Analysis of a New Mannosyltransferase Required for the Synthesis of Phosphatidylinositol Mannosides and Lipoarabinomannan Reveals Two Lipomannan Pools in Corynebacterineae*

David J. Lea-Smith1,2, Kirstee L. Martin9,1, James S. Pyke5,6, Dedreia Tull9, Malcolm J. McConville5,6, Ross L. Coppell3,4, and Paul K. Crellin3,6

From the 4Australian Research Council Centre of Excellence in Structural and Functional Microbial Genomics, and Victorian Bioinformatics Consortium, Department of Microbiology, Monash University, Clayton, Victoria 3800 and the 6Department of Biochemistry and Molecular Biology, Bio21 Institute of Molecular Sciences and Biotechnology, University of Melbourne, Parkville, Victoria 3010, Australia

The cell walls of the Corynebacterineae, which includes the important human pathogen Mycobacterium tuberculosis, contain two major lipopolysaccharides, lipoarabinomannan (LAM) and lipomannan (LM). LAM is assembled on a subpool of phosphatidylinositol mannosides (PIMs), whereas the identity of the LM lipid anchor is less well characterized. In this study we have identified a new gene (Rv2188c in M. tuberculosis and NCgl2106 in Corynebacterium glutamicum) that encodes a mannosyltransferase involved in the synthesis of the early dimannosylated PIM species, acyl-PIM2, and LM. Disruption of the C. glutamicum NCgl2106 gene resulted in loss of synthesis of AcPIM2 and accumulation of the monomannosylated precursor, AcPIM1. The synthesis of a structurally unrelated manno-glycolipid, Gl-X, was unaffected. The synthesis of AcPIM2 in C. glutamicum ΔNCgl2106 was restored by complementation with M. tuberculosis Rv2188c. In vivo labeling of the mutant with [3H]Man and in vitro labeling of membranes with GDP-[3H]Man confirmed that NCgl2106/Rv2188c catalyzed the second mannose addition in PIM biosynthesis, a function previously ascribed to PimB/Rv0557. The C. glutamicum ΔNCgl2106 mutant lacked mature LAM but unexpectedly still synthesized the major pool of LM. Biochemical analyses of the LM core indicated that this lipopolysaccharide was assembled on Gl-X. These data suggest that NCgl2106/Rv2188c and the previously studied PimB/Rv0557 transfer mannose residues to distinct mannoglycolipids that act as precursors for LAM and LM, respectively.

The Corynebacterineae, a suborder of the Actinomycetales, includes bacterial pathogens of medical and veterinary importance, most notably Mycobacterium tuberculosis, Mycobacterium leprae, Mycobacterium avium subspecies paratuberculosis, and Corynebacterium diphtheriae. The growing rise of drug-resistant strains of the devastating pathogen M. tuberculosis, the causative agent of human tuberculosis (TB),7 represents a global health emergency (1). Because existing anti-TB drugs target enzymes involved in the formation of the Corynebacterineae cell wall, the characterization of novel cell wall enzymes as potential drug targets is an area of intense interest.

The cell walls of all Corynebacterineae comprise a giant macromolecule of covalently linked type 4 peptidoglycan, arabinogalactan, and long chain mycolic acids (2). A diverse range of glycolipids coat the surface of this structure and/or are linked to the underlying plasma membrane. The most abundant and highly conserved of these are the phosphatidylinositol mannosides (PIM) and their hyperglycosylated derivatives lipomannan (LM) and lipoarabinomannan (LAM) (2). In pathogenic mycobacteria, these glycolipids are important virulence factors by acting as ligands for host macrophage receptors, preventing maturation of macrophage phagosomes, inhibiting early apoptosis in infected macrophages, and modulating the adaptive immune response (reviewed in Ref. 3).

Considerable progress has been made in delineating steps and genes involved in PIM, LM, and LAM biosynthesis (reviewed in Ref. 4). Most Corynebacterineae (including Corynebacterium glutamicum and pathogenic mycobacteria) accumulate the PIM species AcPIM2 that is synthesized via the sequential transfer of two mannose residues and one fatty acyl chain to phosphatidylinositol. The first and second mannose additions are thought to be catalyzed by two separate mannosyltransferases, PimA and PimB (5, 6). Both mannosyltransferases lack detectable signal sequences and utilize GDP-Man as the mannose donor, suggestive of localization on the cyto-

© 2008 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
Mannosyltransferase Vital for Lipoarabinomannan Biosynthesis

plasmic side of the plasma membrane. AcPIM2 is subsequently elongated with two α1–6-linked mannose residues to form AcPIM4, a likely branch point intermediate in polar PIM and LM/LAM biosynthesis (7, 8). Polar PIMs and the mannan backbone of LM/LAM are formed by the addition of one to two α1–2-linked mannose residues or long (>20) chains of α1–6-mannose residues to AcPIM4, respectively (9, 10). The mannosyltransferases involved in polar PIM and LM/LAM biosynthesis utilize the lipid-linked donor polyprenylphosphomannose and are complex polytopic membrane proteins, indicating that these reactions occur on the outer leaflet of the plasma membrane (10, 11). LM species formed by mannosylation of AcPIM4 are further modified with mannose, arabinose, or complex arabinan side chains to form LAM (12).

