

NOTES

Characterization of the *Corynebacterium glutamicum* Δ pimB' Δ mgtA Double Deletion Mutant and the Role of *Mycobacterium tuberculosis* Orthologues Rv2188c and Rv0557 in Glycolipid Biosynthesis[∇]

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In this study, utilizing a *Corynebacterium glutamicum* Δ pimB' Δ mgtA double deletion mutant, we unequivocally assign the in vivo functions of Rv2188c as an Ac₁PIM₁:mannosyltransferase (originally termed PimB'_{Mt} [*Mycobacterium tuberculosis* PimB']) and Rv0557 as a GlcAGroAc₂:mannosyltransferase (originally termed PimB_{Mt}), which we have reassigned as PimB_{Mt} and MgtA_{Mt}, respectively, in *Mycobacterium tuberculosis*.

The current model of mycobacterial phosphatidyl-*myo*-inositol mannoside (PIM) biosynthesis, supported by biochemical and genetic studies, follows a linear pathway from phosphatidylinositol (PI) → Ac₁PIM₂ → Ac₁PIM₄ → Ac₁PIM₆ (4, 17, 19) as shown in Fig. 1. In this pathway, mycobacterial PI is glycosylated by an α -mannopyranosyl residue at the 2-OH position of inositol, followed by the acylation and mannosylation at the 6-OH position of PI to form Ac₁PIM₂ (3), which is further mannosylated to form Ac₁PIM₄ and Ac₁PIM₆, extending the 6-OH position of Ac₁PIM₂ (19).

In view of the identification of genes involved in PIM, lipomannan (LM), and lipoarabinomannan (LAM) biosynthesis, Schaeffer et al. (22) proposed Rv0557 as an α -D-mannose- α -(1→6)-phosphatidyl-*myo*-inositol-mannosyltransferase that transfers mannose from GDP-Man to Ac₁PIM₁ to form Ac₁PIM₂, a precursor of the immunomodulatory lipoglycans LM and LAM (4, 17). The study was based on a cell-free assay using GDP[¹⁴C]Man, Ac₁PIM₁, *Mycobacterium smegmatis* membranes, and/or partially purified recombinant Rv0557. On the basis of these in vitro studies, Rv0557 was assigned as PimB_{Mt} (*Mycobacterium tuberculosis* PimB) in the synthesis of Ac₁PIM₂. However, on the disruption of Rv0557 in *Mycobacterium tuberculosis*, PIM biosynthesis remains unaffected (G. S. Besra and L. S. Schlesinger, unpublished data), suggesting that either gene duplication or Rv0557 performed another function in *M. tuberculosis*. Interestingly, in a recent study, Rv0557 was also shown to be involved in the biosynthesis of 1,2-di-*O*-C₁₆/C_{18:1}-(α -D-mannopyranosyl)-(1→4)-(α -D-glucopyranosyluronic acid)-(1→3)-glycerol (ManGlcAGroAc₂) and an LM-like molecule in *Corynebacterium glutamicum* and was termed Mg-

tA_{Mt} (*M. tuberculosis* MgtA) (25). More recently, Rv2188c was also proposed to be involved in the synthesis of Ac₁PIM₂ as the second α -D-mannose- α -(1→6)-phosphatidyl-*myo*-inositol-mannosyl transferase (termed PimB'_{Mt}) (13, 16), which has augmented ongoing confusion in the field. Due to the essentiality of *M. tuberculosis* PIM biosynthesis (3) in this study, we have generated *C. glutamicum* Δ pimB' Δ mgtA, deficient in pimB'_{Cg} and mgtA_{Cg} (*C. glutamicum* pimB' and mgtA) and subsequently overexpressed Rv2188c and Rv0557 individually to identify their true in vivo and in vitro biochemical activities.

Construction, growth, and complementation of *C. glutamicum* Δ pimB' Δ mgtA. Rv2188c and Rv0557 both belong to the glycosyltransferase B family (14) and are part of subgroup GT4 according to the Carbohydrate-Active EnZymes (CAZy) classification system (6). Using Rv0557 as a query sequence in a BLAST comparison, the next paralog among the six members of *M. tuberculosis* within the GT4 family was Rv2188c (identity score, 35%), revealing a structural similarity between the two proteins. Both proteins possess orthologs in *C. glutamicum*, and previous genetic and biochemical studies confirmed that the orthologous proteins have identical functions (13, 16, 25). When either pimB'_{Cg} or mgtA_{Cg} was deleted, no reliable growth defect was observed (data not shown). We therefore transformed *C. glutamicum* Δ pimB' (16) with the allele replacement vector pK19mobsacB Δ mgtA (21, 25) (Table 1) to kanamycin resistance, and after two rounds of positive selection, small colonies on brain heart infusion (BHI) medium plates were obtained. The mgtA_{Cg} locus was analyzed by PCR, and finally, 1 of 18 positive clones was identified as *C. glutamicum* Δ pimB' Δ mgtA, exhibiting the double deletion phenotype. We analyzed the growth of this strain on complex medium (BHI) and found a significantly reduced growth rate in the exponential phase from 0.43 h⁻¹ to 0.32 h⁻¹, whereas the final optical density reached was not influenced (data not shown).

