolic flux at this branch point (18, 19). This branch point is structurally viable because the terminal two mannoses of AcPIM6 are α1,2-linked, whereas the LM/LAM mannan chain is α1,6-linked. LM is believed to be the precursor of LAM, but the precursor-product relationship has not been experimentally proven.

The linear α1,6-mannose chain of LM is synthesized by an α1,6-mannosyltransferase encoded by MSMEG_4241 in M. smegmatis (8) or MptA in a related organism, Corynebacterium glutamicum (20). Deletion of MSMEG_4241 results in the accumulation of an intermediate containing 5–20 mannose residues instead of mature LM containing 21–34 mannose residues (Fig. 1A). Accumulation of the LM5–20 intermediate suggests that there is another α1,6-mannosyltransferase that mediates the initial stage of LM synthesis up to LM5–20. Another gene, MSMEG_3111, might potentially play a role in LM/LAM biosynthesis because it is homologous to an α1,6-mannosyltransferase of Corynebacterium LM (21). However, an MSMEG_3111 gene deletion mutant showed no defects in LM/LAM production (21), leaving its precise function unclear.

MSMEG_4247 and its M. tuberculosis ortholog, Rv2181, are α1,2-mannosyltransferases involved in LM/LAM biosynthesis (22, 23). An MSMEG_4247 gene deletion mutant produced LAM lacking the α1,2-monomannose side chains, suggesting that MSMEG_4247 is responsible for the addition of the α1,2-mannose branches onto the α1,6-mannose backbone of LAM. Interestingly, the MSMEG_4247 gene deletion mutant failed to accumulate LM (22), suggesting an essential role for the α1,2-mannose branch in the accumulation of LM.

A number of mannosyltransferases involved in the PIM/LM/LAM biosynthetic pathway have now been identified, but it remains largely unknown how these enzymes coordinate and control the complex and overlapping pathways of PIM/LM/LAM synthesis. As part of our study of the pime gene (17), we generated an MSMEG_4247 gene deletion mutant. Here we report our analyses on the ΔMSMEG_4247 mutant, revealing that controlling expression levels of MSMEG_4247 is critical for the proper synthesis of LM and LAM.

**EXPERIMENTAL PROCEDURES**

**Mycobacterial Strains and Culture Conditions**—Wild-type M. smegmatis strain mc^{	ext{Δ155}} (24) and derived mutants were grown at 30 °C in Middlebrook 7H9 broth (BD Biosciences) supplemented with 0.2% (w/v) glucose, 0.2% (v/v) glycerol, 15 mM NaCl, and 0.05% (v/v) Tween 80. Where appropriate, antibiotics at the following concentrations were used: 20 μg/ml streptomycin, 200 μg/ml hygromycin, or 20 μg/ml kanamycin. Samples were prepared at logaritthic growth phase (A_{600} nm = 0.5–1.0) unless otherwise indicated.

Gene Cloning and Complementation—A fragment containing the MSMEG_4247 gene was PCR-amplified (primers 053/054; supplemental Table S1) from M. smegmatis genomic DNA and cloned into the Mscl and EcoRI sites of the episcopal expression vector, pHBJ334 (25), driven by a strong promoter of a mycobacterial heat shock protein hsp60 (Hsp60), resulting in pYAB143. To create an integrative vector, an XbaI/SpeI fragment of pYAB143, containing Phsp60, the MSMEG_4247 gene, and the streptomycin resistance cassette, was blunt end-ligated with an SpeI/Eam1105I fragment of pMV361 (26), containing the attachment site and integrase gene, resulting in pYAB243. Site-directed mutagenesis was performed as described previously (17), using pYAB143 or pYAB250 as a template and primers 320/321, resulting in pYAB254 or pYAB255. Hygromycin-resistant versions of the expression vectors were constructed as follows: pYAB251 by replacing the streptomycin resistance cassette of pHBj334, pYAB250 by replacing the streptomycin resistance cassette of pYAB143, and pYAB184 by replacing the kanamycin resistance cassette of pMV306 (26). For an expression vector driven by the endogenous promoter, the MSMEG_4247 gene and 200 bp of upstream flanking sequence was PCR-amplified (primers 312/313) and ligated into the ClaI/XbaI sites of pYAB184, resulting in pYAB247. For the acetamide-inducible system, the MSMEG_4247 gene was PCR-amplified (primers 329/330) and ligated into the BamHI/XbaI sites of pJAM2 (27), resulting in pYAB262. To make an acetamide-inducible MSMEG_4247 expression vector (pYAB246), the kanamycin resistance cassette of pJAM2 was first replaced by a streptomycin resistance cassette, resulting in pYAB040, and the HindIII/Bpu1102I fragment of pYAB143 containing the MSMEG_4247 gene was blunt end-ligated into the BamHI site of pYAB040. These constructs were transfigted into M. smegmatis wild-type strain mc^{	ext{Δ155}} and mutant cells by electroporation.

**Lipid Extraction and Analysis**—The cell wall lipids were extracted from the cell pellets as described previously (17). Extracted lipids were analyzed by high performance thin layer chromatography (HPTLC) using chloroform, methanol, 13M ammonia, 1M ammonium acetate, water (180:140:9:9:23, v/v/v/v/v/v/v) as a solvent. Occinol or molybdenum blue staining was used to visualize PIMs or phospholipids, respectively.

**LM and LAM Extraction and Analysis**—After lipid extraction, the delipidated pellets were resuspended in five volumes of phenol saturated with Tris-EDTA buffer (pH 6.6) (Nacalai Tesque, Kyoto, Japan) and five volumes of water and extracted for 2 h at 55 °C. The extract was further purified by dialysis or octyl-Sepharose column chromatography (GE Healthcare) and electroelution (model 422 Electro-Eluter, Bio-Rad) from an SDS-polyacrylamide gel where appropriate. LM/LAM were separated by SDS-PAGE (10–20% gradient gel) and visualized using the ProQ Emerald 488 carbohydrate staining kit (Molecular Probes) and a fluoroimage analyzer (FLA-5000, Fujifilm).