Although the major LM fraction is generally considered to be a precursor of LAM, a recent study has raised the possibility that Corynebacteria may contain two populations of LM with different lipid anchors. Disruption of the C. glutamicum ortholog of PimB had no effect on the synthesis of AcPIM2 and LAM biosynthesis but resulted in loss of synthesis of a novel glycolipid, termed GI–X, and a severe reduction in steady state LM levels (13). GI–X comprised a diacylglycerol lipid modified with a Manα1–4GlcA head group. The coincident loss of GI–X and decrease in LM levels raised the possibility that the major pool of LM in C. glutamicum is assembled on GI–X rather than AcPIM2, although the presence of glucuronic acid in the LM core was not confirmed in this study.

These recent findings suggest that PimB (renamed MgtA) is not required for AcPIM2 biosynthesis. This conclusion is further supported by the absence of a PimB ortholog in the genome of M. leprae (14), a pathogenic mycobacterium that synthesizes mature PIMs and LAM (15). To identify the mannosyltransferase responsible for AcPIM2 biosynthesis, we used a bioinformatic approach to identify essential mycobacterial genes that are conserved in C. glutamicum. C. glutamicum has proved to be a useful model system for studying aspects of cell wall biosynthesis that are common to all members of this group (16–26). Importantly, C. glutamicum mutants lacking cell wall components essential for mycobacterial survival are often viable. Using this approach we identified a new gene in C. glutamicum that is required for the conversion of AcPIM1 to AcPIM2. This enzyme, encoded by the ortholog of the M. tuberculosis gene Rv2188c, has been named PimB'. Remarkably, loss of function of PimB' results in loss of synthesis of LM but not the major pool of LM. Our data provide strong evidence that Corynebacteria contain two pathways for lipopolysaccharide biosynthesis.

MATERIALS AND METHODS

Bioinformatics Analyses—M. tuberculosis strain H37Rv sequences were obtained from the Tuberculist World-Wide Web Server at the Institut Pasteur. C. glutamicum ATCC 13032 Kitasato sequences, in addition to protein alignments, were obtained at The Institute for Genomic Research (TIGR) Comprehensive Microbial Resource web site. Amino acid similarity percentage values were assigned according to the results found in the protein versus all alignment menu located for each gene in the above C. glutamicum ATCC 13032 Kitasato web site. Sequences were compared utilizing ClustalW.

Strains and Culture Conditions—E. coli DH5α was cultured in Luria-Bertani media at 37 °C. All C. glutamicum strains, including the wild-type strain ATCC 13032, were grown in brain heart infusion media (BHI) (Oxoid) at 30 °C. 15 g/liter of agar was used for preparation of solid media, and 10% sucrose (w/v) was added when necessary. C. glutamicum electrocompetent cells were prepared according to a protocol (27) except electroporation was performed at the following settings: 2.5 kV, 200 ohms, and 25 microfarads. Kanamycin (30 μg/ml) and ampicillin (100 μg/ml) were added to media when necessary.

Construction of the C. glutamicum ΔNCgl2106 Mutant and Complementation Plasmids—Gene deletion was performed by amplifying a 2.41-kb fragment containing the entire NCgl2106 gene and flanking sequences. PCR was performed by standard procedures using Proofstart DNA polymerase (Qiagen) and the primers NCgl2106for, incorporating an XbaI site (5′-CCGTCTAGGAACACACGCAAATGACAAA-3′) and NCgl2106rev, incorporating a HindIII site (5′-GCAAGCTTTCCACAAACG-TGAATTGATC-3′). The PCR product was inserted into the Xba1/HindIII sites of pUC18 (28), sequenced, and a 680-bp fragment excised from NCgl2106 by NruI/SphI digestion. The remaining 1.73-kb fragment was then excised and inserted into the Xba1/HindIII sites of pUC18 (28), sequenced, and subcloned into pSM22. Following electroporation, kanamycin-resistant transformants were tested for single homologous recombination events by Southern blot hybridization using a probe prepared from the digoxigenin hybridization kit (Roche Applied Science). A verified single crossover clone was cultured on BHI plates containing 10% sucrose to select for double homologous recombination events. Gene deletion was screened by PCR using the primers NCgl2106for and NCgl2106rev and then confirmed by Southern blot hybridization analysis. One confirmed deletion strain, ΔNCgl2106, was selected for further analysis. Complementation was performed by inserting the NCgl2106 gene into plasmid pSM22 containing the Corynebacterium origin of replication repA and the kanamycin resistance gene aphA3 (30). The entire Rv2188c gene was cloned into pUC18, sequenced, and subcloned into pSM22. The complementation plasmid and empty plasmid control were then electroporated into C. glutamicum ΔNCgl2106 and transformants selected on kanamycin plates.