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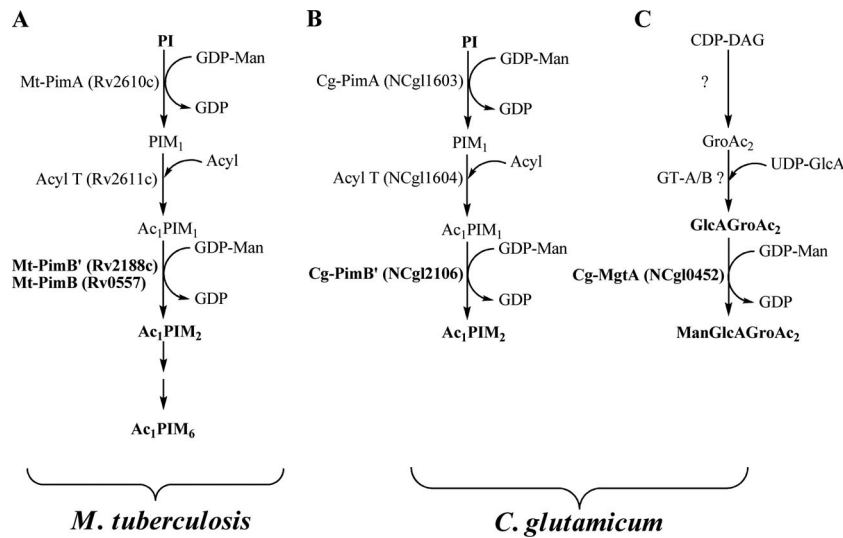


FIG. 1. Glycolipid biosynthetic pathways in *Corynebacterineae*. (A) PIM synthesis in *M. tuberculosis*; (B) PIMs; (C) ManGlcAGroAc₂ synthesis in *C. glutamicum*.

To enable the expression of Rv2188c for functional studies, the open reading frame was amplified and cloned in pEKEx2 (8), producing pEKEx2-Rv2188c (Table 1). This vector, as well as pEKEx3-Rv0557 (25), was introduced into *C. glutamicum* Δ pimB' Δ mgtA via electroporation. Complementation with the Rv0557 gene restored growth, whereas the Rv2188c gene was unable to reverse the growth defect but is apparently expressed based on glycolipid and lipoglycan analysis (see below). The complementation of *C. glutamicum* Δ pimB' Δ mgtA by pimB' _{Cg} and mgtA _{Cg} gave similar phenotypes to those of the Rv2188c and Rv0557 genes. The above growth rates for all strains were similar to those for all strains in salt medium CGXII (data not shown).

In vivo glycolipid and lipoglycan analysis. Polar lipids containing PIMs and other glycolipids were extracted from *C. glutamicum*, *C. glutamicum* Δ pimB' (16), *C. glutamicum* Δ mgtA (25), *C. glutamicum* Δ pimB' Δ mgtA, *C. glutamicum* Δ pimB'

Δ mgtA-pEKEx2-Rv2188c, and *C. glutamicum* Δ pimB' Δ mgtA-pEKEx3-Rv0557, using an established chloroform-methanolic saline procedure (7). The extracted lipids were examined by two-dimensional thin-layer chromatography (2D-TLC) and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). The lipid extracts from *C. glutamicum* possessed a typical profile of ManGlcAGroAc₂, GlcAGroAc₂, Ac₁PIM₂, trehalose monocorynomycolate (TMCM), and glucose monocorynomycolate (GMCM) by α -naphthol/sulfuric acid staining (Fig. 2). As shown previously the corresponding Ac₁PIM₂ (negative-ion-mode MALDI-TOF MS, *m/z* 1398 [M-H]⁻, fatty acyl groups C₁₆ and C_{18:1}; Fig. 3A) and ManGlcAGroAc₂ (positive-ion-mode MALDI-TOF MS, *m/z* 977 [M-H + 2Na]⁺, fatty acyl groups C₁₆ and C_{18:1}; Fig. 3B) (25) were confirmed by MS. In addition, as reported earlier, Ac₁PIM₂ and ManGlcAGroAc₂ were completely absent in *C. glutamicum* Δ pimB' (16) and *C. glutamicum* Δ mgtA (25), re-