**Metabolic Labeling of ΔMSMEG_4247**—Cells were washed and resuspended in a fresh aliquot of Middlebrook 7H9 broth at 0.2 g/ml pellet. Cells were radiolabeled with 50 μCi/ml [2,3H]mannose (20 Ci/mmol; American Radiolabeled Chemicals) for 15 min, washed in 7H9 broth to remove unincorporated radioactive mannosse, and chased in 7H9 broth containing 1 mM...
Role of Mannosyltransferases in Lipoarabinomannan Synthesis

manganese. Extracted LM/LAM were separated by SDS-PAGE and visualized by fluorography.

Mannosidase Treatment—LM/LAM fractions, mixed with PIMs as internal controls, were treated with or without 0.4 milli-units of Aspergillus saitoi α,1,2-mannosidase (Prozyme) for 24 h at 37 °C. The treatment was repeated three times. LM/LAM were repurified by phenol and analyzed by SDS-PAGE as described above. Intensity profiles were obtained by the Multi Gauge software (Fujifilm). PIMs were purified by 1-butanol/water (2:1, v/v) partitioning and analyzed by HPTLC.

High Performance Anion Exchange Chromatography (HPAEC)—Acetolysis was performed following a published protocol (28). Monosaccharides were released by 2 M trifluoro-acetic acid at 100 °C for 2 h. Released carbohydrates were analyzed by a Nanospace high performance liquid chromatography system (Shiseido, Tokyo, Japan) equipped with Serebead II (2.0 × 250 mm) (Shiseido) as an anion exchange column, 50 mM sodium acetate in 0.1 M NaOH as a mobile phase, and a pulsed amperometric detector.

Western Blotting—Rabbit antibodies were raised against a mixture of two peptides from MSMEG_4241 (MTPTETHK-PNPGLAEHVC and CRAPESAETASRG), MSMEG_4247 (RTHTGDHETDEPLVC and MSKRQSPRAGLPC), or PimB’ (CEHLPPGVDTRFAPDPD and CGARLAELLGRR-EARQA) and affinity-purified. Cell lysates were prepared by bead beating as described previously (17). Proteins were fractionated by SDS-PAGE (10–20% gradient gel) under reducing conditions and blotted onto a polyvinylidene difluoride membrane (Millipore). The membrane was blocked with 5% skim milk and then incubated with a primary antibody (rabbit anti-MSMEG_4247, anti-MSMEG_4241, or anti-PimB’ antibodies; 1 μg/ml) for 1 h and a secondary antibody (anti-rabbit IgG antibody, horseradish peroxidase-conjugated; 1:2000 dilution; Amersham Biosciences) and affinity-purified. Cell lysates were prepared by bead beating as described previously (17). Proteins were fractionated by SDS-PAGE (10–20% gradient gel) under reducing conditions and blotted onto a polyvinylidene difluoride membrane (Millipore). The membrane was blocked with 5% skim milk and then incubated with a primary antibody (rabbit anti-MSMEG_4247, anti-MSMEG_4241, or anti-PimB’ antibodies; 1 μg/ml) for 1 h and a secondary antibody (anti-rabbit IgG antibody, horseradish peroxidase-conjugated; 1:2000 dilution; Amersham Biosciences) and affinity-purified. Cell lysates were prepared by bead beating as described previously (17). Proteins were fractionated by SDS-PAGE (10–20% gradient gel) under reducing conditions and blotted onto a polyvinylidene difluoride membrane (Millipore). The membrane was blocked with 5% skim milk and then incubated with a primary antibody (rabbit anti-MSMEG_4247, anti-MSMEG_4241, or anti-PimB’ antibodies; 1 μg/ml) for 1 h and a secondary antibody (anti-rabbit IgG antibody, horseradish peroxidase-conjugated; 1:2000 dilution; Amersham Biosciences) and affinity-purified. Cell lysates were prepared by bead beating as described previously (17). Proteins were fractionated by SDS-PAGE (10–20% gradient gel) under reducing conditions and blotted onto a polyvinylidene difluoride membrane (Millipore). The membrane was blocked with 5% skim milk and then incubated with a primary antibody (rabbit anti-MSMEG_4247, anti-MSMEG_4241, or anti-PimB’ antibodies; 1 μg/ml) for 1 h and a secondary antibody (anti-rabbit IgG antibody, horseradish peroxidase-conjugated; 1:2000 dilution; Amersham Biosciences) and affinity-purified. Cell lysates were prepared by bead beating as described previously (17). Proteins were fractionated by SDS-PAGE (10–20% gradient gel) under reducing conditions and blotted onto a polyvinylidene difluoride membrane (Millipore). The membrane was blocked with 5% skim milk and then incubated with a primary antibody (rabbit anti-MSMEG_4247, anti-MSMEG_4241, or anti-PimB’ antibodies; 1 μg/ml) for 1 h and a secondary antibody (anti-rabbit IgG antibody, horseradish peroxidase-conjugated; 1:2000 dilution; Amersham Biosciences) and affinity-purified. Cell lysates were prepared by bead beating as described previously (17). Proteins were fractionated by SDS-PAGE (10–20% gradient gel) under reducing conditions and blotted onto a polyvinylidene difluoride membrane (Millipore). The membrane was blocked with 5% skim milk and then incubated with a primary antibody (rabbit anti-MSMEG_4247, anti-MSMEG_4241, or anti-PimB’ antibodies; 1 μg/ml) for 1 h and a secondary antibody (anti-rabbit IgG antibody, horseradish peroxidase-conjugated; 1:2000 dilution; Amersham Biosciences) and affinity-purified. Cell lysates were prepared by bead beating as described previously (17). Proteins were fractionated by SDS-PAGE (10–20% gradient gel) under reducing conditions and blotted onto a polyvinylidene difluoride membrane (Millipore). The membrane was blocked with 5% skim milk and then incubated with a primary antibody (rabbit anti-MSMEG_4247, anti-MSMEG_4241, or anti-PimB’ antibodies; 1 μg/ml) for 1 h and a secondary antibody (anti-rabbit IgG antibody, horseradish peroxidase-conjugated; 1:2000 dilution; Amersham Biosciences) and affinity-purified. Cell lysates were prepared by bead beating as described previously (17). Proteins were fractionated by SDS-PAGE (10–20% gradient gel) under reducing conditions and blotted onto a polyvinylidene difluoride membrane (Millipore). The membrane was blocked with 5% skim milk and then incubated with a primary antibody (rabbit anti-MSMEG_4247, anti-MSMEG_4241, or anti-PimB’ antibodies; 1 μg/ml) for 1 h and a secondary antibody (anti-rabbit IgG antibody, horseradish peroxidase-conjugated; 1:2000 dilution; Amersham Biosciences) and affinity-purified. Cell lysates were prepared by bead beating as described previously (17). Proteins were fractionated by SDS-PAGE (10–20% gradient gel) under reducing conditions and blotted onto a polyvinylidene difluoride membrane (Millipore). The membrane was blocked with 5% skim milk and then incubated with a primary antibody (rabbit anti-MSMEG_4247, anti-MSMEG_4241, or anti-PimB’ antibodies; 1 μg/ml) for 1 h and a secondary antibody (anti-rabbit IgG antibody, horseradish peroxidase-conjugated; 1:2000 dilution; Amersham Biosciences) and affinity-purified. Cell lysates were prepared by bead beating as described previously (17). Proteins were fractionated by SDS-PAGE (10–20% gradient gel) under reducing conditions and blotted onto a polyvinylidene difluoride membrane (Millipore). The membrane was blocked with 5% skim milk and then incubated with a primary antibody (rabbit anti-MSMEG_4247, anti-MSMEG_4241, or anti-PimB’ antibodies; 1 μg/ml) for 1 h and a secondary antibody (anti-rabbit IgG antibody, horseradish peroxidase-conjugated; 1:2000 dilution; Amersham Biosciences) and affinity-purified. Cell lysates were prepared by bead beating as described previously (17). Proteins were fractionated by SDS-PAGE (10–20% gradient gel) under reducing conditions and blotted onto a polyvinylidene difluoride membrane (Millipore). The membrane was blocked with 5% skim milk and then incubated with a primary antibody (rabbit anti-MSMEG_4247, anti-MSMEG_4241, or anti-PimB’ antibodies; 1 μg/ml) for 1 h and a secondary antibody (anti-rabbit IgG antibody, horseradish peroxidase-conjugated; 1:2000 dilution; Amersham Biosciences) and affinity-purified. Cell lysates were pre...
Role of Mannosyltransferases in Lipoarabinomannan Synthesis