Extraction of Cell Wall Glycolipids/Lipopolysaccharides—C. glutamicum strains were grown to exponential phase (A560 nm = 7) and cell wall lipids extracted in chloroform:methanol (2:1 v/v) and chloroform:methanol:water (1:2:0.8 v/v) (31). After removal of insoluble material by centrifugation (15,000 × g, 10 min), extracts were dried under nitrogen and subjected to biphasic partitioning in 1-butanol and water (2:1 v/v). The organic phase was dried, and lipids were resuspended in 1-butanol prior to analysis by HPTLC. Glycolipids were analyzed by either one- or two-dimensional HPTLC as indicated in the text using aluminum-backed silica gel sheets (Merck) and stained with orcinol:H2SO4. One-dimensional HPTLCs were developed in chloroform, methanol,13M NH3,1 M ammonium acetate, water (180:140:9:23 v/v), whereas two-dimensional TLCs were developed in the first dimension using chloroform:
Mannosyltransferase Vital for Lipoarabinomannan Biosynthesis

methanol:water (65:25:4 v/v) and chloroform:acetic acid:methanol:water (40:25:3:6 v/v) in the second dimension. Individual glycolipid species were extracted from silica scrapings from one-dimensional HPTLCs using chloroform:methanol:water (10:10:3 v/v), and the supernatant was dried under nitrogen and desalted by 1-butanol:water biphasic partitioning (2:1 v/v). The 1-butanol phase was dried, and purified lipids were resuspended in a minimal volume of 1-butanol.

LM and LAM were extracted from delipidated cell pellets by reflux in 50% ethanol (8). LMs and LAM were further purified by octyl-Sepharose chromatography. Dried extracts were suspended in 0.1 M NH₄OAc containing 5% 1-propanol and loaded onto an octyl-Sepharose column using the same buffer. Following elution of unbound material, LM and LAM were eluted with 30 and 40% 1-propanol. LMs and LAMs were analyzed by SDS-PAGE and periodic acid-Schiff silver staining using reagents from the GelCode SilverSNAP stain kit 2 (Pierce). LAM/LM fractions were exhaustively digested with jack bean α-mannosidase (Sigma) in 0.1 M sodium acetate buffer, pH 5.0, in the presence of 0.1% taurodeoxycholate. The released mannose residues were separated from the residual glycolipid anchor by 1-butanol:water partitioning (2:1 v/v), and the organic phase was dried and resuspended in a minimal volume of 1-butanol. The glycolipids were analyzed by one-dimensional HPLC using chloroform:methanol:water (60:35:8 v/v) and visualized using orcinol:H₂SO₄.

Analytical Procedures—The monosaccharide composition of the purified glycolipids was determined after solvolysis (0.5 M methanolic HCl, 100 °C 16 h) and conversion of the released sugar methyl esters to their trimethylsilyl derivatives with N-(trimethylsilyl) trifluoroacetamide containing 1% methanolic HCl, 100 °C 16 h) and conversion of the released sugar methyl esters to their trimethylsilyl derivatives with N-methyl-N-(trimethylsilyl) trifluoroacetamide containing 1% trichloromethylsilane (Pierce). The trimethylsilyl derivatives were analyzed by GC-MS (32).

LM and LAM fractions were permethylated as described previously (32). For linkage analysis, permethylated LM/LAM were hydrolyzed in 2 M trifluoroacetic acid (100 °C, 2 h), and released permethylated sugars were reduced with NaB₂H₄ and visualized using orcinol:H₂SO₄.

RESULTS

Rv2188c Is a Putative Glycosyltransferase Highly Conserved within Corynebacterineae—As corynebacteria and mycobacteria contain structurally related PIMs, LM and LAM, we undertook a bioinformatics search for genes that were highly conserved in both groups of bacteria. All proteins predicted to be encoded by the C. glutamicum ATCC 13032 Kitasato genome were compared with those encoded by the M. tuberculosis H37Rv genome using the BLAST algorithm (33). Conserved genes of unknown function that are essential in M. tuberculosis, as determined by the transposon mutagenesis studies of Sassetti et al. (34), were considered candidates for cell wall biosynthesis genes of major structural components, because the majority of characterized cell wall genes are refractory to deletion in mycobacteria. Inspection of our list of 83 conserved genes led to our selection of the putative glycosyltransferase Rv2188c for genetic disruption in C. glutamicum.

Rv2188c is a putative CAzy Family 4 glycosyltransferase containing an EXXGXXXXE motif, representing a potential GDP-mannose-binding site, as well as a conserved lysine residue characteristic of many members of this family (Fig. 1). The M. tuberculosis ortholog is predicted to consist of 385 amino acid residues and be 41 kDa in size. In addition, Rv2188c shares some sequence homology, and several potential motifs, with the mannosyltransferases PimA and MgtA. The orthologs of Rv2188c in M. leprae, M. avium subspecies paratuberculosis, C. glutamicum, and C. diphtheriae display 81, 81, 65, and 64% amino acid similarity, respectively, to the M. tuberculosis protein (Fig. 1).

