Genetic Basis for the Synthesis of the Immunomodulatory Mannose Caps of Lipoarabinomannan in Mycobacterium tuberculosis*

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Lipoarabinomannan (LAM) is a high molecular weight, heterogenous oligosaccharide present in abundant quantities in Mycobacterium tuberculosis and many other actinomycetes. In M. tuberculosis, the non-reducing arabinan termini of the LAM are capped with α1→2 mannose residues; in some other species, the arabinan of LAM is not capped or is capped with inositol phosphate. The nature and extent of this capping plays an important role in disease pathogenesis. MT1671 in M. tuberculosis CDC1551 was identified as a glycosyltransferase that could be involved in LAM capping. To determine the function of this protein a mutant strain of M. tuberculosis CDC1551 was studied, in which MT1671 was disrupted by transposition. SDS-PAGE analysis showed that the LAM of the mutant strain migrated more rapidly than that of the wild type and did not react with concanavalin A as did wild-type LAM. Structural analysis using NMR, gas chromatography/mass spectrometry, endoarabinanase digestion, Dionex high pH anion exchange chromatography, and matrix-assisted laser desorption ionization-time-of-flight mass spectrometry demonstrated that the LAM of the mutant strain was devoid of mannose capping. Since an ortholog of MT1671 is not present in Mycobacterium smegmatis mc²155, a recombinant strain was constructed that expressed this protein. Analysis revealed that the LAM of the recombinant strain was larger than that of the wild type, had gained concanavalin A reactivity, and that the arabinan termini were capped with a single mannose residue. Thus, MT1671 is the mannosyltransferase involved in deposition of the first of the mannose residues on the non-reducing arabinan termini and the basis of much of the interaction between the tubercle bacillus and the host cell.

Lipoarabinomannan (LAM) is a high molecular weight amphipathic oligosaccharide, which makes up one of the major components of the cell wall of mycobacteria and exhibits a wide spectrum of immunomodulatory effects. Its structure is complex and heterogeneous with three distinct structural domains, including a phosphatidylinositol anchor (PI anchor), a branched mannan, and a branched arabinan (Fig. 1). The PI anchor is composed of a myo-inositol phosphoryl diacylglycerol substituted at the 2 position with a single mannoyranose (Manp) and at the 6 position with the mannan; this structure is identical to those found in the mycobacterial phosphatidylinositolmannosides and lipomannan (1, 2), which are thought to be biosynthetic precursors of LAM (3). The mannan core is linked to the 6 position of the myo-inositol residue of the PI anchor and consists of a linear α(1→6) Manp chain with varying degrees of α(1→2) Manp branching with single Manp residues (3). This structure is conserved in all mycobacterial species studied with two exceptions (4, 5). The arabinan component consists of linear stretches of α(1→5) arabinofuranose (Araf) residues with some α(1→3) branching. Two distinct non-reducing termini are generated with a β-D-Araf(1→2)-α-D-Araf/D-disaccharide linked to the 5 position of α-D-Araf(1→5)-α-D-Araf(1→ or the 3 and 5 positions of α-D-Araf(1→5)-α-D-Araf(1→, resulting in the formation of well characterized Ara₄ and Ara₅ motifs, respectively, when digested with an endoarabinanase obtained from a Cellulomonas species (Fig. 1; Refs. 3 and 6). In slow growing mycobacteria such as Mycobacterium tuberculosis, Mycobacterium leprae, Mycobacterium bovis, Mycobacterium avium, and Mycobacterium kansasii (2, 5, 7–12) a portion of the non-reducing termini of the Araf chains is capped, to varying degrees, with short α(1→2) Manp chains consisting of one to three residues (Fig. 1), thus the molecule is termed ManLAM. The situation is different in rapidly growing mycobacteria. LAM isolated from Mycobacterium smegmatis and an unidentified Mycobacterium sp. is largely uncapped with a small fraction being capped with inositol phosphate (PILAM) (7, 13) and in Mycobacterium chelonae, no modification exists on the arabinosyl termini (AraLAM) (4).