Controlled expression of MSMEG_4247 is critical for the restoration of LM/LAM biosynthesis. A, MSMEG_4247 expression examined by Western blotting using anti-MSMEG_4247 antibody. Lane 1, wild type; lane 2, ΔMSMEG_4247; lanes 3 and 4, two clones of ΔMSMEG_4247 + Phsp60/MSMEG_4247 (pYAB250); lanes 5 and 6, two clones of ΔMSMEG_4247 + P4247/MSMEG_4247 (pYAB247); lanes 7 and 8, two clones of ΔMSMEG_4247 transfected with empty integrative vector (pYAB184). Loading was adjusted to 5 μg of protein/lane except for lanes 3 and 4, in which 1 μg of protein was loaded per lane. Relative intensities were calculated taking different protein loadings into account. B, LM/LAM profiles of MSMEG_4247 deletion mutants complemented by various expression vectors. LM/LAM were analyzed by SDS-PAGE and visualized by carbohydrate staining. Lanes are arranged in the same order as in A. Faint doublet bands slightly above the 18 kDa marker seen in all lanes are protein contaminants. C, LM or ΔMSMEG_4247 + Phsp60/MSMEG_4247 was treated with or without α1,2-mannosidase (ASAM), analyzed by SDS-PAGE using Tris-Tricine gel (15–20%) to improve the separation, and visualized by carbohydrate staining. Peak of LM in each lane was determined from the intensity profile and is indicated by an arrowhead. PIMs were included as internal controls and showed specific and complete digestion of the terminal two α1,2-mannoses of AcPIM6, producing AcPIM4. D, LM/LAM extracts were acetylated, and released mannose and α1,2-mannobiose were detected by HPAEC. Molar ratios of α1,2-mannobiose to mannose, measured in triplicate, are shown as averages with S.D. values. Column numbers specify the samples analyzed in the corresponding lanes in A and B. E, LAM was purified by electrodialysis and hydrolyzed by 2 μl trifluoroacetic acid. Released carbohydrates were quantified by HPAEC. Data are presented as molar ratio relative to inositol. Averages of triplicate measurements with S.D. values are shown. 

Expression Level of MSMEG_4247 Is Critical for Controlling the Sizes of LM and LAM Produced—We considered the possibility that LM/LAM synthesis was prematurely terminated because of the high expression level of MSMEG_4247. We found that ΔMSMEG_4247 + P4247/MSMEG_4247 expressed MSMEG_4247 at >700 times the levels of the wild-type (Fig. 2A, compare lanes 1 and lanes 3 and 4; note that the protein loading is 5 times less in lanes 3 and 4). To achieve a more controlled expression of MSMEG_4247, the ΔMSMEG_4247 mutant was transfected with a vector carrying MSMEG_4247 with its own upstream region instead of an artificial promoter. This vector lacks the mycobacterial origin of replication and instead carries the attachment site and the integrase gene from the mycobacteriophage L5, allowing for the site-specific integration of a single copy of the MSMEG_4247 gene (26). Western blotting confirmed that the expression levels of MSMEG_4247 in the complemented strains (ΔMSMEG_4247 + P4247/MSMEG_4247) were much closer to those of wild-type (4–5-fold overexpression; Fig. 2A, compare lane 1 and lanes 5 and 6). As shown in Fig. 2B, LM and LAM from ΔMSMEG_4247 + P4247/MSMEG_4247 restored the migration pattern of wild-type LM and LAM (lanes 5 and 6). In contrast, ΔMSMEG_4247 + P4247/MSMEG_4247 reproducibly accumulated faster migrating LM and LAM (lanes 3 and 4). These results suggest that the sizes of LM and LAM are normalized if the ΔMSMEG_4247 mutant is complemented with a wild-type level of MSMEG_4247 expression.