Inactivation of the C. glutamicum NCgl2106 Gene—Because Rv2188c is an essential gene in M. tuberculosis (34), we targeted its ortholog in C. glutamicum, NCgl2106, for disruption using a two-step recombinant strategy. The suicide plasmid pK18mobbacBΔNCgl2106, containing the NCgl2106 sequence and flanking regions but devoid of 680 bp of the 1145-bp gene,
was constructed. Following electroporation into *C. glutamicum*, kanamycin-resistant transformants were selected and examined for the genomic integration of the suicide plasmid via a homologous recombination event using Southern blot hybridizations (data not shown). One of these single crossover recombinants was chosen to generate a knock-out strain by selecting for a second recombination event on sucrose, which is toxic to bacteria carrying the *sacB* gene. After 3 days of growth on BHI:sucrose plates, hundreds of colonies were obtained. A large number were examined by PCR using the NCgl2106for and NCgl2106rev primers, and all were found to be wild-type revertants. After growth for a further 5 days, a second population of colonies was evident, indicating a growth defect. These small colonies were examined by PCR and displayed the expected knock-out profile (data not shown). Deletion of NCgl2106 in these strains was confirmed by Southern blot hybridization following digestion of genomic DNA with Clal. Bands of 1.9 and 6.0 kb in the wild-type sample hybridized to the probe, whereas a single band of 7.2 kb was observed for the potential knock-outs, because a Clal site was present in the excised region of NCgl2106 (Fig. 2, A and B). These results confirmed inactivation of NCgl2106. Complementation of the mutant by electroporation with the plasmids pSM22:NCgl2106 and pSM22:Rv2188c, but not with the empty pSM22 vector, restored the colony size to wild-type dimensions (data not shown). This slow growth phenotype was maintained in liquid BHI media and was also restored to wild-type levels by both complementation plasmids (Fig. 2C).

Characterization of PIMs in *C. glutamicum ΔNCgl2106 and Complementation Strains*—Two-dimensional HPTLC analysis of the lipid fractions of wild-type *C. glutamicum* and the
NCgl2106 mutant strain revealed a marked change in the steady state levels of two glycolipid species (Fig. 3), provisionally identified as AcPIM1 and AcPIM2, based on their co-migration with authentic standards. Cellular levels of the putative AcPIM1 were markedly elevated in the ΔNCgl2106 mutant, whereas the predominant AcPIM2 was missing. A third glycolipid species, with a slightly faster HPTLC mobility than AcPIM2, was expressed at similar levels in both wild-type and mutant strain. The HPTLC mobility of this glycolipid was consistent with it being the recently characterized Gl-X glycolipid. The synthesis of the putative Gl-X glycolipid was consistent with it being the recently characterized Gl-X glycolipid. The synthesis of the putative AcPIM1 bands from both wild type and ΔNCgl2106 mutant revealed only mannose and glucuronic acid (molar ratio 1:1) and lacked myo-inositol in a 1:1 molar ratio (data not shown). Negative ion MALDI-TOF analysis of these fractions gave pseudomolecular ions at m/z 1239 consistent with the presence of an AcPIM1 species with C16:0 and C18:1 fatty acids (Fig. 4B). Comparable analysis of the glycolipid doublet (comprising putative AcPIM2 and Gl-X) from wild-type cells revealed the presence of mannose, glucuronic acid, and myo-inositol in the molar ratio 1:0.5:1. MALDI-TOF analysis in positive mode clearly showed that this fraction comprised two distinct lipid species. The pseudomolecular ions at m/z 955 (M-H + Na) and 977 (M-H + 2Na) indicate the presence of GI-X with the structure Manα1–4GlcAα1–3diacylglycerol (13). This fraction also contained AcPIM2 based on the presence of characteristic and prominent ions at m/z 1422 (M-H + Na) and m/z 1444 (M-H + 2Na) (Fig. 4C). The major lower band in this doublet was missing in the ΔNCgl2106 mutant. GC-MS compositional analysis of the remaining upper band indicated that it comprised only mannose and glucuronic acid (molar ratio 1:1) and lacked myo-inositol-containing glycolipids. This was confirmed by positive ion MALDI-TOF analysis which revealed the presence of Gl-X (m/z 955 and m/z 977), and the complete absence of ions corresponding to AcPIM2 (m/z 1422 and m/z 1444) (Fig. 4D). Collectively, these analyses suggest that deletion of NCgl2106 in C. glutamicum prevents the synthesis of AcPIM2 and results in the accumulation of AcPIM1, but it has no effect on the synthesis of Gl-X.
In Vivo and in Vitro Enzyme Assays Reveal a Specific Defect in \textit{AcPIM1} to \textit{AcPIM2} Conversions in \textit{C. glutamicum} \textit{ΔNGc2106}—To further confirm that the synthesis of \textit{AcPIM2} is disrupted in the \textit{ΔNGc2106} mutant, wild-type and mutant cells were pulse-labeled with [3H]Man and labeled glycolipids extracted from cells after a short chase and analyzed by HPTLC. As shown in Fig. 5A, several bands were labeled during the pulse, including species that co-migrated with \textit{AcPIM1}, \textit{AcPIM2}, and Gl-X (an additional band with a faster HPTLC mobility than \textit{AcPIM1} corresponds to the polyprenylphosphate mannose donor). During the chase, label in \textit{AcPIM1} was lost, whereas label in \textit{AcPIM2} increased (Fig. 5A). In contrast, labeled \textit{AcPIM2} was not detected in the \textit{ΔNGc2106} mutant, and labeled \textit{AcPIM1} accumulated during the chase. Lower but increased labeling of the Gl-X band was also observed in the \textit{ΔNGc2106} mutant (Fig. 5A). A similar accumulation of label in \textit{AcPIM1} and Gl-X was observed in the \textit{ΔNGc2106} mutant complemented with the empty vector, whereas synthesis of \textit{AcPIM2} and depletion of label in \textit{AcPIM1} was observed in \textit{ΔNGc2106} mutant complemented with \textit{NCgl2106} and \textit{Rv2188c} genes (Fig. 5A).