LAM has been implicated in a plethora of biological functions; typically ManLAM is thought to be anti-inflammatory, while PILAM is thought to be pro-inflammatory. ManLAM has...
Mannose Capping of Mycobacterial LAM

FIGURE 1. Schematic diagram of ManLAM. The diagram shown is consistent with published structural analysis and relative amounts of each sugar. The exact number, length, and point of attachment of the arabian chains are not known; thus, the arabian is not shown attached to the mannan core. The origins of Man_Ara, and Man_Ara motifs generated by C. gellida endoarabinanase digestion are indicated. *, the indicated Ara4 and Ara6 motifs are only generated by the endoarabinanase if the β-Araf residue is uncapped. **, if the β-Araf residue is unsubstituted with Manp, the residue designated in the diagram as 5-β-Araf is t-β-Araf.

been implicated in inhibition of phagosomal maturation, apoptosis, and interferon-γ signaling in macrophages and interleukin-12 secretion of dendritic cells (reviewed in Refs. 3, 14, and 15). It has also been suggested that the type of LAM capping is a major structural feature in determining how the immune system is modulated (14), and a recent publication suggests that dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) may act as a pattern recognition receptor and discriminate between Mycobacterium species through selective recognition of the Manp caps on LAM molecules (16). Thus, LAM structure is generally considered to be a crucial factor in mycobacterial pathogenesis.

Despite the level of understanding of the structure of LAM and the variety of immunomodulatory effects it mediates, nothing was known of the enzymes involved in capping of ManLAM. However, the facts that decaprenylphosphorylarabinofuranose is the only known donor of the Ara residues in the mycobacterial cell wall (17), and synthesis of polar phosphatidylinositolmannosides and linear LM can be inhibited by amphotycin (18, 19), suggested that the glycosyltransferases (GTs) involved in the later steps of ManLAM synthesis likely utilize prenylphosphorylglycoses as sugar donors. This idea was supported by the fact that in prokaryotes with the exception of mycobacteria (22). Approximately 40 putative members of the GT-C superfamily were identified in the genomes of M. tuberculosis H37Rv, M. tuberculosis CDC1551, M. leprae, and M. smegmatis (22). Of these putative GTs 16 were identified in M. tuberculosis H37Rv; these included putative arabinosyltransferases, the mycobacterial Emb proteins, and Rv1002c, which was subsequently shown to catalyze the initial Manp transfer in mycobacterial protein mannosylation (23). It was hypothesized that the mannosyltransferases (MTs) involved in capping of ManLAM would be among these 16 putative GTs and would have homologs in M. leprae, M. avium, and M. bovis but would not have an ortholog in M. smegmatis. Based on this hypothesis MT1671 from M. tuberculosis was identified as a gene encoding a potential GT involved in LAM capping. Therefore, LAM was isolated and structurally characterized from a transposition mutant in which the gene encoding the protein was disrupted and from a recombinant strain of M. smegmatis that expresses the protein.

EXPERIMENTAL PROCEDURES

Materials—All chemical reagents were of the highest grade from Sigma unless otherwise specified. M. smegmatis mc²155 was obtained from the American Type Culture Collection. M. tuberculosis CDC1551 (ΔMT1671) strain was obtained by inactivation of the MT1671 gene in a M. tuberculosis ΔSigF background by transposition (24, 25); these strains along with the parental strain, M. tuberculosis CDC1551, were generously provided by Dr. W. R. Bishai (Center for Tuberculosis Research, The Johns Hopkins University School of Medicine, Baltimore, MD). The insertion of the kanamycin cassette in MT1671 gene was checked by PCR (using primers MT1671f (5′-ATG TTG CTG TGT AAC GCT-3′) and MT1671r (5′-TTA CGG CCT TGA CTT GAC-3′) specific to the MT1671 gene; TSPf (5′-CGC TTC CTC GTG CCT TTC TAC GGT ATC G-3′) and TSPr (5′-CCC GAA AAG TGC CAC CTA AAT TGT AAG CG-3′) specific to the transposon).

Culture Conditions—M. smegmatis mc²155 and M. tuberculosis CDC1551 were grown in 7H9 containing oleic acid, albumin, dextrose, and catalase and 0.05% Tween 80. M. smegmatis mc²155-pVV16 and M. smegmatis mc²155-pVV16-ΔMT1671 were grown with kanamycin (50 μg/ml) and hygromycin (50 μg/ml) selection. M. tuberculosis ΔSigF was cultured with hygromycin selection (50 μg/ml), and M. tuberculosis ΔMT1671 was cultured with kanamycin and hygromycin (50 μg/ml each). All bacteria strains were cultured and harvested at late log phase.

Construction of Recombinant M. smegmatis mc²155—The 1671-bp open reading frame of MT1671 from M. tuberculosis CDC1551 was amplified from genomic DNA by PCR using primers (MT1671f (5′-TTT TTT CAT ATG CAT GCG AGT CTG TGT AAC GCT-3′) and MT1671r (5′-TTT TTG AAG CTT ACC GGT TTT ACT TGA CTT GAC CCA C-3′)) engineered to include NdeI and HindIII restriction sites (underlined), respectively. The PCR product was cloned into the vector pGEM (Promega) for sequence confirmation and subsequently ligated into pVV16 derived from pMV261 (26) after digestion with NdeI and HindIII to create plasmid pVV16-ΔMT1671. M. smegma-
tis mc²155 was then transformed with pVV16 or pVV16-MT1671 by electroporation.