TABLE 1. Strains, plasmids, and oligonucleotides used in this study

Strain, plasmid, or oligonucleotide ^a	Relevant characteristics or sequence ^a	Source, reference, or purpose
<i>C. glutamicum</i> strains		
ATCC 13032	Wild type	Culture collection
<i>C. glutamicum</i> Δ mgtA	<i>C. glutamicum</i> devoid of mgtA _{Cg}	25
<i>C. glutamicum</i> Δ pimB'	<i>C. glutamicum</i> devoid of pimB' _{Cg}	16
<i>C. glutamicum</i> Δ pimB' Δ mgtA	<i>C. glutamicum</i> devoid of pimB' _{Cg} and mgtA _{Cg}	This work
Plasmids		
pK19mobsacB Δ mgtA	Vector enabling deletion of 1,094 bp of mgtA _{Cg}	25
pEKEx3-Rv0557	Expression of Rv0557	25
pEKEx2-Rv2188c	Expression of Rv2188c	16
pET16b	Expression vector, His-tag fusion	Novagen
pET16b-Cg-pimB'	Expression of His-PimB' _{Cg}	This work
Primers		
Cg-pimB' forprot	CTCCATATGTCTGCATCCCGAAAACTCTCGTTG	Expression of His6-PimB' _{Cg} (NdeI) ^b
Cg-pimB' revprot	GAGCATATGTTATCGTGGTTCACCTCTGCAAAA TATTG	Expression of His6-PimB' _{Cg} (NdeI) ^c

^a Primers are given in their 5' to 3' direction.
^b The linker endonuclease restriction site in the previous column is italicized.
^c The restriction endonuclease restriction site in the previous column is italicized.