To further confirm that the \textit{ΔNGc2106} mutant lacks the mannosyltransferase activity responsible for the synthesis of \textit{AcPIM2}, membrane fractions from wild-type and \textit{ΔNGc2106} were incubated with GDP-[3H]Man, the likely sugar nucleotide donor for this enzyme (7, 35). As shown in Fig. 5B, the synthesis of \textit{AcPIM1}, \textit{AcPIM2}, and Gl-X is reconstituted in this cell-free system when membranes from wild-type \textit{C. glutamicum} are used. In contrast, membranes from the \textit{ΔNGc2106} mutant only synthesize \textit{AcPIM1} and Gl-X and fail to synthesize detectable levels of \textit{AcPIM2}. These data strongly suggest that \textit{NCgl2106} catalyzes the transfer of mannose from GDP-Man to \textit{AcPIM1} to form \textit{AcPIM2}. Loss of this enzyme does not prevent the synthesis of Gl-X suggesting that it does not act on the Gl-X precursor Gl-A (GlcA-diacylglycerol). Finally, the absence of detectable synthesis of \textit{AcPIM2} in \textit{ΔNGc2106} indicates that the previously assigned PimB/MgtA (Rv0557) is not involved in \textit{AcPIM2} synthesis.

Characterization of Lipoglycans from \textit{C. glutamicum} \textit{ΔNGc2106} and Complementation Strains—\textit{AcPIM2} is thought to form the lipid anchor for LAM and LM species that act as precursors for LAM. Disruption of \textit{AcPIM2} biosynthesis...
should therefore result in loss of LAM and LM synthesis. To investigate the downstream consequences of loss of AcPIM2 synthesis, the major lipopolysaccharides of wild-type and NCgl2106 mutant cells were purified and analyzed by SDS-PAGE. As shown in Fig. 6, wild-type C. glutamicum expressed high levels of LAM as well as significant but lower levels of LM. In contrast, the NCgl2106 mutant lacked species that co-migrated with LAM but expressed higher levels of the faster migrating LM (Fig. 6A). Expression of “LAM” was restored following complementation of ΔNCgl2106 mutant with functional NCgl2106 or Rv2188c (Fig. 6A).

To confirm that the major lipopolysaccharide in the ΔNCgl2106 mutant was indeed LM and not a truncated LAM, the combined LM/LAM fractions from wild-type and mutant cells were subjected to methylation linkage analysis. As shown in Fig. 6C, the LM fraction from the ΔNCgl2106 mutant lacked the terminal arabinose residues characteristic of mature LAM in this species (Fig. 6B) (12). To investigate whether the LM fraction contained an inositol phospholipid (i.e. AcPIM2) or Gl-X lipid anchor, the LM fraction was purified by octyl-Sepharose chromatography, exhaustively extracted with 1-butanol to remove any contaminating inositol phospholipids, and then digested with jack bean α-mannosidase. This treatment converted the long chain LM to a series of smaller mannoglycolipids that were recovered by 1-butanol:water partitioning. GC-MS analysis of these species from wild-type cells revealed the presence of mannose, glucuronic acid, and myo-inositol in a molar ratio of 4:1:0.03, suggesting that in these cells PI-based LM constitutes ~3% of the total LM pool. In comparison, GC-MS analysis of the ΔNCgl2106 mutant showed that although mannose and glucuronic acid were still present (molar ratio 4:1), there was no detectable myo-inositol, confirming that these mutant cells are devoid of PI-based LM. Together these results show that deletion of ΔNCgl2106 renders the cells unable to synthesize PI-based LM and LAM. Also, these data strongly suggest that the major C. glutamicum LM is assembled on a distinct lipid anchor from LAM and that this anchor is the same or structurally related to Gl-X.