**Extraction of LAM and LM**—A quick method was used when LAM and LM were extracted from small samples of mycobacteria (50 mg) as described previously (27). Briefly, a mixture of chloroform/methanol/water (10:10:3) was added to the cell pellet and incubated 30 min at 55 °C. The sample was centrifuged, and the organic solvent was removed. Water and phenol saturated with phosphate-buffered saline (1:1) were added to the pellet and then incubated at 80 °C for 2 h. Chloroform was added, and the sample was centrifuged. The supernatant containing LAM and LM was dialyzed against water overnight, and the LAM and LM were analyzed by SDS-PAGE.

When LAM and LM were extracted from large quantities of *M. tuberculosis* for structural analysis the cell pellets from 5-liter cultures were delipidated by serial extractions of 2:1 chloroform/methanol and 10:10:3 chloroform/methanol/water. Subsequently, LAM and LM were extracted essentially as described (8). Wet, delipidated cells were resuspended in breaking buffer (phosphate-buffered saline containing 8% Triton X-114 (Sigma), pepstatin, phenylmethylsulfonyl fluoride, leupeptin, DNase, and RNase). Cells were then disrupted using a French pressure cell. The resulting suspension was centrifuged at 2000 × g for 10 min, and the pellet was discarded. The supernatant was rocked overnight at 4 °C and then centrifuged at 27,000 × g for 15 min. The resulting 27,000 × g pellet was resuspended in breaking buffer and centrifuged as before. The combined supernatant was placed at 37 °C to generate a biphasic and centrifuged at 27,000 × g for 15 min at room temperature. The aqueous layer and the detergent layer were back extracted twice, and 9 volumes of cold 95% ethanol was added to the combined detergent layers and incubated at −20 °C overnight. The ethanol precipitate was collected, dried, resuspended in water at 50 mg/ml, digested with 1 mg/ml Pronase (Roche Applied Science), and dialyzed against water.

Extraction of LAM and LM from large quantities of *M. smegmatis* for structural analysis was done as described previously (28). Cultures (10 liters) in late log phase were harvested by centrifugation. The cells were delipidated using organic solvents (29) and subjected to several freeze-thaw cycles before mechanical disruption by sonication. The resulting suspension was refluxed in 50% ethanol three times. The extracts were pooled and debris removed by centrifugation. The solvent was evaporated, and the sample was resuspended in water at ∼50 mg/ml and digested with 1 mg/ml Proteinase K (Invitrogen). After dialysis the LAM/LM fractions from either *M. tuberculosis* CDC1551 or *M. smegmatis* were further purified by size fractionation and analyzed by SDS-PAGE and Western or lectin blotting.

**Size Fractionation, SDS-PAGE, and Blotting**—HPLC size fractionation was performed on a Rainin SD 200 series LC system fitted with a Sephacryl S-200 HiPrep 16/60 column in tandem with a HiPrep 16/60 Sephacryl S-100 column (Amersham Biosciences) at a flow rate of 1 ml/min (29). SDS-PAGE (6% stacking gel and 15% resolving gel) followed by periodic acid-Schiff base (PAS) staining (9) was used to monitor the elution profile of the fractions containing LAM and LM, which were then pooled and dialyzed. Pooled fractions were re-analyzed by SDS-PAGE to check for purity prior to detailed analysis. Samples were also analyzed by Western blot using monoclonal antibody CS-35 (provided by National Institutes of Health/NIAID contract AI-25469) or lectin blot with concanavalin A (ConA) conjugated to peroxidase (Sigma). LAM and LM were electroeluted from 15% SDS-PAGE to Protran nitrocellulose membranes (Whatman Schleicher and Schuell Bioscience), which were then blocked and incubated with CS-35 (9) or conjugated ConA. In the case of the Western blots, membranes were incubated with the secondary antibody (anti-mouse IgG coupled to alkaline phosphatase), and color was developed using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) substrate (Sigma). In the case of the lectin blots, compounds were visualized using the 4-chloro-1-naphthol/3,3′-diaminobenzidine, tetrahydrochloride (CN/DAB) substrate kit according to manufacture’s instructions (Pierce).

**Monosaccharide Composition and Linkage Analysis**—LAM samples were hydrolyzed with 2 M trifluoroacetic acid, converted to alditol acetates, and analyzed by GC using scylo-inositol as an internal standard (30). GC analysis of the alditol acetates was performed on an Hewlett Packard gas chromatograph model 5890 fitted with a SP 2380 column (30 m, 0.25-μm film thickness, 0.25-mm inner diameter; Supelco) using a temperature gradient of 50 °C for 1 min, 30 °C/min to 170 °C, and then 4 °C/min to 260 °C.