MSMEG_4247 is an α1,2-mannosyltransferase involved in the
transfer of the monomannose side chain. It was therefore perplexing that its overexpression resulted in smaller LM and LAM. To investigate whether the smaller sized LM and LAM produced in MSMEG_4247-overexpressing strains were modified by α1,2-mannose side chains, we removed the α1,2-mannose branches of LM by α1,2-specific mannosidase treatment and analyzed the size changes by SDS-PAGE (Fig. 2C). A shift of LM was detectable upon digestion of LM from both the wild-type and overexpressing strains. To confirm further, we acetylated the LM/LAM mixture from select strains and quantified the frequencies of α1,2-mannose branching. Acetylation cleaves α1,6-mannose linkages without digesting α1,2-mannose linkages, producing α1,2-mannobiose and mannosone. The ratio of α1,2-mannobiose to mannosone indicates the ratio of mannosines with α1,2-mannose branches to those without. As shown in Fig. 2D, whereas the ΔMSMEG_4247 mutant completely lacked the α1,2-mannose branch, LM/LAM from all complemented strains showed frequencies of α1,2-mannose branches comparable with those of the wild-type LM/LAM (i.e. ~45–50% of backbone mannosones were branched).

Despite the restoration of α1,2-mannose branching, LM and LAM from the ΔMSMEG_4247+Phsp60MSMEG_4247 strain appeared small on SDS-PAGE. To determine which component of LAM was responsible for the SDS-PAGE mobility shift, we purified LAM from an SDS-polyacrylamide gel by electrophoresis and analyzed its sugar composition. As shown in Fig. 2E, the ΔMSMEG_4247 mutant did not show significant changes in the sugar compositions of LAM despite the lack of α1,2-mannose branches, suggesting that α1,6-mannose backbone is longer than that of wild-type LAM, and arabinosylation is occurring normally. The numbers of arabinose and mannosone residues relative to inositol were somewhat smaller than previously reported (~70 arabinose and 21–34 mannosone residues; see Introduction). This might be due to the contribution of inositol from inositol phosphate capping, a minor modification of LAM arabinan in M. smegmatis and other fast growing mycobacteria (31, 32). Strikingly, LAM from the ΔMSMEG_4247+Phsp60MSMEG_4247 strain showed greatly reduced numbers of arabinose and mannosone. The mannan core carried less than half the number of mannosone residues found in wild-type LAM. Because the frequency of the side-chain mannose is not significantly different between the dwarfed and wild-type LAM, these data suggest that the length of mannan backbone is less than half the length of wild-type LAM. Furthermore, arabinan was found to be 4 times smaller. This gross dwarfing is apparently the cause of faster migration on the SDS-polyacrylamide gel, although the protein molecular weight standards do not precisely reflect the actual molecular weight shifts of LM/LAM (see also Ref. 33 for another example). Taken together, our data suggested that overexpression of MSMEG_4247 restored the branches of α1,2-monomannose but prematurely terminated the elongation of the α1,6-mannose backbone and dwarfed the arabinan size.

Next we investigated whether the effect of MSMEG_4247 overexpression is specific to the LM/LAM pathway. Because LM/LAM biosynthetic pathways are thought to diverge from PIM biosynthesis at the AcPIM4 intermediate (see Fig. 1A), we examined the lipid fractions to see if there are any changes in the PIM or phospholipid profiles. We found no significant differences in either PIMs or phospholipids (supplemental Fig. S2), suggesting that the deletion or overexpression of MSMEG_4247 primarily affects LM/LAM biosynthesis.

**Dwarfing LM/LAM Requires and Correlates with MSMEG_4247 Enzyme Activity**—If the expression levels of MSMEG_4247 affect the elongation of the α1,6-mannose backbone, overexpression of MSMEG_4247 can affect the profile of LM and LAM in wild-type cells as well. To test this prediction, we introduced integrative or episomal MSMEG_4247 overexpression vectors and examined the effect of expression in the wild-type cells. Indeed, overexpression of MSMEG_4247 resulted in smaller LM and LAM in wild-type cells (Fig. 3A). A higher expression level was achieved using an episomal vector compared with an integrative vector. The hyperexpression in cells transfected with the episomal vector led to the production of LM and LAM, which appeared even smaller than the LM and LAM from cells transfected with the integrative vector (Fig. 3A, compare lane 2 with lanes 3 and 4). To examine whether the production of smaller LM and LAM was dependent on the enzyme activity of MSMEG_4247, we created an MSMEG_4247 expression vector, in which the gene carried a site-directed mutation at a conserved aspartic acid residue. The aspartic acid residue 45 was conserved among mycobacterial species and aligned with the aspartic acid residue of PimE that is essential for enzymatic activity (supplemental Fig. S3A) (17). The expression of D45A-mutated MSMEG_4247 could not alter the LM/LAM profile of the ΔMSMEG_4247 mutant, suggesting the loss of enzyme activity (supplemental Fig. S3, B and C). When introduced in the wild-type cells, the D45A mutant of MSMEG_4247 did not show a shift in the migration patterns of wild-type LM and LAM (Fig. 3A, lanes 5 and 6). The lack of effect was not due to the lack of expression of MSMEG_4247 D45A mutant because it was expressed at a level comparable with that of wild-type MSMEG_4247 (Fig. 3B, compare lane 2 with lanes 5 and 6). These data further support our notion that the enzyme activity of MSMEG_4247 needs to be properly controlled to produce normal sized LM and LAM.