DISCUSSION

In this study we show that NCgl2106/Rv2188c encodes the mannosyltransferase that catalyzes the conversion of AcPIM1 to AcPIM2 and that this step is essential for the synthesis of the major lipopolysaccharide, LAM. Remarkably, disruption of this gene did not prevent synthesis of the dominant pool of LM, providing strong evidence that a major fraction of LM is


Assembled on a distinct glycolipid anchor from LAM in C. glutamicum. These findings indicate unanticipated complexity in the pathways of assembly of these major cell wall lipopolysaccharides.

AcPIM2 is ubiquitously expressed in all Corynebacterineae. It appears to be both a metabolic end product, accumulating to high steady state levels, as well as being a precursor for more polar PIMs and the complex lipopolysaccharides, LM and LAM. The addition of the second mannose residue to AcPIM1 to form AcPIM2 was initially ascribed to the M. tuberculosis gene Rv0557, which was found to confer resistance to mannosamine, an inhibitor of PIM biosynthesis (6). Overexpression of Rv0557 in M. smegmatis resulted in a small (<2-fold) increase in AcPIM2 biosynthesis, whereas a recombinant enzyme was reported to display some capacity to convert AcPIM1 to AcPIM2 (6). However, more recent studies indicated that Rv0557 was unlikely to catalyze this conversion of AcPIM1 to form AcPIM2 (14, 15). Second, Tatituri et al. (13) recently provided strong evidence that the C. glutamicum ortholog of Rv0557 (Mt-PimB), NCgl0452 (Cg-PimB), catalyzed the addition of mannose to the novel glycolipid, Gl-A (GlcA-diacylglycerol). Significantly, disruption of Cg-PimB had no effect on AcPIM2 or LAM levels but resulted in loss of synthesis of a more polar glycolipid, Gl-X, and a concomitant decrease in LM levels. The latter studies suggested that Cg-PimB was not required for AcPIM2 biosynthesis and that the Gl-A/Gl-X glycolipids may be required for synthesis of the major pool of LM. As such, the authors renamed this enzyme α-mannosylglucopyranosyluronic acid-transferase A (MgtA). In this study we provide compelling evidence that NCgl2106/Rv2188c encodes the mannosyltransferase involved in AcPIM2 synthesis, and we have assigned it the name PimB'. Targeted disruption of Cg-PimB' resulted in complete loss of AcPIM2 and accumulation of the AcPIM1 precursor. 

In vivo metabolic labeling confirmed that the absence of AcPIM2 in the ΔCg-PimB mutant was because of a defect in AcPIM2 biosynthesis rather than increased turnover. The synthesis of AcPIM1, AcPIM2, and Gl-X was reconstituted in vitro using crude membrane preparations charged with GDP-[3H]mannose. As previous studies have shown that the synthesis of AcPIM2 is not dependent on polyprenylphosphomannose, it is likely that the mannosyltransferase involved utilizes the supplied GDP-[3H]mannose directly. Although synthesis of AcPIM2 was clearly detected in wild-type membranes, no activity was detected in the membranes prepared from the mutant. The absence of AcPIM2 biosynthesis in both the in vivo and in vitro labeling experiments suggested that MgtA is unable to compensate for loss of Cg-PimB' in vivo. Finally, an ortholog for Cg-PimB' is present in all sequenced species of Corynebacteriae that synthesize PIMs, including M. leprae (designated ML0886) (14).

It has generally been assumed that LM is a precursor form of LAM. Although LM synthesis was essentially completely inhibited in the ΔNCgl2106 mutant, levels of LM increased compared with wild-type bacteria. Further analysis of the LM glycolipid core in the ΔNCgl2106 mutant indicated that it comprised the same sugars as Gl-X, mannose, and glucuronic acid, leading us to refer to this dominant LM pool as GlcA-LM. These data suggest that the major pools of GlcA-LM and LAM are assembled on Gl-X and AcPIM2, respectively (Fig. 7). In this new model, GlcA-LM and LAM are the end products of two different biosynthetic pathways. In support of this model, Tatituri et al. (13) showed that disruption of MgtA (required for synthesis of Gl-X) resulted in greatly reduced levels of LM. The residual LM in this mutant may correspond to true LM precursors of LAM that contain a PIM anchor (which we term PI-LM). As genes encoding MgtA and PimB' are present in both the Corynebacteria and Mycobacteria, it is possible that both pathways of lipopolysaccharide biosynthesis exist in all the Corynebacterineae, except M. leprae. Whether the relative abundance of PI-LM or GlcA-LM varies between different species or different growth stages remains to be determined. Similarly, nothing is known about the function of GlcA-LM.