For linkage analysis, LAM was permethylated using the NaOH/dimethyl sulfoxide slurry method (31), hydrolyzed with 2 M trifluoroacetic acid, and acetylated (30). GC/MS of the partially methylated alditol acetates was carried out using a ThermoQuest Trace gas chromatograph 2000 (ThermoQuest) connected to a GCQ/Polaris MS mass detector (ThermoQuest). Sample was dissolved in chloroform prior to injection on a DB-5 fused silica capillary column (10 m, 0.18-μm film thickness, and 0.18-mm inner diameter (J&W Scientific)) at an initial temperature of 50 °C, which was held for 1 min. The temperature was then increased to 180 °C over 20 min and then to 250 °C over 8 min.

**NMR Spectroscopy**—Spectra were acquired after several lyophilizations in D₂O of 4 mg/0.6 ml in 100% D₂O. Two-dimensional ¹H,¹³C heteronuclear single quantum correlation spectroscopy (HQC) NMR spectra were acquired on a Varian Inova 500-MHz NMR spectrometer using the supplied Varian pulse sequences. The HSQC data were acquired with a 7-kHz window for proton in F2 and a 15-kHz window for carbon in F1. The total recycle time was 1.65 s between transients. Adiabatic decoupling was applied to carbon during proton acquisition. Pulsed field gradients were used throughout for artifact suppression but were not used for coherence selection. The data set consisted of 1000 complex points in t₂ by 256 complex points in t₁ using States-TPPI (time proportional phase incrementation). Forward linear prediction was used for resolution enhancement to expand t₁ to 512 complex points. A cosine-squared weighting function and zero filling were applied to both t₁ and t₂ prior to the Fourier transformation. The final resolution was 3.5 Hz/point in F2 and 15 Hz/point in F1.

**Endoarabinanase Digestion and Analysis by HPAEC**—LAM (20 μg) was incubated for 16 h at 37 °C with an endoarabinanase...
Mannose Capping of Mycobacterial LAM

isolated from Cellulomonas gelida (3, 6). An aliquot of the digestion mixtures containing both the mannan core and the released oligosaccharides was analyzed directly by Dionex analytical HPAEC performed on a Dionex liquid chromatography system fitted with a Dionex Carbopac PA-1 column. The oligosaccharides were detected with a pulse-amperometric detector (PAD-II) (Dionex). The remaining mixtures were peracetylated as described above and analyzed by MALDI-TOF mass spectrometry.

Matrix-assisted Laser Desorption Ionization-Time-of-Flight Mass Spectrometry—The peracetylated oligosaccharides (10 µg/µl) or the aqueous solutions of the native LAM (10 µg/µl) were mixed with an equal volume of matrix (2,5-dihydroxybenzoic acid dissolved in 10 mg/ml acetonitrile/water, 50:50, 0.1% trifluoroacetic acid) prior to analysis and the molecular mass was measured in negative ion mode by MALDI-TOF on a Bruker Ultraflex TOF/TOF mass spectrometer (Bruker Daltonics).

Other Techniques—Protein sequences were obtained from the National Institute for Biotechnology Information (NCBI) world wide web site. BLAST searches were performed at the NCBI site or the TB Structural Genomics Consortium web site. Amino acid sequence alignments were performed using the MultAlin (32) interface on the L’institut National de la Recherche Agronomique Chemin de Bourde-Rouge-Auzeville web site. Standard molecular biology techniques were done as described previously (33).

RESULTS AND DISCUSSION

Identification of MT1671 as a GT Potentially Involved in LAM Capping—MT1671 from M. tuberculosis CDC1551 met all of the criteria proposed for a GT involved in Manp capping of LAM; it is classified as a putative GT-C GT (22), species of mycobacteria known to have ManLAM have orthologs of this protein, and M. smegmatis, which has PILAM, does not (Fig. 2A). The amino acid sequences of the orthologs in M. tuberculosis H37Rv (Rv1635c) and M. bovis AF2122/97 (Mb1661c) are identical to that of MT1671. M. leprae TN and Mycobacterium avium paratuberculosis also have genes that encode proteins, ML1389 and MAP1338c, respectively, with a high degree of similarity (Fig. 2B). MT1671 is predicted to be a 60.3-kDa protein with an isoelectric point at pH 10.66. The protein is also predicted to have 10 (TMHMM 2.0, PHDhtm) or 11 (SOSUI) transmembrane helices. The program PHDhtm was utilized by Oriol et al. (21) to predict the secondary structure of GTs, which use dolichylphosphorylmonosaccharides as the donor substrate, and when used to predict the secondary structure of MT1671, a model was generated in which there were 10 transmembrane helices with the first loop on the extracellular face of the membrane. This loop contains an Asp residue and a Glu residue (DE motif) that are consistent with conserved amino acids in the first, extracellular loop of proteins in the α 2/6 MT superfamily (21) and is similar to that predicted for Rv1002c, an enzyme responsible for the addition of a Manp residue to protein acceptors in M. tuberculosis H37Rv (23).

