The biosynthesis of lipoarabinomannan (LAM), a key mycobacterial lipoglycan that has been implicated in numerous immunoregulatory functions, was examined utilizing D-mannosamine (ManN) as a tool to identify mannosyltransferase genes involved in LAM synthesis. Cell-free reactions utilizing cellular membranes of mycobacteria as the enzyme source indicated that ManN inhibited the synthesis of phosphatidylinositol mannosides, early precursors to LAM. A selection strategy was devised to screen a Mycobacterium tuberculosis genomic library in Mycobacterium smegmatis for clones conferring conditional resistance to ManN, with the rationale that overexpression of the gene(s) encoding a target of ManN would impart a ManN-resistant phenotype under these conditions. This strategy led to the identification of pimB, whose deduced amino acid sequence shows similarity to mannosyltransferases and other glycosyltransferases. Partially purified recombinant PimB protein from Escherichia coli or membranes from M. smegmatis overexpressing the pimB gene were used in cell-free assays to show that PimB catalyzes the formation of triacylphosphatidylinositol dimannoside from GDP-mannose and triacylphosphatidylinositol monomannoside.

The emergence of multidrug-resistant strains of Mycobacterium tuberculosis and the increased incidence of tuberculosis, especially in developing countries, have made it clear that there is a need for new chemotherapeutic agents (1). In this regard, knowledge of the genetics and biochemistry of key biochemical pathways in mycobacteria will provide a basis for the rational design of new drugs (2). The mycobacterial cell wall (reviewed in Ref. 3) is the site of action of many of the first-line antimycobacterial agents (4), and it contains numerous immunoregulatory functions, was examined utilizing D-mannosamine (ManN) as a tool to identify mannosyltransferase genes involved in LAM synthesis. Cell-free reactions utilizing cellular membranes of mycobacteria as the enzyme source indicated that ManN inhibited the synthesis of phosphatidylinositol mannosides, early precursors to LAM. A selection strategy was devised to screen a Mycobacterium tuberculosis genomic library in Mycobacterium smegmatis for clones conferring conditional resistance to ManN, with the rationale that overexpression of the gene(s) encoding a target of ManN would impart a ManN-resistant phenotype under these conditions. This strategy led to the identification of pimB, whose deduced amino acid sequence shows similarity to mannosyltransferases and other glycosyltransferases. Partially purified recombinant PimB protein from Escherichia coli or membranes from M. smegmatis overexpressing the pimB gene were used in cell-free assays to show that PimB catalyzes the formation of triacylphosphatidylinositol dimannoside from GDP-mannose and triacylphosphatidylinositol monomannoside.

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Although little is known about the biosynthesis of LAM, structural similarities have suggested that the early precursors are phosphatidyl-myoinositol (PI) and certain phosphatidylinositol mannosides (PIMs) (12–14), and this hypothesis is now supported by recent direct biosynthetic evidence (15, 16). The proposed sequence of PI → PIM → lipomannan (LM) → LAM (15) builds on work that started over 30 years ago with the PI/S (17–19) and that led to the identification of two distinct mannosyltransferase activities (20) that transfer Man from GDP-Man to the 2-position of the myo-inositol ring of PI to form phosphatidylinositol monomannoside (PIM3) and then to the 6-position to form phosphatidylinositol dimannoside (PIM2) (14, 15). From here, it has been proposed that PIM2 is further glycosylated with Man to form LM, which is characterized by a linear α(1→6)-linked mannan backbone punctuated by α(1→2)-linked mannoxyranose side chains, and LM is further glycosylated with arabinan to form LAM (13).

The PIM intermediates between PIM2 and larger LM-like molecules are most likely PIM3–PIM6 on the basis of their structures (14, 15), and these PIMs are a subset of the so-called higher PIMs (PIM4–PIM6) that all lack the branched configuration of LM (i.e. the α(1→2)-linked Man side chains) (14). PIM6 appears to be a terminal product because it contains linear α(1→2)-linked Man, which is not found in LM or LAM (14). Besra et al. (15) have used a cell-free assay to show that PIM2 (or PIM4) may be extended by the addition of Man residues from an alkali-stable polypropen-based mannoolipid (polypropenolipomannan (PPI)) (15) to form “linear LM” containing only α(1→6)-linked Man. These authors also suggest that this linear
LM is then further mannosylated to form mature, branched LM (15). If this is the case, then PIM₂ would also have to be a terminal product because it contains α(1→2)-linked Man (14).

Further evidence that PIM₂ is the biosynthetic precursor to LAM comes from the location of the acyl functions elaborating these molecules. The PIMs can vary in the extent of acylation, from the lyso form with only C16:0 palmitate on the 1-position of the glycerol to the multiply acylated forms. The latter are composed of the predominant diacyl form containing C₁₆:₀ palmitate and C₁₉:₀ tuberculostearate fatty acids esterified to the 1- and 2-positions of the glycerol, respectively, as well as the triacyl and tetraacyl forms that contain additional palmitate moieties on specific Man residues (14). Several studies (20, 21) indicate that the triacyl form of PIM₂ serves as the substrate for the subsequent mannosylation step leading to triacyl-PIM₃. and Kho et al. (14) have confirmed that it is the triacyl form of PIM₂ that is the predominant structural feature found within LM and LAM.