The Cg- and Mt-PimB' proteins share low sequence homology with PimA and MgtA, lack detectable signal sequences, and utilize GDP-Man rather than polyprenylphosphomannose as sugar donors. These characteristics suggest that the conversion of AcPIM1 to AcPIM2 occurs on the inner leaflet of the plasma membrane. A similar subcellular localization and topology have been suggested for PimA and the downstream enzyme PimC (an apparently redundan-
Mannosyltransferase Vital for Lipoarabinomannan Biosynthesis

A dant enzyme that may catalyze the conversion of AcPIM2 to AcPIM3 in some strains (7, 38). All of the early steps in PIM (and possibly Gl-X) biosynthesis are thus localized inside the cell. In contrast, the conversion of AcPIM4 to polar PIM and LAM appears to be mediated by mannosyltransferases that utilize polypropenylphosphate mannoside (10, 11). These reactions are predicted to occur in the outer leaflet of the plasma membrane. It will be intriguing to investigate whether the presence of different anchors on PI-LM and GlcA-LM are responsible for defining the access of the growing mannan chains to enzymes involved in arabinosylation and chain capping.

The proximity of Rv2188c to Rv2181, recently identified as encoding an LM mannosyltransferase (39), might suggest the presence of a LAM biosynthetic cluster. Another essential M. tuberculosis gene Rv2174, encoding a mannosyltransferase responsible for LM elongation (10), and Rv2182c, a nonessential M. tuberculosis gene encoding a putative acyltransferase, may also form part of the cluster. As other unidentified glycosyltransferases are thought to catalyze later steps in LAM biosynthesis, all should be amenable to disruption in C. glutamicum. Combined with a recent bioinformatics breakthrough identifying the polypropenyl mannosyltransferase motif (11), the tools to characterize the complete biosynthesis of the mannan portion of LAM are now available.

As Rv2188c/Mt-PimB is an essential enzyme in M. tuberculosis, it represents a potential drug target. Current studies are focused on protein expression, crystallization, and structural determination with the ultimate aim of rational inhibitor design. The recent emergence of drug-resistant strains of M. tuberculosis, particularly multiply (MDR-TB) and extremely (XDR-TB) drug-resistant forms, highlights the importance of such targets in the treatment of this devastating disease.

Acknowledgments—We thank Andreas Schafer for pK18mobsacB, Margaret Britz for C. glutamicum ATCC 13032 and Tim Stinear and Sandra McKean for pSM22.