**LM/LAM Biosynthesis Is Immediately Affected by Induced Expression of MSMEG_4247**—Secondary mutations sometimes occur to compensate for an existing mutation in the genome, and secondary mutants with improved growth fitness can outgrow the original mutant. For example, deletion of the lpgW gene leads to spontaneous mutations in the pimE gene, resulting in an improved growth fitness of the mutant (18). We therefore wanted to exclude the possibility that the constitutive overexpression of MSMEG_4247 resulted in secondary mutations and that smaller LM/LAM was the consequence of these secondary mutations. To address this possibility, we created a vector, in which MSMEG_4247 expression was controlled by an acetamide-inducible promoter (27) and introduced this vector into wild-type cells. As shown in Fig. 4A, the acetamide-inducible promoter is leaky and expresses MSMEG_4247 at a level significantly higher than the wild-type level (compare lanes 1 and 3). Nevertheless, a much higher expression level was achieved after 4 h of acetamide induction (lane 6). At the 4-h time point, cells were metabolically pulse-
labeled with [3H]mannose and chased for 25 min in the presence of excess non-radioactive mannose (Fig. 4B). LM and LAM were relatively small in size immediately after pulse (lanes designated P). After the chase (lanes designated C), increased LM/LAM sizes were detected in control strains (compare lanes 5 and 6 or lanes 7 and 8). Compared with control strains, the sizes of LM and LAM after chase remained smaller in the cells carrying acetamide-inducible MSMEG_4247 even without acetamide induction due to leaky expression of MSMEG_4247 (compare lanes 2 and 6; white arrowheads (LAM) and white lines (LM) indicate the peaks of the intensity profile shown in supplemental Fig. S4). Upon induction of MSMEG_4247 expression, production of normal sized LAM became even less efficient (compare black arrowheads in lanes 2 and 4), dwarfed LAM became even smaller (compare white arrowheads in lanes 2 and 4), and dwarfed LM became greater in intensity and slightly smaller in size (compare white lines in lanes 2 and 4). These observations suggest less efficient elongation of LM and LAM, being similar to those seen under constitutive overexpression of MSMEG_4247 (see Fig. 3A). Thus, MSMEG_4247 can exert its effect on LM/LAM biosynthesis immediately, and the timing appears to be too fast to be explained by secondary mutations.

The Effect of Overexpressed MSMEG_4247 Can Be Alleviated by the Overexpression of MSMEG_4241—Our data suggested that overexpressed MSMEG_4247 dominates the elongating enzymes, preventing the efficient elongation of the linear α1,6-mannose chain. We wanted to test if this effect of MSMEG_4247 overexpression can be competed by the overexpression of an elongating mannosyltransferase, MSMEG_4241. Therefore, we created another mutant in which an episomal vector for acetamide-inducible MSMEG_4241 expression was transfected into the MSMEG_4247 overexpressing strain (used in lane 3 of Fig. 3). MSMEG_4241 expression was maximized at 4 h after the addition of acetamide (Fig. 5A). At this time point, we metabolically labeled cells with [3H]mannose in a pulse-chase experiment. In control cells overexpressing MSMEG_4247 only, dwarfed LM immediately accumulated, and dwarfed
LAM also accumulated over time (Fig. 5B). When MSMEG_4241 overexpression was induced, we found little accumulation of dwarfed LM and identified a radiolabeled species migrating at around 35 kDa, which was comparable with wild-type LAM (Fig. 5B). The synthesis of normal size LM (~20 kDa) was less prominent, which might be due to the relative overexpression of MSMEG_4241. Next, we examined if the biosynthetic changes induced by the overexpression of MSMEG_4241 can lead into changes in cell wall composition of LM/LAM. Without acetamide induction, dwarfed LM and dwarfed LAM persisted over the 12-h time course (Fig. 5C, left). Being consistent with the metabolic labeling experiments, a species comparable with wild-type LAM started to appear at around 35 kDa after the 4-h induction and accumulated over the 12-h induction (Fig. 5C, right). In contrast to metabolic labeling, dwarfed LM persisted during the time course, suggesting a slow turnover rate of this species. We purified dwarfed LAM (from 0-h induction) and LAM-like species (from 12-h induction) by electroelution (Fig. 5D) and examined the sugar composition (Fig. 5E). The LAM-like species carried a mannan moiety with a size comparable with the wild-type LAM (see Fig. 2E for comparison). In contrast, arabinan size was only partially restored, suggesting that the rate of arabinan biosynthesis falls short of that of mannan biosynthesis when both elongating and branching mannosyltransferases are overexpressed in wild-type cells. These data together suggest that the dwarfing effect of overexpressed MSMEG_4247 can be partially overridden by MSMEG_4241 overexpression.

Spatial and Temporal Control of MSMEG_4241 and MSMEG_4247 Expressions in Wild-type Cells—The above results suggested that MSMEG_4247 and MSMEG_4241 function in close collaboration with each other during the synthesis of LM/LAM. Therefore, we predicted that these two enzymes are located...
Role of Mannosyltransferases in Lipoarabinomannan Synthesis

In the in vitro assay, the incorporation of radiolabeled mannose into LAM was examined in the presence of excess non-radioactive mannose for up to 2 h. As expected from the enriched PIM2 biosynthetic activities in PM fractions (Fig. 6B), PimB’ was specifically localized to these fractions (Fig. 6C). In contrast, MSMEG_4247 and MSMEG_4241 were both enriched in PM-CW fractions, suggesting that LM/LAM biosynthesis takes place in PM-CW fractions. These data are consistent with MSMEG_4247 and MSMEG_4241 acting in proximity to coordinate the LM/LAM biosynthesis. We have also tested co-immunoprecipitation of these two proteins to show their physical interaction, but it was not successful (data not shown; also see “Discussion”). Another interesting feature of LM/LAM biosynthesis is that it appears to be down-regulated in the late stationary phase (17) (Fig. 6D). Because imbalanced expressions of MSMEG_4247 and MSMEG_4241 can result in the production of aberrant forms of LM/LAM, we thought it important for the cells to down-regulate the expression levels of both enzymes in a concerted manner. Indeed, the expression levels of these two proteins were reduced simultaneously in the stationary phase (Fig. 6D), in correlation with the reducing levels of LM/LAM. Taken together, MSMEG_4247 and MSMEG_4241 were expressed in a temporally and spatially controlled manner.