The above structural and biosynthetic studies have established that PIM₂ is an intermediate in the LAM biosynthetic pathway and provided the tools needed to initiate our analysis of the genetics of LAM biosynthesis. In this report, we describe the biochemical and genetic characterization of the pimB gene from M. tuberculosis. Evidence is presented that PimB is the α-D-mannose-(1→6)-phosphatidyl-myo-inositol-monomannoside transferase responsible for the formation of triacyl-PIM₂ from GDP-Man and triacyl-PIM₁.

**Experimental Procedures**

Bacterial Strains and Growth Conditions—Mycobacterium smegmatis strain mc²155 (22) was propagated in Middlebrook 7H11 broth or agar medium (Difco). Escherichia coli strains XL1-Blue and XL2-Blue (both from Stratagene) and TB1 (New England Biolabs, Inc.) were grown in LB broth or agar medium (Life Technologies, Inc.) that was supplemented with 20 mg glucose for strain TB1. All cultures were incubated at 37 °C except when noted, and all broth cultures were aerated by shaking. Media for the propagation of recombinant E. coli or M. smegmatis strains contained 25 µg/ml kanamycin, 12.5 µg/ml tetracycline, or 100 µg/ml ampicillin (all antibiotics were from Sigma). M. smegmatis cells utilized in growth curve, whole-cell labeling, or cell-free experiments were grown in glycerol alanine salts medium (23). Unless otherwise noted, 2- mannosamine (ManN) (ICN) was used at a concentration of 5 mg/ml, a concentration that was empirically determined to completely inhibit the growth of freshly diluted (10⁶ cfu) 0.010–0.030) cells grown overnight in 7H11 broth. When necessary, cultures were centrifuged every 18–24 h, and fresh MAN and glycerol alanine salts medium were added because ManN has been shown to be relatively unstable due to nonenzymatic modifications of the amino sugar in vitro (24). For growth assays with glucose (GlcN) and ManN, the empirically determined concentration of 0.2 mg/ml GlcN was used in addition to 5 mg/ml ManN. All assays were done in triplicate and repeated at least three times.

Cell-free Assay for Mannolipid Synthesis Using GDP-[¹⁴C]Man—The incorporation of GDP-[¹⁴C]Man into membrane lipids was assayed according to Besra et al. (15) using membranes isolated as described previously (25). Briefly, 15–20 g of cells were broken in a French pressure cell and centrifuged at 27,000 × g, and the supernatant was subjected to ultracentrifugation at 100,000 g for 15 min. The pellet membrane fraction was suspended in Buffer A (50 mM MOPS and 10 mM MgCl₂, pH 7.9); and the protein concentration, determined by the method of Bradford (28), was adjusted to 20 mg/ml. Prior to the addition of 0.25 µCi of GDP-[¹⁴C]Man (321.4 µCi/mmol; NEN Life Science Products), membranes (2 mg of protein) were preincubated for 10 min with varying concentrations of ManN and, in some cases, amphotericin (100 µg/ml). Reactions were stopped by the consecutive additions of H₂O, CH₃OH, and CHCl₃, to give a final ratio of 1:2:4 and extracted for 15 min. The lipids were Folch-washed five times with CHCl₃/CH₃OH/H₂O (4:2:1) (27) and dried before reconstituting in CHCl₃/CH₃OH (2:1) for analysis by TLC. The total cpm of radioactivity incorporated into the extracted lipids was measured by scintillation counting of 10% of the labeled material. Another 10% of the labeled material was subjected to TLC analysis in CHCl₃/ CH₃OH/NH₄OH/H₂O (65:25:0.5:3.6) (15) on aluminum-backed plates of Silica Gel 60 F₂₅₄ (E. Merck, Darmstadt, Germany). Autoradiograms were obtained by exposing thin-layer chromatomographs to X-ray film at -70 °C for 2–5 days. Reactions were run on the TLC plate were scraped off, dissolved in CHCl₃/CH₃OH, and analyzed by scintillation counting. All assays were done in triplicate and repeated at least three times.

Cell-free Assay for Mannolipid Synthesis Using [¹⁴C]PIM₁—Triacyl-[¹⁴C]PIM₁ was purified from a scaled-up cell-free reaction by TLC as described previously (25). The dry protein fraction was suspended by sonication in Buffer A containing 100 mM CHAPS. Reaction mixtures contained 1000 epm [¹⁴C]PIM₁, 62.5 µM ATP, 10 µg GDP-Man, and either nonbacterial membranes (2 mg) or partially purified recombinant protein (0.5 mg). The reactions were incubated at 37 °C for 30 min, extracted with CHCl₃/CH₃OH/H₂O (4:2:1), and analyzed by TLC autoradiography as described above. All assays were done in triplicate and repeated at least three times.