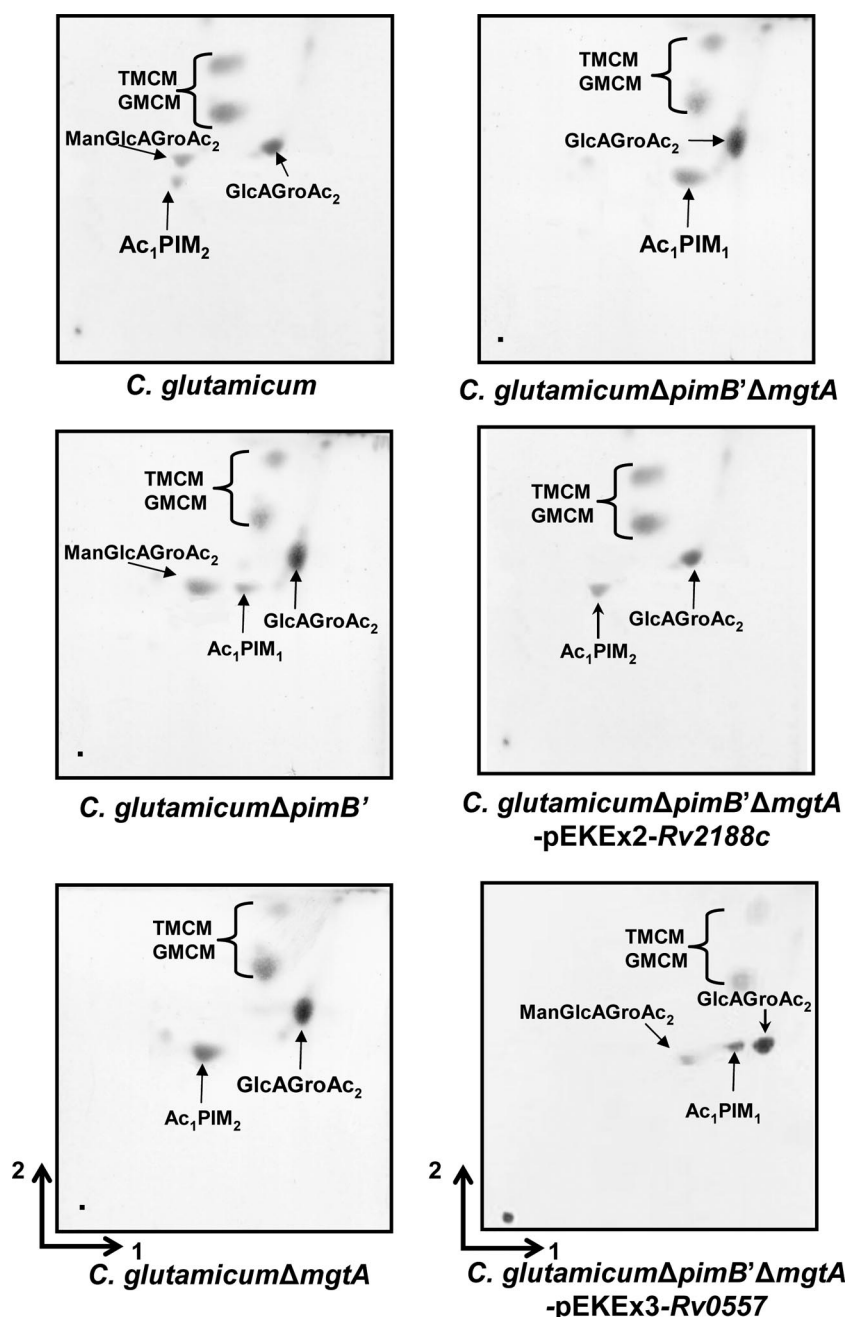


FIG. 2. Glycolipid profiles of *C. glutamicum*, *C. glutamicum* Δ *pimB'*, *C. glutamicum* Δ *mgtA*, *C. glutamicum* Δ *pimB'* Δ *mgtA*, *C. glutamicum* Δ *pimB'* Δ *mgtA*-pEKEx2-Rv2188c, and *C. glutamicum* Δ *pimB'* Δ *mgtA*-pEKEx3-Rv0557. The polar lipid extracts were examined by 2D-TLC on aluminum-backed plates of silica gel 60 F₂₅₄ (Merck 5554), using CHCl₃/CH₃OH/H₂O (60:30:6, vol/vol/vol) in the first direction and CHCl₃/CH₃COOH/CH₃OH/H₂O (40:25:3:6, vol/vol/vol/vol) in the second direction. Glycolipids were visualized by spraying plates with α -naphthol/sulfuric acid, followed by gentle charring of the plates.

spectively. Therefore, the absence of both types of lipids in *C. glutamicum* Δ *pimB'* Δ *mgtA* would be anticipated. Indeed, lipid extracts from the *C. glutamicum* Δ *pimB'* Δ *mgtA* double knock-out were found to be devoid of both Ac₁PIM₂ and ManGlcAGroAc₂ by 2D-TLC (Fig. 2) and MALDI-TOF MS (Fig. 3A and B) and accumulated Ac₁PIM₁ (negative-ion-mode MALDI-TOF MS, m/z 1236 [M-H]⁻; Fig. 3A). Therefore, *C. glutamicum* Δ *pimB'* Δ *mgtA* was utilized to study the role of the

orthologous Rv2188c and Rv0557 proteins in this background strain. In the current study, we again establish the inherent usefulness of *C. glutamicum* in the identification of genes involved in indispensable biochemical pathways in mycobacteria (1–2, 5, 9, 16–18, 23–24).

The plasmid-borne overexpression of Rv2188c in *C. glutamicum* Δ *pimB'* Δ *mgtA* restored the synthesis of Ac₁PIM₂ by 2D-TLC (Fig. 2) and MALDI-TOF MS analysis (Fig. 3A), while