**DISCUSSION**

Our analysis on MSMEG_4247, a branch-forming α,1,2-mannosyltransferase involved in LM/LAM synthesis, revealed unexpected effects of its expression levels on the sizes and distributions of LAM-like species. The expression levels of MSMEG_4247 and MSMEG_4241 were both enriched in PM-CW fractions, as expected from the enriched PIM2 biosynthetic activities in PM fractions (Fig. 6B). PimB’ was specifically localized to these fractions (Fig. 6C). In contrast, MSMEG_4247 and MSMEG_4241 were both enriched in PM-CW fractions, suggesting that LM/LAM biosynthesis takes place in PM-CW fractions. These data are consistent with MSMEG_4247 and MSMEG_4241 acting in proximity to coordinate the LM/LAM biosynthesis. We have also tested co-immunoprecipitation of these two proteins to show their physical interaction, but it was not successful (data not shown; also see “Discussion”). Another interesting feature of LM/LAM biosynthesis is that it appears to be down-regulated in the late stationary phase (17) (Fig. 6D). Because imbalanced expressions of MSMEG_4247 and MSMEG_4241 can result in the production of aberrant forms of LM/LAM, we thought it important for the cells to down-regulate the expression levels of both enzymes in a concerted manner. Indeed, the expression levels of these two proteins were reduced simultaneously in the stationary phase (Fig. 6D), in correlation with the reducing levels of LM/LAM. Taken together, MSMEG_4247 and MSMEG_4241 were expressed in a temporally and spatially controlled manner.

**FIGURE 5. Dwarfing effect of MSMEG_4247 overexpression on LM/LAM can be competed by overexpression of MSMEG_4241.** A, acetic acid induction of MSMEG_4241 examined by Western blotting using anti-MSMEG_4241 antibody. The MSMEG_4247 overexpression mutant was transfected with either acetic-acid-inducible MSMEG_4241 expression vector (pPACE4241) (transfected with pYAB262) or empty vector (Vec) (transfected with pJAM2) and incubated with (+) and without (−) acetic acid for the indicated period to induce MSMEG_4241 expression. The loading was adjusted to 5 μg of protein/lane. B, metabolic labeling with [3H]mannose. Cells were preincubated with (+) or without (−) acetic acid for 4 h, pulsed for 15 min with [3H]mannose, and then chased in the presence of excess non-radioactive mannose for up to 2 h. C, changes in total LM/LAM profiles after induction of MSMEG_4241 expression in MSMEG_4247 overexpression mutant. LM/LAM were separated by SDS-PAGE and visualized by carbohydrate staining. D, dwarfed LM at 0 h and LM-like species at 12 h after acetic acid induction, shown in C, were purified by electrophoresis, and the purities were confirmed by SDS-PAGE and carbohydrate staining. E, compositional analysis of LM-like species produced after MSMEG_4241 overexpression. Trifluoroacetic acid-hydrolyzed carbohydrates were quantified by HPAEC. Data are presented as molar ratio relative to inositol. Averages of triplicate measurements with S.D. values are shown.

**FIGURE 6. Subcellular localization and growth phase-dependent expression of MSMEG_4247 and MSMEG_4241 in wild-type cells.** A, sucrose density (dashed line) and protein concentration (open circle) profiles of wild-type cell lysate fractionated by a density sedimentation. B, PIM biosynthetic activities of each fraction measured by GDP-[3H]mannose radiolabeling, PPM, polyprenol-phosphate-mannose. C, Western blotting using anti-MSMEG_4247 (top), anti-MSMEG_4241 (middle), or anti-PimB’ (bottom) antibodies. D, growth phase-dependent changes in LM/LAM levels of wild type cells grown in Middlebrook 7H9 broth. Culture was initiated by 1:100 dilution of a confluent starter culture, and aliquots were collected at the indicated time points, which correspond to logarithmic (18 h), early stationary (41 h), and late stationary (90 h) phases. Purified LM/LAM were separated by SDS-PAGE and visualized by carbohydrate staining. Loading was adjusted for equal cell pellet equivalents. E, growth phase-dependent changes in levels of MSMEG_4247 and MSMEG_4241 detected by Western blotting using anti-MSMEG_4247 or anti-MSMEG_4241 antibodies. Loading was adjusted to 15 μg of protein/lane.
quantities of LM/LAM (Fig. 8). Overexpression of MSMEG_4247 resulted in the production of dwarfed LM/LAM. In contrast, the deletion of MSMEG_4247 resulted in the disappearance of LM and accumulation of branchless LAM. We suggest that overexpression of the branching mannosyltransferase results in premature chain termination, whereas in the absence of branching, transiently synthesized branchless LM is either converted to LAM or degraded. Below, we discuss possible control mechanisms of mannan chain length and why branchless LM cannot be stably maintained.

Most important, the following two pieces of evidence suggested that MSMEG_4247 affects the sizes of LM/LAM primarily by competing with the elongating α1,6-mannosyltransferase. First, we showed that 15-fold or more overexpression of MSMEG_4247 resulted in short 1,6-mannose backbone (see Figs. 2 and 3). Second, overexpression of elongating α1,6-mannosyltransferase (MSMEG_4241) alleviated the dwarfing effect of MSMEG_4247 overexpression (see Fig. 5). Dwarfed LM/LAM are unlikely to be caused by substrate depletion because MSMEG_4241 overexpression can at least partially override the dwarfing effect of MSMEG_4247 overexpression. It is tempting to speculate that MSMEG_4241 and MSMEG_4247 compete for the growing non-reducing terminus of mannan polymer, and MSMEG_4247-mediated α1,2-mannosylation of the non-reducing end blocks further elongation. Future studies using a cell-free assay system and synthetic substrates will address these possibilities.