REFERENCES

1. Raviglione, M. C. (2003) Tuberculosis (Edinb.) 83, 4–14
2. Brennan, P. J. (2003) Tuberculosis (Edinb.) 83, 91–97
3. Koul, A., Herget, T., Klebl, B., and Ullrich, A. (2004) Nat. Rev. Microbiol. 2, 189–202
4. Berg, S., Kaur, D., Jackson, M., and Brennan, P. J. (2007) Glycobiology 17, 35–56
5. Kordulakova, J., Gilleron, M., Mikusova, K., Puzo, G., Brennan, P. J., Georgiou, B., and Jackson, M. (2002) J. Biol. Chem. 277, 31335–31344
6. Schaeffler, M. L., Khou, K. H., Besra, G. S., Chatterjee, D., Brennan, P. J., Belisle, J. T., and Inamine, J. M. (1999) J. Biol. Chem. 274, 31625–31631
7. Morita, Y. S., Patterson, J. H., Billman-Jacobe, H., and McConville, M. J. (2004) Biochem. J. 378, 589–597
8. Kovacevic, S., Anderson, D., Morita, Y. S., Patterson, J., Haines, R., McMillan, B. N., Copple, R., McConville, M. J., and Billman-Jacobe, H. (2006) J. Biol. Chem. 281, 9011–9017
9. Khou, K. H., Dell, A., Morris, H. R., Brennan, P. J., and Chatterjee, D. (1995) Glycobiology 5, 117–127
10. Kaur, D., McNeil, M. R., Khou, K. H., Chatterjee, D., Crichton, D. C., Jackson, M., and Brennan, P. J. (2007) J. Biol. Chem. 282, 27133–27140
11. Morita, Y. S., Sena, C. B., Waller, R. F., Kurokawa, K., Sernee, M. F., Nakatani, F., Haines, R. E., Billman-Jacobe, H., McConville, M. J., Maeda, Y., and Kinoshita, T. (2006) J. Biol. Chem. 281, 25143–25155
12. Tatituri, R. V., Alderwick, L. J., Mishra, A. K., Nigou, J., Gilleron, M., Krumhach, K., Hitchen, P., Giordano, A., Morris, H. R., Dell, A., Eggeling, L., and Besra, G. S. (2007) Microbiology 153, 2621–2629
13. Tatituri, R. V., Illarionov, P. A., Dover, L. G., Nigou, J., Gilleron, M., Krumhach, K., Morris, H. R., Spencer, N., Dell, A., Eggeling, L., and Besra, G. S. (2007) J. Biol. Chem. 282, 4561–4572
14. Cole, S. T., Eiglmeyer, K., Parkhill, J., James, D. K., Thomson, N. R., Wheeler, P. R., Honore, N., Garnier, T., Churcher, C., Harris, D., Mungall, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R. M., Devlin, K., Duthoy, S., Feltwell, T., Fraser, A., Hamlin, M., Holroyd, S., Hornsby, T., Jagels, K., Lacroix, C., Maclean, J., Moule, S., Murphy, L., Oliver, K., Quail, M. A., Rajandream, M. A., Rutherford, K. M., Rutter, S., Seeger, K., Simon, S., Simmonds, M., Skelton, J., Squares, R., Squares, S., Stevens, K., Taylor, K., Whitehead, S., Woodward, J. R., and Barrell, B. G. (2001) Nature 409, 1007–1011
15. 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
16. Gibson, K. J., Eggeling, L., Maughan, W. N., Krumhach, K., Gurza, S., Nigou, J., Puzo, G., Sahm, H., and Besra, G. S. (2003) J. Biol. Chem. 278, 40842–40850
17. Alderwick, L. J., Radmacher, E., Seidel, M., Gande, R., Hitchen, P. G., Morris, H. R., Dell, A., Sahm, H., Eggeling, L., and Besra, G. S. (2005) J. Biol. Chem. 280, 32362–32371
18. Alderwick, L. J., Seidel, M., Sahm, H., Besra, G. S., and Eggeling, L. (2006) J. Biol. Chem. 281, 15653–15661
19. Puech, V., Bayan, N., Salim, K., Lebbon, G., and Daffe, M. (2000) Mol. Microbiol. 35, 1026–1041
20. Tropis, M., Meniche, X., Wolf, A., Gehhardt, H., Strelkov, S., Chami, M., Schomburg, D., Kramer, R., Morbach, S., and Daffe, M. (2005) J. Biol. Chem. 280, 26573–26585
21. Kacem, R., De Sousa-D’Auria, C., Tropis, M., Chami, M., Goumon, P., Lebon, G., Houssin, C., and Daffe, M. (2004) Microbiology 150, 73–84
22. Portevin, D., de Sousa-D’Auria, C., Montoziero, H., Houssin, C., Stella, A., Lanelle, M. A., Bardou, F., Guilhot, C., and Daffe, M. (2005) J. Biol. Chem. 280, 8862–8874
23. Portevin, D., de Sousa-D’Auria, C., Houssin, C., Grimaldi, C., Chami, M., Daffe, M., and Guilhot, C. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 314–319
24. Gande, R., Gibson, K. J., Brown, A. K., Krumhach, K., Dover, L. G., Sahm, H., Shioyama, S., Oikawa, T., Besra, G. S., and Eggeling, L. (2004) J. Biol. Chem. 279, 44847–44857
25. Lea-Smith, D. J., Pyke, J. S., Tull, D., McConville, M. J., Copple, R. L., and Creltin, P. K. (2007) J. Biol. Chem. 282, 11000–11008
26. Kalinowski, J., Barbe, B., Berthaud, M., Jaffre, B., Biscaretti, B., Mentré, C., Danchin, A., Wieland, M., Marchesan, M., Rapin, M., and Besra, G. S. (2003) J. Biol. Chem. 278, 1352–1363
27. Schafer, A., Tauch, A., Berthou, M., Arroyo, J., Brennen, P. J., and Besra, G. S. (1999) J. Mol. Microbiol. Biotechnol. 5, 103–119
28. Schafer, A., Tauch, A., Willems, A., Besra, G. S., and Eggeling, L. (1999) J. Mol. Microbiol. Biotechnol. 5, 11000–11008
29. Schafer, A., Tauch, A., Willems, A., Besra, G. S., and Eggeling, L. (1999) J. Mol. Microbiol. Biotechnol. 5, 11000–11008
30. Schafer, A., Tauch, A., Willems, A., Besra, G. S., and Eggeling, L. (1999) J. Mol. Microbiol. Biotechnol. 5, 11000–11008
Mannosyltransferase Vital for Lipoarabinomannan Biosynthesis

36. Kremer, L., Gurcha, S. S., Bifani, P., Hitchen, P. G., Baulard, A., Morris, H. R., Dell, A., Brennan, P. J., and Besra, G. S. (2002) *Biochem. J.* **363**, 437–447

37. Gurcha, S. S., Baulard, A. R., Kremer, L., Locht, C., Moody, D. B., Muhslecker, W., Costello, C. E., Crick, D. C., Brennan, P. J., and Besra, G. S. (2002) *Biochem. J.* **365**, 441–450

38. Morita, Y. S., Velasquez, R., Taig, E., Waller, R. F., Patterson, J. H., Tull, D., Williams, S. I., Billman-Jacobe, H., and McConville, M. J. (2005) *J. Biol. Chem.* **280**, 21645–21652

39. 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