Identification of Mannolipids—The initial characterization of the mannolipids affected by ManN was carried out by subjecting the mannosides to mild acid (in 0.5 N HCl) or mild alkali (in 0.1 N NaOH) hydrolysis as described previously (15). The presence of Man was demonstrated by hydrolyzing the [¹⁴C]Man-labeled lipids with 2.0 N trifluoroacetic acid, followed by TLC analysis of the Folch wash in comparison with sugar standards (28). For structural analyses, nonradioactive mannolipids were synthesized in vitro as described above using unlabeled GDP-Man and isolated by preparative TLC using the radiolaabeled mannolipids as markers. Following autoradiography, the relevant regions of the TLC plate were scraped off and extracted with CHCl₃/CH₃OH (2:1), and the organic phase of the Folch wash (CHCl₃/CH₃OH/H₂O (4:2:1)) was collected and dried under nitrogen.

Mannolipids were analyzed by fast atom bombardment mass spectrometry (FAB-MS) (14) either directly in negative ion mode or as perdeuterocoxyl acetate derivatives (100 µl of pyridine-d₆/acetic anhydride (1:1, v/v) for 2 h) in positive ion mode. Samples were redissolved in CH₃OH for loading onto the probe tip coated with triethanolamine or m-nitrobenzyl alcohol as matrix for negative and positive ion modes, respectively. FAB mass spectra were acquired on an Autospec orthogonal acceleration-time-of-flight mass spectrometer (Micromass, Manchester, United Kingdom) fitted with a cesium ion gun operating at 26 kV. Collision-induced desorption (CID) MS-MS was performed by introducing argon gas to the collision cell to a reading of ~1.2 × 10⁻⁶ mbar during the time of flight analysis. The source accelerating voltage was at 8 kV, and the laboratory frame collision energy was maintained at 800 eV, with a push-out frequency of 56 kHz for orthogonal sampling. A 1-s integration time per spectrum was chosen for the time of flight analyzer with a 0.1-s interscan delay. Individual spectra were summed for data processing.

Whole-cell Radiolabeling Experiments—The effect of ManN on PIM/LM/LAM synthesis in M. smegmatis was determined by the whole-cell radiolabeling procedure described by Mikušová et al. (28). Briefly, cultures (100 ml) were grown to mid-log phase (A₆₀₀nm = 0.20–0.40) prior to the addition of 1 µCi/ml of [¹⁴C]glucose (296 µCi/mmol; NEN Life Science Products) in the presence or absence of 5 mg/ml ManN and then incubated for 8 h. In certain experiments, the radiolabel was [¹⁴C]ManN (55 µCi/mmol; ICN). Cells were then harvested and washed, and the cell pellets were delipidated with CHCl₃/CH₃OH (2:1) to provide the extractable lipids containing the PIMs (29) that were analyzed by TLC autoradiography as described above. The delipidated cell pellet was then subjected to 50% aqueous ethanol reflux, followed by partitioning between hot phenol and water (12). The aqueous phase, containing the LM/LAM population, was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) on 13.5% gels (28). Gels were either stained with periodic acid and silver nitrate or blotted onto nitrocellulose and autoradiographed.

Selection of ManN-resistant Clones—A genomic library of M. tuberculosis strain H37Rv was screened for cosmid clones conferring ManN resistance in the presence of exogenous GlcN (defined as the ability of the bacteria to grow, as determined by growth curve experiments, in the presence of 1 mg/ml of ManN and 0.5 mg/ml of GlcN). The library (generated by Dr. Aimee E. Belanger) was constructed by cloning 35–40-kilobase cosmids into E. coli and sequenced. A representative plasmid (pYUB18) was then used to transform M. tuberculosis strain H37Rv to ManN resistance, and the library was screened for cosmid clones conferring ManN resistance. Following electroporation of cosmids pYUB18 (30, 31). Following electroporation of M. smegmatis (31), the cells were diluted to a low A₆₀₀nm (0.010–0.030) and subjected to passage in 7H11 broth prior to plating on 7H11 agar while maintaining continuous selection with kanamycin, ManN, and GlcN. M. smegmatis was then used to transform M. tuberculosis strain H37Rv to ManN resistance, and the library was screened for cosmid clones conferring ManN resistance.
ManN transformed with pYUB18 alone was utilized as a control during the selection procedure. To ensure that ManN resistance (in the presence of GlcN) was conferred by the cosmid, putative ManN-resistant cosmids were electroted into E. coli (32) and purified with QIAprep columns, and the phenotype was retested after electroporating into M. smegmatis.