Interestingly, overexpression of MSMEG_4247 resulted in the dwarfing of not only the mannan chain but the arabinan moiety of LAM (see Fig. 2). Because both MSMEG_4241 and MSMEG_4247 are mannosyltransferases, it is unclear how the expression levels of these enzymes affect the arabinan size. One possibility is that multiple arabinans are attached to a single LAM molecule, and a short mannan chain limits the sites available for arabinosylation. In another experiment, we showed that overexpressed MSMEG_4241 competed with overexpressed MSMEG_4247 in terms of the LAM mannan size, but the restoration of the arabinan size was only partial (see Fig. 5). We speculate that relatively low (wild-type) expression levels of arabinosyltransferases compared with overexpressed mannosyltransferases have led to the reduction in arabinan size. Based on these observations, it is conceivable that the expression levels of arabinosyltransferases are also controlled in concert with the two mannosyltransferases to coordinate LAM synthesis in wild-type cells. Identification and characterization of the arabinosyltransferase that initiates the arabinan synthesis will provide further mechanistic insight into LAM biosynthesis.

The lack of α1,2-monomannose branching resulted in the complete absence of LM in both M. smegmatis and M. tuberculosis (see Figs. 1B and 7) (22), suggesting a conserved function of α1,2-mannose branching in LM accumulation. There are
several possibilities for why branchless LM cannot accumulate. First, in the absence of branching-dependent chain termination, MSMEG_4241 may be incapable of terminating the mannan polymerization by itself. We then speculate that arabinosylation acts as an alternative termination signal, thus resulting in the production of LAM only. Second, contrary to the first possibility, MSMEG_4241 may be able to terminate the polymer elongation after reaching a certain chain length, producing branchless LM. However, branchless LM cannot accumulate stably, perhaps because it is either efficiently converted to LAM or degraded. These possibilities are not exclusive to each other, and in either case, there appears to be an alternative chain termination mechanism in addition to mannose branching to control the mannan chain length. We consistently observed more enriched accumulation of branchless LAM in the ΔMSMEG_4247 mutant compared with the wild-type levels. Although the precursor-product relationship of LM and LAM is not established, this observation favors the possibility that branchless LM intermediate is more avidly converted to LAM rather than being degraded. Curiously, although our M. tuberculosis ΔRv2181 mutant showed a complete lack of LM, another recently reported ΔRv2181 mutant accumulated small size LM (23). Further studies are necessary to identify factors that have led these two ΔRv2181 mutants into different phenotypes.

At least a fraction of LAM synthesized in the plasma membrane appears to be transported to the surface layer of the cell wall (34), suggesting a dynamic spatial control of LAM biosynthesis. We demonstrated that both MSMEG_4247 and MSMEG_4241 are topologically confined in a membrane domain known as PM-CW (see Fig. 6, A–C), suggesting that LAM is synthesized in a spatially controlled manner, and these two enzymes may function in topological proximity. If MSMEG_4247 and MSMEG_4241 form a heterodimer, overexpression of kinetically inactive MSMEG_4247 may interfere with the formation of a functional dimer in the wild-type cells, leading to a phenotype similar to ΔMSMEG_4247 mutant. However, such dominant negative effects were not observed (see Fig. 3A), making heterodimer formation less likely. We do not know if the levels of overexpression achieved in this study occur in physiological situations. Nevertheless, our results suggest that the expression levels of MSMEG_4247 and MSMEG_4241 need to be controlled in concert to avoid the production of aberrant LM/LAM. Being consistent with this suggestion, disappearance of LM/LAM in the stationary phase was closely correlated with concerted down-regulation of both enzymes (see Fig. 6, D and E). Interestingly, truncated structural variants of LAM have been identified in Mycobacterium leprae and clinical isolates of M. tuberculosis (33), implying that the polymer length of LM/LAM can vary among species/strains and may be controlled in response to environmental stimuli. Controlling the expression levels of MSMEG_4247 and MSMEG_4241 in M. smegmatis or their orthologs in other species may represent a key feature of the polymer length control of mycobacterial LM/LAM. Understanding the mechanisms of transcriptional and post-transcriptional controls of MSMEG_4247 and MSMEG_4241 expressions may provide further clues to understand the regulatory mechanisms of LM/LAM biosynthesis.

Role of Mannosyltransferases in Lipoarabinomannan Synthesis

Acknowledgments—We thank Hidekazu Murakami, Dr. Morihisa Fujita, and Dr. Matthew Stokes for critical reading of the manuscript, Keiko Kinoshita for technical assistance, Dr. William R. Jacobs, Jr. (Albert Einstein College of Medicine) for kindly providing vectors pMV306 and pMV361, and Dr. Helen Billman-Jacobe (University of Melbourne) for kindly providing pJAM2 vector.