Characterization of LAM from M. tuberculosis CDC1551 with a Disrupted Copy of MT1671—Since a M. tuberculosis CDC1551 transposon mutant in which MT1671 was disrupted had already been constructed in a ΔSigF background (24, 25), and MT1671 had 100% identity with Rv1635c, LAM from this mutant was subjected to structural analysis. LAM was purified from equal amounts of M. tuberculosis CDC1551 and ΔMT1671 and accounted for 0.03% of dry weight in M. tuberculosis ΔMT1671 but made up 0.3% of dry weight in M. tuberculosis CDC1551; thus, a 10-fold reduction in LAM content could be attributed to the disruption of MT1671. When LAM from each strain was analyzed by SDS-PAGE (Fig. 3A) it was apparent that ΔMT1671 LAM migrated more rapidly than that extracted from either wild-type M. tuberculosis CDC1551 or the parental strain M. tuberculosis CDC1551 ΔSigF (ΔSigF), indicative of a smaller size. Indeed, MALDI-TOF mass spectrometry confirmed that the average mass of the heterogenous LAM from ΔMT1671 was smaller (m/z = 22 kDa) than that from wild-type bacteria (m/z = 25 kDa). The extracted LAM and LM were also analyzed by SDS-PAGE and visualized by Western or lectin blot (Fig. 3, B and C). ManLAM from M. tuberculosis CDC1551 and ΔSigF both react to CS-35 (a monoclonal antibody that recognizes the nonreducing arabinan termini of LAM (34)) and ConA (a lectin that recognizes terminal Manp residues). The LAM isolated from ΔMT1671 also reacted with Cs-35 but did not react with ConA, even though LM isolated from both strains did react with ConA. In addition, PILAM, isolated from M. smegmatis mc²155 and devoid of Manp caps, did not react with ConA (Fig. 3F). These results strongly suggested that LAM from the ΔMT1671 strain was not Manp capped.

Construction of a Recombinant Strain of M. smegmatis mc²155 Expressing MT1671—Preliminary results using the CDC1551 transposon mutant clearly suggested that MT1671 and its orthologs are involved in Manp capping of LAM in slow growing mycobacteria. However, since there are likely to be multiple enzymatic activities associated with LAM, capping it was hypothesized that expression of one of these proteins in a Mycobacterium sp. devoid of ManLAM could shed light on the exact function. Therefore, to elucidate the specific role of the MT1671 protein, M. smegmatis mc²155 was transformed with an expression plasmid harboring MT1671 from M. tuberculosis CDC1551, pVV16-MT1671, or empty vector, pVV16. Transformation with either plasmid did not alter bacterial growth in liquid culture or colony morphology relative to the wild-type strain (data not shown).

Characterization of LAM from M. smegmatis mc²155 Expressing MT1671—Transformation with pVV16-MT1671 reduced the amount of LAM in M. smegmatis harvested in late log-phase by about 3-fold, i.e. 0.25% of the dry weight of M. smegmatis mc²155 and 0.08% of M. smegmatis mc²155-pVV16-MT167 could be accounted for by LAM. Extracted LAM and LM were analyzed by SDSL-PAGE and visualized by PAS staining. Western blot using CS-35, or lectin blot with ConA (Fig. 3, D–F). The most striking result came from the ConA blot (Fig. 3F). The LAM isolated from M. smegmatis mc²155 does not react with ConA; however, it became ConA positive when M. smegmatis was transformed with MT1671. This result clearly indicated that one or more Manp residues had likely been added to the PILAM normally synthesized by the fast growing Mycobacterium sp. Consistent with the addition of extra Manp residues, the LAM of the recombinant strain mc²155-pVV16-
MT1671 migrated more slowly than the LAM of the wild-type strain indicative of increased size. This putative size differential was confirmed by MALDI-TOF mass spectrometry. LAM isolated from the recombinant strain mc2155-pVV16-MT1671 (m/z 22 kDa) was significantly larger than LAM isolated from the wild-type strain (m/z 19.5 kDa); therefore, the average mass of the heterogeneous population of LAM molecules had increased by 2.5 kDa, a value that was consistent with the 3-kDa decrease in size observed for the MT1671 disruption mutant, suggesting that as many as 18–20 glycosyl residues could be involved. This number is consistent with the facts that LAM exists in a mixed population of linear and branched molecules, and there can be up to three Manp residues capping multiple terminal β-D-Araf residues per molecule (Fig. 1) as well as published estimates of the number of Manp residues capping LAM from M. tuberculosis Erdman (35), making a 2–3-kDa change in mass due to Manp capping plausible. Thus, all of the results suggest that the proteins under study are involved in capping of LAM. However, the data did not shed light on where the glycosylation occurred or how many glycosyl residues were added.