DNA Sequencing, PCR, and Cloning Procedures—DNA sequencing was performed by Macromolecular Resources (Colorado State University) using an ABI Prism 377 Automated DNA Sequencer. DNA sequence comparisons were done by BLAST analysis (National Center for Biotechnology Information). Alignments of deduced amino acid sequences were performed using the MULITALIGN program. Standard PCR strategies (33) with Vent DNA polymerase (New England Biolabs, Inc.) were used to amplify the putative mannosyltransferase gene MTCY25D10.36 with primers (5′-AATTATCCACGGGATCCGGC-3′ (sense primer for cloning into pYUB18), 5′-GGGATCCGTCGTCGGT-GGGATCCGGC-3′ (sense primer for cloning into pMV261), 5′-GGTGGTGG-TGGTGCCGGTCGGGCTG-3′ (sense primer for cloning into pMAL-c2), and 5′-GGGGATCCCTTGGTCGAGAC-3′ (antisense primer)) derived from the M. tuberculosis H37Rv genome sequence (34). PCR programs consisted of an initial 4 min denaturation step (94°C; followed by 30 cycles of 1) 94°C for 30 s, 2) 66.6°C for 30 s, and 3) 72°C for 1 min; and then a final elongation period at 72°C for 7 min. PCR amplification of MTCY25D9.12 was facilitated using Pfu DNA polymerase and a program similar to that described above with primers 5′-GGCGGGATCCTTGTC- CAGCTACG-3′ (sense primer for cloning into pYUB18) and 5′-GGTGTCACGGGTCAAGGC-3′ (antisense primer). Amplified genes were then cloned into pYUB18 (31), pMV261 (35), or pMAL-c2 (New England Biolabs, Inc.) for further analysis.

Partial Purification of Active Recombinant PimB—Active recombinant PimB protein was partially purified using the pMAL fusion protein system (New England Biolabs, Inc.) according to the manufacturer’s instructions. The pimB gene was amplified by PCR and cloned into the XmaI site of pMAL-c2 to generate pMAL:pimB2, encoding a maltose-binding protein (MalE)-PimB fusion protein. E. coli TB1(pMal:pimB2) was grown at 37°C to A600~0.5 and then induced by the addition of 0.3 mM isopropyl-1-thiogalactopyranoside with incubation at room temperature for 10–12 h. The cells were harvested, resuspended in Buffer A, and frozen at −20°C overnight. Cells were broken in a French pressure cell and centrifuged at 27,000×g of protein. The supernatant was loaded onto an anamyllose resin column, and the fusion protein eluted with Buffer A and maltose was concentrated 8-fold. The eluate was loaded onto an amylose resin column, and the fusion protein was then eluted with Buffer A and maltose was concentrated 8-fold. The eluate containing PimB was concentrated 8-fold. The eluate was then cloned into pYUB18, pMV261, or pMAL-c2 (New England Biolabs, Inc.) for further analysis.

RESULTS

Effect of Mannosamine on In Vitro PIM Synthesis—ManN (2-deoxy-2-amino-2-mannose) inhibits the synthesis of glycosylphosphatidylinositol anchors in Trypanosoma by chain termination, forming ManN-Man-GlcN-PI that cannot be further mannosylated at the 2-position of ManN (36). This mode of inhibition of ManN into any of these products when [14C]Man was used in whole-cell radiolabeling experiments (see below).

![Fig. 1. Effect of ManN on the in vitro incorporation of [14C]Man into mannolipids](Image)

M. tuberculosis ManNiosyltransferase and LAM Biosynthesis

ManNolipid-1 and -2 were mild acid-stable and mild alkalilabile, indicating that they are members of the PIM family as opposed to PPMs. Both were also shown to contain the hexose Man by TLC analysis of the trifluoroacetic acid-hydrolyzed sugars in comparison with sugar standards. The chemical identities of mannolipid-1 and -2 were further determined by mass spectrometry. Direct FAB-MS analysis (Fig. 2) of the isolated mannolipid-1 in negative ion mode afforded a major [M − H]− molecular ion at m/z 1251, whereas mannolipid-2 gave a molecular ion at m/z 1413, corresponding to a difference of one hexose increment from that of mannolipid-1. Based on previous studies (14) and the TLC mobility in comparison with standards, the molecular ion signal afforded by mannolipid-1 and -2 can be assigned as triacylated PIM1 (with a total of three fatty acyl chains, namely two C16:0 fatty acyl chains and one C19:0 tuberculostearate) and PIM2 (also triacylated with C16:0, C16:0, and C19:0), respectively. Supporting evidence was provided by the fragment ions observed directly (Fig. 2, A and B) as well as those from CID-MS-MS (Fig. 3, A and B). In the former, the key fragment ion at m/z 689 corresponds to the phosphoglycerol moiety diacylated with C16:0 and C19:0. Both fatty acyl functions afforded strong carbonyl ions in the CID daughter spectra at m/z 255 and 297. Loss of one or both fatty acyl functions by CID generated a number of other fragment ions as indicated in Fig. 3. An additional C16:0 substituent on Man was confirmed by several complementary fragment ions observed. In direct FAB-MS analysis (Fig. 2), loss of a C18:0-Man from PIM1 gave the ions at m/z 851 and 879, whereas similar loss from PIM2 afforded the ions at m/z 1013 and 1041. Significantly, both PIM1 and PIM2 can lose a single C18:0 function (m/z 1013/995 and 1175/1157, respectively), whereas only PIM2 can lose a single Man (m/z 1251) without also losing a C18:0. Direct loss of a Man residue from PIM1, to give the expected m/z 1089 ion was not observed, and this is consistent with the additional C18:0 being carried on the single Man residue on PIM1. Further evidence for the assigned composition was derived from FAB-MS analysis of the perdeuteroacetyl derivatives, in which mannolipid-1 and -2 afforded molecular ions at m/z 1590 and
1887, corresponding to [M + Na]^+ of triacylated PIM1 and PIM2, respectively, with similar fatty acyl content (data not shown).