REFERENCES

1. Brennan, P. J. (2003) Tuberculosis 83, 91–97
2. Briken, V., Porcelli, S. A., Besra, G. S., and Kremer, L. (2004) Mol. Microbiol. 53, 391–403
3. Alderwick, L. J., Birch, H. L., Mishra, A. K., Eggeling, L., and Besra, G. S. (2007) Biochem. Soc. Trans. 35, 1325–1328
4. Khoo, K. H., Dell, A., Morris, H. R., Brennan, P. J., and Chatterjee, D. (1995) Glycobiology 5, 117–127
5. Lee, Y. C., and Ballou, C. E. (1964) J. Biol. Chem. 239, 1316–1327
6. Brennan, P., and Ballou, C. E. (1967) J. Biol. Chem. 242, 3046–3056
7. Khoo, K. H., Douglas, E., Azadi, P., Inamine, J. M., Besra, G. S., Mikusová, K., Brennan, P. J., and Chatterjee, D. (1996) J. Biol. Chem. 271, 28682–28690
8. Kaur, D., McNeil, M. R., Khoo, K. H., Chatterjee, D., Crick, D. C., Jackson, M., and Brennan, P. J. (2007) J. Biol. Chem. 282, 27133–27140
9. Chatterjee, D., Hunter, S. W., McNeil, M., and Brennan, P. J. (1992) J. Biol. Chem. 267, 6228–6233
10. Shi, L., Berg, S., Lee, A., Spencer, J. S., Zhang, J., Vissa, V., McNeil, M. R., Khoo, K. H., and Chatterjee, D. (2006) J. Biol. Chem. 281, 19512–19526
11. Besra, G. S., Morehouse, C. B., Rittner, C. M., Waechter, C. J., and Brennan, P. J. (1997) J. Biol. Chem. 272, 18460–18466
12. Brennan, P., and Ballou, C. E. (1968) J. Biol. Chem. 243, 2975–2984
13. Morita, Y. S., Patterson, J. H., Billman-Jacobe, H., and McConville, M. J. (2004) Biochem. J. 378, 589–597
14. Guerin, M. E., Kaur, D., Somashekar, B. S., Gibbs, S., West, P., Chatterjee, D., Brennan, P. J., and Jackson, M. (2009) J. Biol. Chem. 284, 25687–25696
15. Korduláková, J., Gilleron, M., Mikusová, K., Puzo, G., Brennan, P. J., Gicquel, B., and Jackson, M. (2003) J. Biol. Chem. 277, 31335–31344
16. Korduláková, J., Gilleron, M., Puzo, G., Brennan, P. J., Gicquel, B., Mikusová, K., and Jackson, M. (2003) J. Biol. Chem. 278, 36285–36295
17. Morita, Y. S., Sena, C. B., Waller, R. F., Kurokawa, K., Sernée, M. F., Nakatani, F., Haites, R. E., Billman-Jacobe, H., McConville, M. J., Maeda, Y., and Kinoshita, T. (2006) J. Biol. Chem. 281, 25143–25155
18. Crelier, P. K., Kovacevic, S., Martin, K. L., Brammananth, R., Morita, Y. S., Billman-Jacobe, H., McConville, M. J., and Coppell, R. L. (2008) J. Bacteriol. 190, 3690–3699
19. Kovacevic, S., Anderson, D., Morita, Y. S., Patterson, J., Haites, R., McMillan, B. N., Coppell, R., McConville, M. J., and Billman-Jacobe, H. (2006) J. Biol. Chem. 281, 9011–9017
20. Mishra, A. K., Alderwick, L. J., Rittmann, D., Tatitturi, R. V., Nigou, J., Gilleron, M., Eggeling, L., and Besra, G. S. (2007) Mol. Microbiol. 65, 1503–1517
21. Mishra, A. K., Alderwick, L. J., Rittmann, D., Wang, C., Bhatt, A., Jacobs, W. R., Takayama, K., Eggeling, L., and Besra, G. S. (2008) Mol. Microbiol. 68, 1595–1613
22. Kaur, D., Berg, S., Dinadayala, P., Gicquel, B., Chatterjee, D., McNeil, M. R., Vissa, V. D., Crick, D. C., Jackson, M., and Brennan, P. J. (2006) Proc. Natl. Acad. Sci. U.S.A. 103, 13664–13669
23. Kaur, D., Obergöhr-Henao, A., Pham, H., Chatterjee, D., Brennan, P. J., and Jackson, M. (2008) Proc. Natl. Acad. Sci. U.S.A. 105, 17973–17977
24. Snapper, S. B., Lugosi, L., Jekkel, A., Melton, R. E., Kieser, T., Bloom, B. R., and Jacobs, W. R., Jr. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 6987–6991
25. Haites, R. E., Morita, Y. S., McConville, M. J., and Billman-Jacobe, H. (2005) J. Biol. Chem. 280, 10981–10987
26. Stover, C. K., de la Cruz, V. F., Fuerst, T. R., Burlein, J. E., Benson, L. A., Bennett, L. T., Banskal, G. P., Young, J. F., Lee, M. H., and Hadfield, G. F. (1991) Nature 351, 456–460
27. Triccas, J. A., Parish, T., Britton, W. J., and Gicquel, B. (1998) FEMS Mi
Role of Mannosyltransferases in Lipoarabinomannan Synthesis

crobiol. Lett. 167, 151–156
28. Schneider, P., Ralton, J. E., McConville, M. J., and Ferguson, M. A. (1993) Anal. Biochem. 210, 106–112
29. Sauton, B. (1912) C. R. Acad. Sci. III 155, 860–863
30. Morita, Y. S., Velasquez, R., Taig, E., Waller, R. F., Patterson, J. H., Tull, D., Williams, S. J., Billman-Jacobe, H., and McConville, M. J. (2005) J. Biol. Chem. 280, 21645–21652
31. Khoo, K. H., Dell, A., Morris, H. R., Brennan, P. J., and Chatterjee, D. (1995) J. Biol. Chem. 270, 12380–12389
32. Gilleron, M., Himoudi, N., Adam, O., Constant, P., Venisse, A., Rivière, M., and Puzo, G. (1997) J. Biol. Chem. 272, 117–124
33. Torrelles, J. B., Khoo, K. H., Sieling, P. A., Modlin, R. L., Zhang, N., Marques, A. M., Treumann, A., Rithner, C. D., Brennan, P. J., and Chatterjee, D. (2004) J. Biol. Chem. 279, 41227–41239
34. Pitarque, S., Larrouy-Maumus, G., Payré, B., Jackson, M., Puzo, G., and Nigou, J. (2008) Tuberculosis 88, 560–565