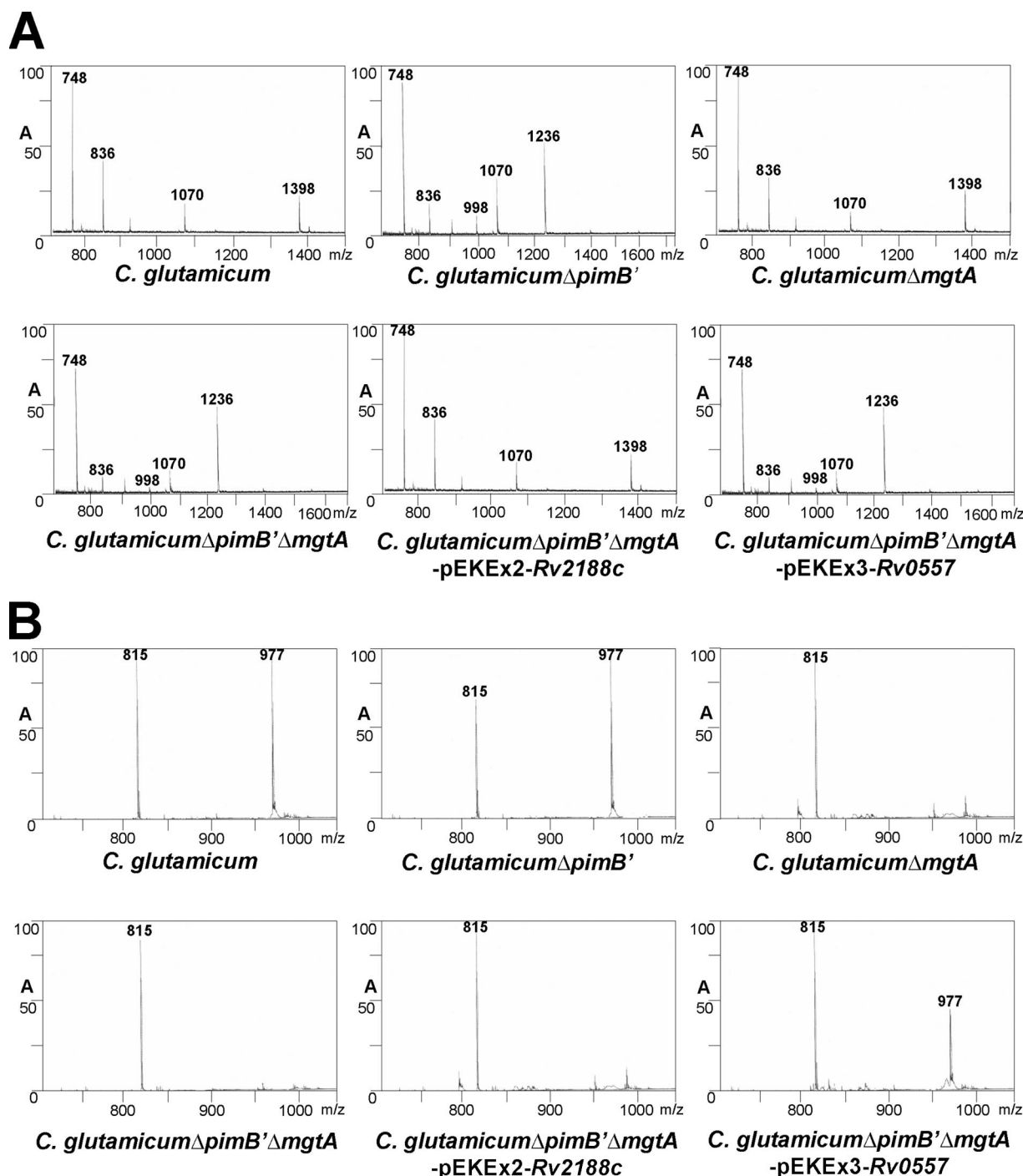


FIG. 3. MALDI-TOF MS analyses of glycolipids from *C. glutamicum*, *C. glutamicum* Δ pimB', *C. glutamicum* Δ mgtA, *C. glutamicum* Δ pimB' Δ mgtA, *C. glutamicum* Δ pimB' Δ mgtA-pEKEx2-Rv2188c, and *C. glutamicum* Δ pimB' Δ mgtA-pEKEx3-Rv0557. (A) Negative-ion-mode MALDI-TOF MS analysis of total glycolipid extract from strains. The peaks observed are m/z 836 (M-H)⁻ [PI with C₁₆/C_{18:1} fatty acyl groups], m/z 998 (M-H)⁻ [PIM₁ with C₁₆/C_{18:1} fatty acyl groups], m/z 1236 (M-H)⁻ [Ac₁PIM₁ with 2C₁₆/C_{18:1} fatty acyl groups], and m/z 1398 (M-H)⁻ [Ac₁PIM₂ with 2C₁₆/C_{18:1} fatty acyl groups]. The peak m/z 748 was not attributable to any PIM species and, as such, may represent unidentified lipid species and/or plasticizer. (B) Positive-ion MALDI-TOF MS spectrum of the cationized, sodiated precursor ion (M-H + 2Na)⁺ of GlcAGroAc₂ and ManGlcAGroAc₂ at m/z 815 and m/z 977, respectively.