NMR Analysis of LAM Variants—Purified LAM samples were analyzed by two-dimensional 1H-13C HSQC experiments;
Mannose Capping of Mycobacterial LAM

![Diagram](https://via.placeholder.com/150)

**FIGURE 3.** SDS-PAGE analysis of mycobacterial LAM. **A–C,** similar amounts of LAM and LM extracted from *M. tuberculosis* CDC1551, *M. tuberculosis* ΔsigF, and *M. tuberculosis* ΔMT1671 were loaded in lanes 1–3, respectively. **D–E,** similar amounts of LAM and LM extracted from *M. smegmatis* mc^2^155-pVV16 (empty vector) and *M. smegmatis* mc^2^155-pVV16-MT1671 were loaded in lanes 1 and 2, respectively. **A** and **D,** SDS-PAGE gels visualized using PAS-staining. **B** and **E,** Western blots using monoclonal antibody CS-35. **C** and **F,** lectin blots using ConA. Positions of molecular mass markers (kDa) are indicated by the arrows.

Resonances for anomeric regions were assigned by referring to a body of published NMR data on the structure of LAM (2, 13, 36, 37) and endoarabinanase-generated fragments (38). Initial analysis of the heterogeneous population of LAM molecules from wild-type CDC1551 showed no marked discrepancies from published NMR spectra of LAM from *M. tuberculosis* H_{37}Rv (37), *M. tuberculosis* CSU20 (37), *M. leprae* (37), *M. bovis* BCG (2), or *M. kansasi** (5). Therefore, chemical shifts for ManLAM isolated from CDC1551 were used as a standard for simplifying in presentation of the data.

Based on the literature referred to above, evidence for the presence or absence of Manp capping was sought from the ^1^H resonance at δ 101.1 ppm, correlating to the anomeric protons at δ 5.17 of 2-α-Manp from the Manp caps and anomeric protons at δ 5.14 for the core 2,6-α-Manp residues. In addition, overlapping anomeric carbon signals at δ 105.1 ppm correlated to proton signals at δ 5.07 ppm were assigned to terminal-α-Manp (t-α-Manp) residues belonging to both the Manp caps and mannan core. The glycosyl residue composition and peak volumes are summarized in Fig. 4.

The volume of the cross-peaks for 2-α-Manp and t-α-Manp generated from LAM samples isolated from ΔMT1671 was markedly reduced, 9.5- and 5.9-fold respectively, relative to those from wildtype CDC1551 LAM. These results suggested that the extent of Manp capping had been greatly reduced or eliminated. Conversely, the volume of the cross-peaks for 2-α-Manp and t-α-Manp generated from LAM samples isolated from *M. smegmatis* expressing MT1671 were somewhat increased, 1.4- and 1.3-fold, respectively, relative to those from wild-type *M. smegmatis*. Thus, it appeared that there was a gain of function, Manp capping, in *M. smegmatis* that was transformed with pVV16-MT1671. Unfortunately, the exact degree of Manp capping cannot unambiguously be determined from these experiments due to the strong overlapping of the resonances from the 2-α-Manp and 2,6-α-Manp residues as well as resonances from the t-α-Manp residues located in the mannan core with those on the cap.

The NMR spectra show two additional cross-peaks that appear to be affected by the deletion of MT1671. One of these peaks, with ^1^C resonance at δ 105.2 ppm, correlating to the anomeric protons at δ 5.42, is characteristic of 5-deoxy-5-methyl-5-thio-α-xylofuranose (MTX (37, 38)). The fact that this peak is not present in the NMR spectrum shown in Fig. 4B is not surprising, in that this compound has unambiguously been shown to be linked to a capping Manp in LAM from *M. tuberculosi**s* (38). Thus, if MT1671 is involved in Manp capping, and LAM from ΔMT1671 is either devoid of or greatly reduced in Manp capping, a reduction in MTX would also be expected. Another cross-peak with ^1^C resonance at δ 103.6 ppm, correlating to the anomeric protons at δ 5.31 ppm in Fig. 4A is also missing from the spectrum shown in Fig. 4B. This signal is unassigned, but it is possible that it is associated with an as yet undescribed modification associated with the caps of ManLAM.