The results of the cell-free experiments and structural analyses of mannolipid-1 and -2 were consistent with the model that ManN inhibits the synthesis of PIM1 and PIM2. Specifically, the hypothesis was that ManN was affecting the mannosyltransferase activity or activities responsible for the addition of Man to either PI (to form PIM1) or PIM1 (to form PIM2). This model was also consistent with the observed decrease in the in vitro synthesis of the higher PIMs since they are derived from PIM1/PIM2.
Feasibility of Cloning the Putative Mannosyltransferase Gene(s) by Selection for ManN Resistance—In conjunction with the above cell-free assays, whole-cell radiolabeling experiments were conducted by growing *M. smegmatis* with d-[14C]glucose in the presence or absence of an empirically determined, growth inhibitory concentration of 5 mg/ml ManN. Treatment with ManN decreased the incorporation of radiolabel into PIM₂, but not PIM₁, as compared with the untreated controls and had no effect on the incorporation of radiolabel into LM/LAM as judged by SDS-PAGE followed by autoradiography (data not shown). The difference between the cell-free and whole-cell results regarding the effect of ManN on PIM₁ synthesis may reflect the availability of PI precursors in purified membrane preparations versus whole cells. In agreement with the cell-free results, ManN did not inhibit the synthesis of the PPMs (DPM/HPM). These studies indicated that it might be feasible to clone the gene(s) for the ManN-sensitive mannosyltransferase, whose activity could be studied in the cell-free assays, by screening a *M. tuberculosis* genomic library in *M. smegmatis* for clones that confer a ManN-resistant phenotype by virtue of overexpression of the ManN target on a multicopy plasmid. Preliminary experiments indicated the need to add exogenous GlcN (0.2 mg/ml) along with ManN to avoid cloning another ManN target that resides in the GlcN synthase pathway rather than the LAM biosynthetic pathway.³

Cloning of Putative Mannosyltransferase Genes by Selection for ManN Resistance in the Presence of GlcN—The above strategy was used to isolate two different cosmids clones (pMLS45 and pMLS47) from the H37Rv genomic library by screening *M. smegmatis* transformants for ManN resistance in the presence of GlcN. The availability of the complete *M. tuberculosis* genome sequence (34) facilitated the identification of the genes of interest: the genomic regions present in the cosmids were delineated by sequencing the cosmid insert junctions, and then those regions were subjected to BLAST analysis. Each cosmid was found to contain one putative mannosyltransferase. That on pMLS45 corresponded to gene MTCY20G9.12, whereas that on pMLS47 corresponded to gene MTCY25D10.36. Each of these genes was individually cloned into pYUB18 via PCR to generate pMLS46 and pMLS48, respectively, and both imparted the phenotype associated with their parental cosmids, namely ManN resistance in the presence of GlcN. Gene MTCY25D10.36 was selected for this study, whereas gene MTCY20G9.12 (which appears to be involved in polyprenol-mannose synthesis) is the subject of a separate investigation.⁴

The 1134-base pair MTCY25D10.36 gene, designated *pimB*, is predicted to encode a 41.2-kDa protein that is 378 amino acids in length. BLAST analysis of this amino acid sequence revealed similarity to several mannosyltransferases and other glycosyltransferases, including MTH173 (GenBank™/EBI accession number AE000805) and MTH450 (AE000829) from *Methanobacterium* sp., which are related to E. coli β-glucosyltransferase involved in lipopolysaccharide biosynthesis, and GPI-3 (GenBank™/EBI accession number P32363) from *Saccharomyces cerevisiae*, which is believed to be involved in the transfer of a glycosyl residue to PI. PimB contains the sequence EXFCXXXXE (amino acids 282–290), which differs by one amino acid from that (EXFGxxxxxe) present in several bacterial α-mannosyltransferases (37). Analysis of PimB using TM-Fred or MacVector (Oxford Molecular Group) predicted a single transmembrane region from amino acids 224 to 239, suggesting that PimB may be a membrane protein or membrane-associated protein. This is consistent with the observed co-localization of the associated mannosyltransferase activity (see below) with the membrane fraction.