ManGlcAGroAc₂ was still absent (Fig. 2 and 3B), which suggests that Rv2188c is solely involved in the synthesis of Ac₁PIM₂. In contrast, the plasmid-borne overexpression of Rv0557 in *C. glutamicum* Δ pimB' Δ mgtA restored the synthesis

of only ManGlcAGroAc₂ as observed by 2D-TLC (Fig. 2) and MALDI-TOF MS analysis (Fig. 3B), which suggests a specific role in ManGlcAGroAc₂ synthesis. Surprisingly, it did not complement the synthesis of Ac₁PIM₂ (Fig. 2 and 3A), a func-

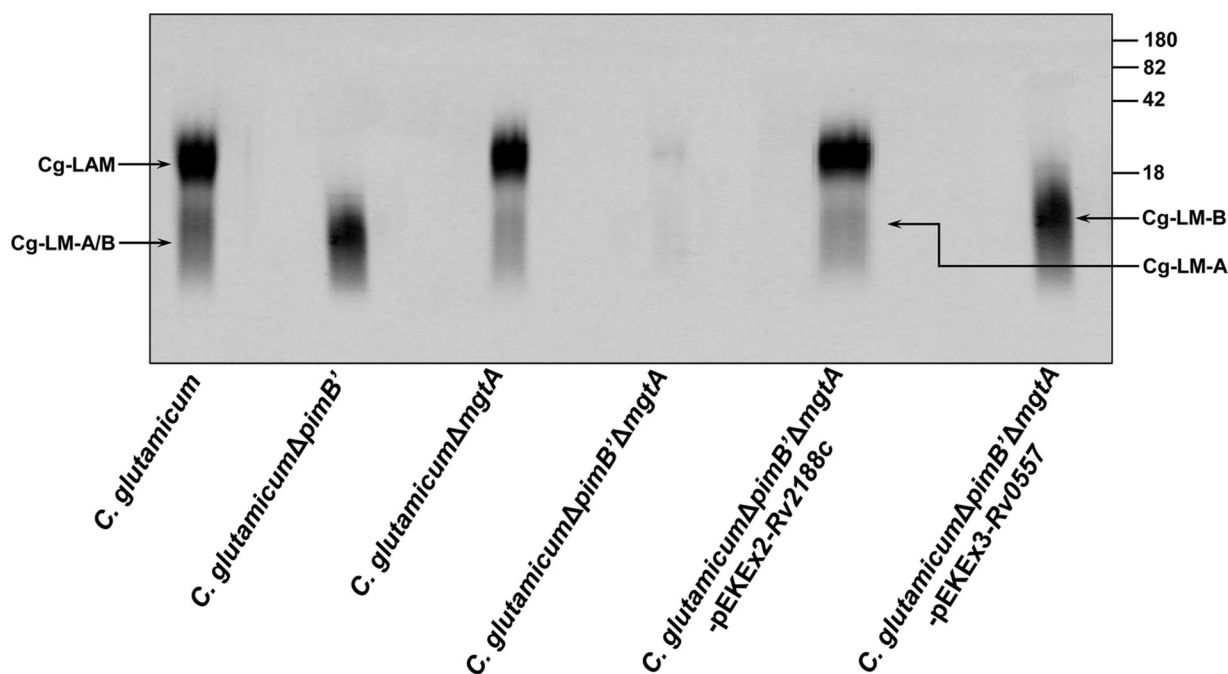


FIG. 4. Lipoglycan profiles of *C. glutamicum*, *C. glutamicum* Δ *pimB'*, *C. glutamicum* Δ *mgtA*, *C. glutamicum* Δ *pimB'* Δ *mgtA*, *C. glutamicum* Δ *pimB'* Δ *mgtA*-pEKEEx2-Rv2188c, and *C. glutamicum* Δ *pimB'* Δ *mgtA*-pEKEEx3-Rv0557. Lipoglycans were analyzed using SDS-PAGE and visualized using a Pro-Q emerald glycoprotein stain (Invitrogen) specific for carbohydrates. The three major bands represented by LAM_{Cg}, LM-A_{Cg}, and LM-B_{Cg} (which comigrates with LM-A_{Cg}) are indicated. The CandyCane glycoprotein molecular weight standards (Invitrogen) are provided on the right for comparison. The four major bands represent glycoproteins of 180, 82, 42, and 18 kDa, respectively.

tion previously assigned by Schaeffer et al. using in vitro studies (22).