**Linkage and Endoarabinanase Analysis**—Linkage of the sugars in the LAM samples was determined by GC/MS analysis of alditol acetates derived from partially per-O-methylated LAM from the various strains (data not shown). Results corroborated the NMR results in that the quantity of 2-Manp was reduced to the limits of detection in ΔMT1671-LAM. In addition, the t-Manp was also slightly reduced from an abundance of 17.8% to 14.6% in the ΔMT1671-LAM. The only observable difference in the linkage analyses of LAM isolated from the recombinant *M. smegmatis* strain, and the wild type was an increase in the relative abundance of t-Manp residues from 6.9 to 15.6%.

To further analyze the extent of Manp capping, LAM isolated from the bacterial strains was digested by endoarabinanase from *C. gelida* (3, 6, 37), and the products were separated by Dionex HPAEC (Fig. 5), and digested material was also per-acetylated and analyzed by MALDI-TOF mass spectrometry to confirm the structures shown in Fig. 5. The HPAEC profiles for *M. tuberculosis* CDC1551 (Fig. 5A) and ΔSigF (data not shown) are similar; LAM from both strains was predominantly capped with a dimannoside as demonstrated by the prominent Man_{2}Ara_{4} (peracetylated [M+Na]^+ m/z = 1565) and Man_{2}Ara_{4} (peracetylated [M+Na]^+ m/z = 2573) peaks, a result that was consistent with previously published data for *M. tuberculosis* H_{37}Rv (8, 37). However, the profile of the digested ΔMT1671-LAM (Fig. 5B) most closely resembles the LAM of *M. smegmatis* mc^2^155 (Fig. 5C), which has no Manp capping (the small amount of inositol phosphate-capped material is not...
detected by HPAEC) indicating that ΔMT1671-LAM is completely devoid of Manp capping. On the other hand, the profile for *M. smegmatis* mc²155-pVV16-MT1671 Fig. 5D is consistent with that seen for LAM in which the Ara₄ and Ara₆ motifs are substituted with a single Manp residue on each terminal β-Araf (peracetylated [M+Na]⁺ m/z values of 1277 and 1997, respectively).

Function of MT1671—Despite the lack of a formal direct enzymatic assay, the data presented here strongly indicate that MT1671 and its orthologs are mannosyltransferases and, more specifically, catalyze the addition of the first Manp residue on each terminal β-Araf (peracetylated [M+Na]⁺ m/z values of 1277 and 1997, respectively).

The NMR ¹H-¹³C HSQC spectra of LAM from *M. tuberculosis* CDC1551 (A) and *M. tuberculosis* CDC1515-ΔMT1671 (B) were acquired in D₂O. Only the expanded anomeric regions are shown. The relative intensities of the peak volumes were measured, and the data are presented in C. Signal volumes were integrated and normalized to the sum of the overlapping signals from t-β-Araf and t-5-β-Araf. The peaks annotated with a question mark have not been assigned. MTX indicates a cross-peak characteristic of 5-deoxy-5-methyl-5-thio-α-xyllofuranose. *The 6-α-Manp peak in B is very weak due to the small quantity of LAM-ΔMT1671 available for analysis. NF, not found.*

**FIGURE 4. Comparative partial two-dimensional NMR spectra of LAM.** The NMR ¹H-¹³C HSQC spectra of LAM from *M. tuberculosis* CDC1551 (A) and *M. tuberculosis* CDC1515-ΔMT1671 (B) were acquired in D₂O. Only the expanded anomeric regions are shown. The relative intensities of the peak volumes were measured, and the data are presented in C. Signal volumes were integrated and normalized to the sum of the overlapping signals from t-β-Araf and t-5-β-Araf. The peaks annotated with a question mark have not been assigned. MTX indicates a cross-peak characteristic of 5-deoxy-5-methyl-5-thio-α-xyllofuranose. *The 6-α-Manp peak in B is very weak due to the small quantity of LAM-ΔMT1671 available for analysis. NF, not found.*

**FIGURE 5. Consistent with that seen for LAM in which the Ara₄ and Ara₆ motifs are substituted with a single Manp residue on each terminal β-Araf (peracetylated [M+Na]⁺ m/z values of 1277 and 1997, respectively).**