Evidence That PimB Catalyzes the Formation of PIM₂ from GDP-Man and PIM₁—The function of PimB was examined by overexpressing the *pimB* gene in both *M. smegmatis* and *E. coli* and assaying the membrane fraction or partially purified protein, respectively, in the cell-free system. For expression in *M. smegmatis*, *pimB* was cloned downstream of the hsp60 heat shock promoter in vector pMV261 (35) to generate pMV36Short. Membranes from heat-shocked (42 °C) cells (35) were assayed in the cell-free system, and representative results are shown in Fig. 4A. The incorporation of radiolabel from GDP-[14C]Man into PIM₂ was 27% with the vector control as compared with 49% with pMV36Short (the difference of 22% incorporation represents a 1.8-fold increase in relative activity), whereas there was no significant change in the amount of PIM₁ synthesized. As expected, there was also no apparent change in the incorporation of [14C]Man into the DPM/HPM populations. Since the cell-free assay supports a number of mannosyltransferase activities (15), there was concern that the major products (notably the PPMs) might obscure any other results on the chromatogram. Therefore, amphomycin (which inhibits polyphosphatirequiring transglycosylases) was added to inhibit the synthesis of DPM/HPM (15) and thus restrict product formation. Under these conditions, there was again no significant change in the incorporation of radiolabel from GDP-[14C]Man into PIM₂ compared with that obtained with the control, and all of the observed mannosyltransferase activity was associated with PIM₁ synthesis (Fig. 4B). The difference between the control and the *pimB* construct remained close to 1.8-fold, although the overall efficiency of the

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³ M. L. Schaeffer and J. M. Inamine, manuscript in preparation.

⁴ S. Gurcha and G. S. Besra, manuscript in preparation.
reactions increased (55 and 96% incorporation, respectively, for a difference of 41%).

These results showed that PimB was associated with the addition of Man to PI in the formation of PIM₂, but it could not be discerned if it was involved in the first or second Man transfer because any newly synthesized PIM₁ might be chased directly into PIM₂. Since Takayama and Goldman (20) provided convincing evidence that two distinct mannosyltransferase activities are required for the mannosylation of PI to form PIM₂, it was highly unlikely that PimB was involved in both reactions, and so the cell-free assays were repeated using TLC-purified triacyl-[¹⁴C]PIM₁ as the sole source of radiolabel. Initial attempts to use the M. smegmatis membranes in these assays indicated that the reaction was inefficient (∼10% maximal incorporation of radiolabel) for three reasons. First, and probably most important, any endogenous, unlabeled triacyl-PIM₁ in the membrane will compete with triacyl-[¹⁴C]PIM₁. A second problem arises because triacyl-[¹⁴C]PIM₁ is purified by preparative TLC, and the residual silica was found to inhibit the reaction. Finally, the in vitro synthesized radiolabel is of low specific activity. The major problem was addressed by partially purifying the active recombinant enzyme from E. coli. The pMAL fusion protein system was used to generate a fusion protein between maltose-binding protein (MalE) and PimB, which could then be purified via an amylase column. As shown in Fig. 5A, SDS-PAGE analysis of the column eluate showed that a major band of ∼90 kDa and a minor band of ∼48 kDa are visible by silver staining. The 90-kDa band is close to the predicted size of the MalE-PimB fusion protein (82 kDa), and both the 90- and 48-kDa bands were recognized by anti-MalE antibodies in Western blot analysis, indicating that the latter is probably a breakdown product of the fusion. The intact fusion protein was not active in the cell-free assay; however, once it was digested with Factor Xa (to precisely cleave off MalE and thus produce a mixture of PimB and MalE as well as Factor Xa), it catalyzed the transfer of Man from GDP-Man to triacyl-[¹⁴C]PIM₁ to form triacyl-[¹⁴C]PIM₂ (representative results are shown in Fig. 5B). In this case, the incorporation of radiolabel was 26%. Further purification of PimB in an active form has not yet been achieved, but this work is currently in progress. Although this reaction was sensitive to ManN (Fig. 5B), only 56% inhibition was observed (the incorporation of radiolabel in the presence of ManN was 12%). This suggests that ManN does not bind directly or efficiently to the active site of the enzyme, but interferes indirectly with the enzyme’s access to GDP-Man, and this conclusion is supported by the high concentration of ManN (5 mg/ml) that is required for inhibition. It should be emphasized that the reaction mediated by recombinant PimB occurs in the complete absence of any mycobacterial membrane, thus addressing any concerns that may arise regarding the validity of using recombinant M. smegmatis membrane preparations to study the activity of a M. tuberculosis protein.