Ac₁PIM₂ and ManGlcAGroAc₂ have been shown to be precursors of the cell wall components LM and LAM in *Corynebacterineae*. Therefore, lipoglycans were extracted by refluxing delipidated cells in 50% ethanol, followed by hot-phenol treatment, protease digestion, and dialysis. The extracted lipoglycans were examined on 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (Fig. 4) using a Pro-Q emerald glycoprotein stain according to an established protocol (15, 25). Extracts from *C. glutamicum* showed the presence of LAM_{Cg} as well as LM-A_{Cg} and LM-B_{Cg} (which comigrates with LM-A_{Cg}), as shown previously (16, 25). The lipoglycan extract from *C. glutamicum* Δ *pimB'* showed the absence of LAM_{Cg} and LM-A_{Cg} and the presence of ManGlcAGroAc₂-based LM-B_{Cg} (16), while *C. glutamicum* Δ *mgtA* showed the presence of the PI-based lipoglycans LAM_{Cg} and LM-A_{Cg} and the absence of LM-B_{Cg} (25). Interestingly, *C. glutamicum* Δ *pimB'* Δ *mgtA* was shown to be devoid of all three species of lipoglycans (Fig. 4). The lipoglycans from *C. glutamicum* Δ *pimB'* Δ *mgtA*-pEKEEx2-Rv2188c were analyzed, and as expected, the synthesis of PI-based LAM_{Cg} was restored by Rv2188c, supporting the in vivo lipid studies and the specific role of Rv2188c (Fig. 4). Similarly, *C. glutamicum* Δ *pimB'* Δ *mgtA*-pEKEEx3-Rv0557 restored the synthesis of ManGlcAGroAc₂-based LM-B_{Cg} akin to the phenotype of *C. glutamicum* Δ *pimB'*, again illustrating the specific role of Rv0557 with respect to LM-B_{Cg} synthesis.

In vitro mannanolipid biosynthesis. Reaction mixtures containing GDP[¹⁴C]Man, ATP, and membrane protein from

wild-type, mutant, and/or complemented *C. glutamicum* strains were incubated at 37°C for 30 min as described previously (10). Membrane preparations from wild-type *C. glutamicum* synthesized C₅₀-polyprenylmonophospho[¹⁴C]mannose (PP[¹⁴C]M), Ac₁PI[¹⁴C]M₂, and [¹⁴C]ManGlcAGroAc₂ utilizing endogenous acceptors and GDP[¹⁴C]Man as a sugar donor, consistent with previous studies (Fig. 5A) (10, 17, 25). In assays performed with *C. glutamicum* Δ *pimB'* membranes, an additional minor species migrating between Ac₁PI[¹⁴C]M₂ and [¹⁴C]ManGlcAGroAc₂ was observed and was confirmed as PI[¹⁴C]M₁ based on previous studies and in comparison with authentic standards (11). Surprisingly, a radiolabeled band corresponding to Ac₁PI[¹⁴C]M₂ was also detected, which suggests a relaxed acceptor specificity for MgtA_{Cg} in a *C. glutamicum* Δ *pimB'* background. Assays utilizing membrane preparations from *C. glutamicum* Δ *mgtA* synthesized Ac₁PI[¹⁴C]M₂ but surprisingly also possessed a faint radiolabeled band corresponding to [¹⁴C]ManGlcAGroAc₂, again due to the relaxed substrate specificity of PimB'_{Cg} present in membrane preparations of *C. glutamicum* Δ *mgtA* (Fig. 5B). The synthesis of Ac₁PI[¹⁴C]M₂ and [¹⁴C]ManGlcAGroAc₂ was totally abrogated in assays with membranes prepared from *C. glutamicum* Δ *pimB'* Δ *mgtA* (Fig. 5B), while the accumulation of PI[¹⁴C]M₁ and PP[¹⁴C]M was observed. Interestingly, Rv2188c from membrane preparations from *C. glutamicum* Δ *pimB'* Δ *mgtA*-pEKEEx2-Rv2188c showed substrate specificity toward Ac₁PIM₁ and also a weak recognition for the substrate GlcAGroAc₂, resulting in the synthesis of Ac₁PI[¹⁴C]M₂ and [¹⁴C]ManGlcAGroAc₂ (Fig. 5B), respectively. In contrast with the above studies of Rv2188c, assays performed using membranes prepared from