**TABLE**

| Residues          | ppm values | CDC1551 | ΔMT1671 | mc²155 | pVV16-MT1671 |
|-------------------|------------|---------|---------|--------|--------------|
|                   | ¹³C        | ¹H      | ¹³C     | ¹H     | ¹³C          | ¹H     | ¹³C     | ¹H     |
| 5-α-Araf          | 110.4      | 5.10    | 240     | 193    | 291          | 374    |
| 5-α-Araf          | 110.0      | 5.18    | 70      | 61     | 83           | 109    |
| 5-α-Araf          | 109.6      | 5.28    | NF      | NF     | 14.6         | 12     |
| 3,5-α-Araf        | 110.3      | 5.13    | 80.82   | 68.3   | 56           | 67.6   |
| 2,5-α-Araf        | 108.6      | 5.19    | 77.4    | 68.4   | 75           | 74.3   |
| 2,3-α-Araf        | 108.4      | 5.26    | 27.5    | 27.3   | 19.3         | 22.3   |
| t-β-Araf + 5-β-Araf| 103.5     | 5.16    | 100     | 100    | 100          | 100    |
| t-α-Manp (core + cap) | 105.1     | 5.07    | 137.4   | 23.4   | 37           | 47.2   |
| 2,6-α-Manp +      | 101.1      | 5.14    | 136.4   | 14.3   | 28.5         | 38.5   |
| 2-α-Manp cap      | 102.4      | 4.93    | 4.46    | 1.54*  | 7.7          | 9      |

The peaks annotated with a question mark have not been assigned. MTX indicates a cross-peak characteristic of 5-deoxy-5-methyl-5-thio-α-xyllofuranose. *The 6-α-Manp peak in B is very weak due to the small quantity of LAM-ΔMT1671 available for analysis. NF, not found.*

**FIGURE 4. Comparative partial two-dimensional NMR spectra of LAM.** The NMR ¹H-¹³C HSQC spectra of LAM from *M. tuberculosis* CDC1551 (A) and *M. tuberculosis* CDC1515-ΔMT1671 (B) were acquired in D₂O. Only the expanded anomeric regions are shown. The relative intensities of the peak volumes were measured, and the data are presented in C. Signal volumes were integrated and normalized to the sum of the overlapping signals from t-β-Araf and t-5-β-Araf. The peaks annotated with a question mark have not been assigned. MTX indicates a cross-peak characteristic of 5-deoxy-5-methyl-5-thio-α-xyllofuranose. *The 6-α-Manp peak in B is very weak due to the small quantity of LAM-ΔMT1671 available for analysis. NF, not found.*
Mannose Capping of Mycobacterial LAM

FIGURE 5. HPAEC profiles of the endoarabinanase digestion products of LAM. Shown are LAM from \textit{M. tuberculosis} CDC1551 (A), \textit{M. tuberculosis} CDC1551-\textit{pMT1671} (B), \textit{M. smegmatis} mc\textsuperscript{5}155 (C), and \textit{M. smegmatis} mc\textsuperscript{155}\textit{pVV16-\textit{pMT1671}} (D). Samples were digested with endoarabinanase from \textit{C. gelida} for 16 h at 37 °C. For each strain, an aliquot was analyzed by HPAEC, and the remaining material was peracetylated and analyzed by MALDI-TOF mass spectrometry to verify the identification of the peaks.

been defined in precise molecular terms. An early hypothesis linking Manp cap to virulence was weakened by the observation that LAM from all strains of \textit{M. tuberculosis} and \textit{M. bovis}, including attenuated ones, studied were Manp capped regardless of virulence status (3, 7). Recently, several groups have utilized LAM isolated from organisms with varying types of capping with, and without, chemical and/or enzymatic modifications in efforts to define the importance of the Manp cap in mycobacterial pathogenesis (16, 39, 40).

These experiments led to the suggestions that: 1) the ultimate response of the host to the pathogen may depend on the LM or LAM in the mycobacterial envelope (40); 2) the caps of ManLAM are likely not necessary to modulate the induction of IL-12 and apoptosis (39); and 3) that DC-SIGN may act as a pattern recognition receptor and discriminate between mycobacterium species through selective recognition of the Manp caps on LAM molecules (16). In addition, it has been shown that the selective removal of Manp caps from ManLAM completely abrogates the ability of LAM to bind to phagocyte mannose receptors (41). Thus, it is likely that the presence of one, two, or three Manp residues in the cap has profound biological consequences \textit{in vitro} as they do \textit{in vivo}.

To date it has not been possible to test this hypothesis, but with the identification of the function of MT1671 we now have the ability to generate slow growing \textit{Mycobacterium} strains lacking Manp caps and fast growing strains with Manp caps, which can be used in detailed immunological studies aimed at defining the precise role of ManLAM caps in the pathogenesis of \textit{M. tuberculosis} \textit{in vivo}.

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