**DISCUSSION**

The biochemical analysis of pimB indicates that the gene product is involved in the utilization of GDP-Man as the donor for Man in the LAM biosynthetic pathway. Specifically, the cell-free results with partially purified recombinant PimB provide evidence that pimB encodes the α-6-mannose-α(1→6)-phosphatidylinositol-monomannoside transferase that mediates the transfer of Man from GDP-Man to triacyl-PIM₁ to form triacyl-PIM₂. Sequence analysis of PimB originally suggested that the gene encoded a glycosyltransferase based on the presence of the deduced amino acid sequence EFXXXXE, which differs by one amino acid (C instead of G) from the sequence found in bacterial α-mannosyltransferases (37), as well as similarity to other glycosyltransferases and certain enzymes that transfer glycosyl residues to PI.

The analysis of glycosyltransferases from eukaryotic sources indicates that the majority of the enzymes have a single transmembrane region with a C-terminal catalytic domain, and they are oriented with the catalytic domain within a membrane-bound compartment (40). As noted earlier, a single transmembrane region is predicted for PimB, and the preferred orientation would place the amino terminus outside the cell. Assuming that the glycosyltransferase domain of PimB resides in the C terminus, it would be on the cytoplasmic side of the membrane. This would be consistent with PimB being an enzyme that utilizes GDP-Man, a cytoplasmic component.

BLAST analysis of the M. tuberculosis genome sequence data base shows similarity between PimB (Rv0557) and seven predicted M. tuberculosis proteins. Rv0486 (the mannosyltransferase encoded by MTCY20G9.12), Rv0302, Rv2610c, and Rv3709c all contain the sequence EXFXXXXE, whereas Rv2188c, Rv0225, and Rv1212c contain variant sequences (EXL/W/GXXXXE). This similarity to PimB suggests that these proteins are potential candidates for the other mannosyltransferases that must be involved in LAM biosynthesis, although it should be noted that the EXFXXXXE motif is also found in bacterial glycosyltransferases other than mannosyltransferases (37).

The total number of mannosyltransferases that are needed for LAM biosynthesis is a matter of speculation, depending on how the α(1→6)-linked mannan backbone and α(1→2)-linked Man side chains are assembled. However, one can predict that numerous enzymes are needed for the formation of the different linkages as well as the different acceptor and donor specificities and the possibility that certain Man residues might be added sequentially while others might be derived from intermediates built up on lipid carriers. One should also expect there to be a few species-specific enzyme requirements given that, beyond the structurally conserved PI/PIMs, the mature LM/LAMs from different mycobacteria species have been found to differ in the extent of glycosylation and modification (38). For example, the synthesis of the Man caps on LAM will certainly involve enzymes that are distinct from those required to form the mannan core. Other unrelated biosynthetic pathways in M. tuberculosis will also need specific mannosyltransferases, most notably those involved in producing the mannosyl portions of the glycoproteins (39).

The mannosyltransferases involved in the PIM/LM/LAM biosynthetic pathway are potential targets for the rational design of novel chemotherapeutic agents against M. tuberculosis. The evidence that PIMs are early precursors to LM and LAM is well established (14, 15), and drugs that inhibit PIM synthesis could
have an effect on the organism’s ability to produce LAM. Although the importance of LAM in retaining the structural integrity of the cell wall can only be speculated, its potent immunomodulatory effects show that LAM clearly plays a role in the pathogenesis of mycobacterial disease, and so agents that disrupt its biogenesis will undoubtedly affect the ability of *M. tuberculosis* to survive within the host. This identification of PimB provides groundwork for determining if this particular enzyme and this biosynthetic pathway are viable drug targets in *M. tuberculosis*.

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REFERENCES

1. Blanchard, J. S. (1996) *Annu. Rev. Biochem.* 65, 215–239
2. Young, D. B., and Duncan, K. (1995) *Annu. Rev. Microbiol.* 49, 641–673
3. Brennan, P. J., and Nikaido, H. (1995) *Annu. Rev. Biochem.* 64, 29–63
4. Chopra, I., and Brennan, P. (1998) *Tuberculosis Lung Dis.* 78, 89–98
5. Chan, J., Fan, X., Hunter, S. W., Brennan, P. J., and Bloom, B. R. (1991) *Infect. Immun.* 59, 1755–1761
6. Chatterjee, D., Roberts, A. D., Lowell, K., Brennan, P. J., and Orme, I. M. (1992) *Infect. Immun.* 60, 1249–1253
7. Kaplan, G., Gandhi, R. R., Weinstein, D. E., Levis, W. R., Patarroyo, M. E., Brennan, P. J., and Cohn, Z. A. (1987) *J. Immunol.* 138, 3028–3034
8. Moreno, C., Mehlert, A., and Lamb, J. (1988) *Clin. Exp. Immunol.* 74, 206–210
9. Sibley, L. D., Hunter, S. W., Brennan, P. J., and Krahenbuhl, J. L. (1988) *Infect. Immun.* 56, 1232–1236
10. Chatterjee, D., Lowell, K., Rivoire, B., McNeil, M. R., and Brennan, P. J. (1992) *J. Biol. Chem.* 267, 6234–6239
11. Schlesinger, L. S., Hull, R. S., and Kaufman, T. M. (1993) *J. Immunol.* 152, 4670–4679
12. Hunter, S. W. and Brennan, P. J. (1990) *J. Biol. Chem.* 265, 9272–9279
13. Chatterjee, D., Hunter, S. W., McNeil, M. R., and Brennan, P. J. (1992) *J. Biol. Chem.* 267, 6228–6233
14. Khos, K.-H., Delling, A., Morris, H. R., Brennan, P. J., and Chatterjee, D. (1995) *Glyobiology* 5, 117–127, and references cited therein
15. Besra, G. S., Morehouse, C. B., Rittner, C. M., Waechter, C. J., and Brennan, P. J. (1997) *J. Biol. Chem.* 272, 18460–18466, and references cited therein
16. Salaman, M., Lonsdale, J. T., Besra, G. S., and Brennan, P. J. (1999) *Biochem. Biophys. Acta* 146, 437–450
17. Pangborn, M., and McKinney, J. A. (1966) *J. Lipid Res.* 7, 627–633
18. Brennan, P., and Ballou, C. R. (1967) *J. Biol. Chem.* 242, 3046–3056
19. Brennan, P., and Ballou, C. R. (1968) *J. Biol. Chem.* 242, 2975–2984
20. Takayama, K., and Goldman, D. S. (1969) *Biochem. Biophys. Acta* 176, 196–198
21. Ballou, C. E. (1972) *Methods Enzymol.* 28, 493–500
22. Snapper, S. B., Melton, R. E., Kieser, T., Mustafa, S., and Jacobs, W. R., Jr. (1990) *Mol. Microbiol.* 4, 1911–1919
23. Takayama, K., Schoes, H. K., Armstrong, E. L., and Boyle, R. W. (1975) *J. Lipid. Res.* 16, 308–317
24. Horowitz, M. J. (1991) *Arch. Biochem. Biophys.* 288, 317–323
25. Belanger, A. E., Besra, G. S., Ford, M. E., Mikulova, K., Belisle, J. T., Brennan, P. J., and Inamine, J. M. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 11919–11924
26. Bradford, M. M. (1976) *Anal. Biochem.* 72, 248–254
27. Folch, J., Lees, M., and Sloane Stanley, G. H. (1957) *J. Biol. Chem.* 226, 497–509
28. Mikulova, K., Sladen, R. A., Besra, G. S., and Brennan, P. J. (1995) *Antimicrob. Agents Chemother.* 39, 2484–2489
29. Deng, L., Mikulova, K., Robuck, K. G., Scherman, M., Brennan, P. J., and McNeil, M. R. (1995) *Antimicrob. Agents Chemother.* 39, 694–701
30. Belisle, J. T., Pascopella, L., Inamine, J. M., Brennan, P. J., and Jacobs, W. R., Jr. (1991) *J. Bacteriol.* 173, 6991–6997
31. Jacobs, W. R., Jr., Kalpana, G. V., Cirillo, J. D., Pascopella, L., Snapper, S. B., Udani, R. A., Jones, W., Barletta, R. G., and Bloom, B. R. (1991) *Methods Enzymol.* 204, 537–555
32. Baulard, A., Jordan, C., Mercenier, A., and Locht, C. (1992) *Nucleic Acids Res.* 20, 4105
33. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1991) *Current Protocols in Molecular Biology*, Greene & Wiley, New York
34. Cole, S. T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S. V., Eiglmeier, K., Gas, S., Barry, C. E., II, Tekaia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentle, S., Hamlin, N., Holroyd, S., Hornby, T., Jagels, K., Krogh, A., McLean, J., Moule, S., Murphy, L., Oliver, S., Osborne, J., Quail, M. A., Rajandream, M. A., Rogers, J., Rutter, S., Seeger, K., Skelton, S., Squares, S., Squares, R., Sulston, J. E., Taylor, K., Whitehead, S., and Barlow, B. G. (1998) *Nature* 393, 537–544
35. Stover, C. K., de la Cruz, V. F., Fuerst, T. R., Burlein, J. E., and Barry, C. E., III (1991) *Antimicrob. Agents Chemother.* 35, 24183–24189
36. Galton, J. E., Milne, K. G., Guther, M. L. S., Field, R. A., and Ferguson, M. A. J. (1993) *J. Biol. Chem.* 268, 24183–24189
37. Geremia, R. A., Petroni, E. A., Ielpi, E., and Henrissat, B. (1996) *Biochem. J.* 318, 133–138
38. Khos, K.-H., Delling, A., Morris, H. R., Brennan, P. J., and Chatterjee, D. (1995) *J. Biol. Chem.* 270, 12380–12389
39. Döös, K. M., Döös, K.-H., Swiderek, J. M., Brennan, P. J., and Belisle, J. T. (1996) *J. Bacteriol.* 178, 2498–2506
40. Field, M. C., and Wainwright, L. J. (1995) *Glyobiology* 5, 463–472