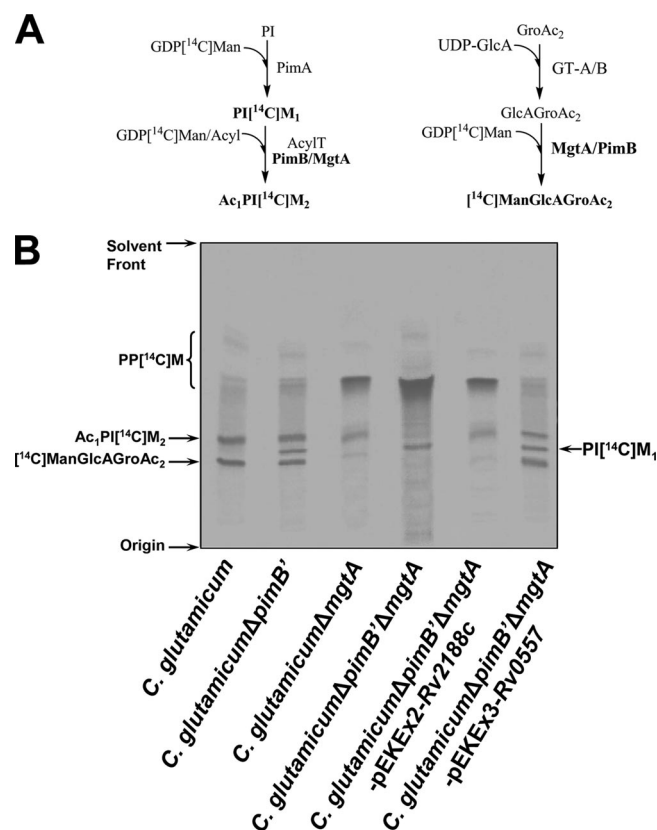


FIG. 5. In vitro mannolipid biosynthesis. (A) Biosynthetic reaction scheme of products formed in in vitro assays utilizing GDP[¹⁴C]Man and corynebacterial membranes. (B) TLC-autoradiography of synthesized mannolipids, using GDP[¹⁴C]Man and membrane extracts from *C. glutamicum*, *C. glutamicum* ΔpimB', *C. glutamicum* ΔmgtA, *C. glutamicum* ΔpimB' ΔmgtA, *C. glutamicum* ΔpimB' ΔmgtA-pEKEx2-Rv2188c, and *C. glutamicum* ΔpimB' ΔmgtA-pEKEx3-Rv0557. Enzymatically synthesized products PP[¹⁴C]M, [¹⁴C]ManGlcAGroAc₂, Ac₁PI[¹⁴C]M₂, and PI[¹⁴C]M₁ were isolated and subjected to TLC/autoradiography using CHCl₃/CH₃OH/NH₄OH/H₂O (65:25:0.4:3.6, vol/vol/vol/vol).

C. glutamicum ΔpimB' ΔmgtA-pEKEx3-Rv0557 illustrated that Rv0557 possessed a broader relaxed substrate specificity, as both Ac₁PIM₁ and GlcAGroAc₂ were equally efficient substrates for the enzyme affording Ac₁PI[¹⁴C]M₂ and [¹⁴C]ManGlcAGroAc₂ synthesis (Fig. 5B). These results explain the previous misinterpretation of the function of Rv0557 on the basis of in vitro data that Rv0557 was involved in the synthesis of Ac₁PIM₂ and annotated as PimB_{Mt} (22).

Mannolipid synthesis using recombinant PimB'_{Cg}. Initial attempts to develop an in vitro assay using either purified recombinant Rv2188c or Rv0557 have thus far proved unsuccessful. Therefore, their *C. glutamicum* orthologs were cloned into pET16b and transformed into *Escherichia coli* BL21(DE3); cultures were grown at 30°C in Luria-Bertani medium (Difco) supplemented with ampicillin (100 μg/ml). The expression of PimB'_{Cg} was induced by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at an A₆₀₀ of 0.4 to 0.6 for 4 h and purified to near homogeneity (>95%) as observed on a 12% SDS-PAGE gel using Ni²⁺-affinity chromatography with a negligible effect on activity (see below).

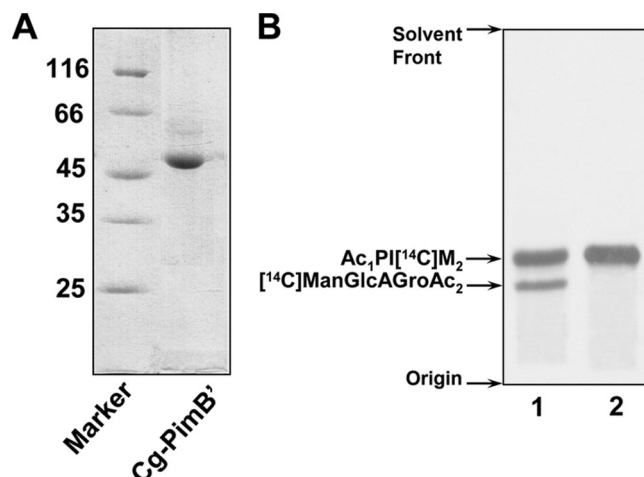
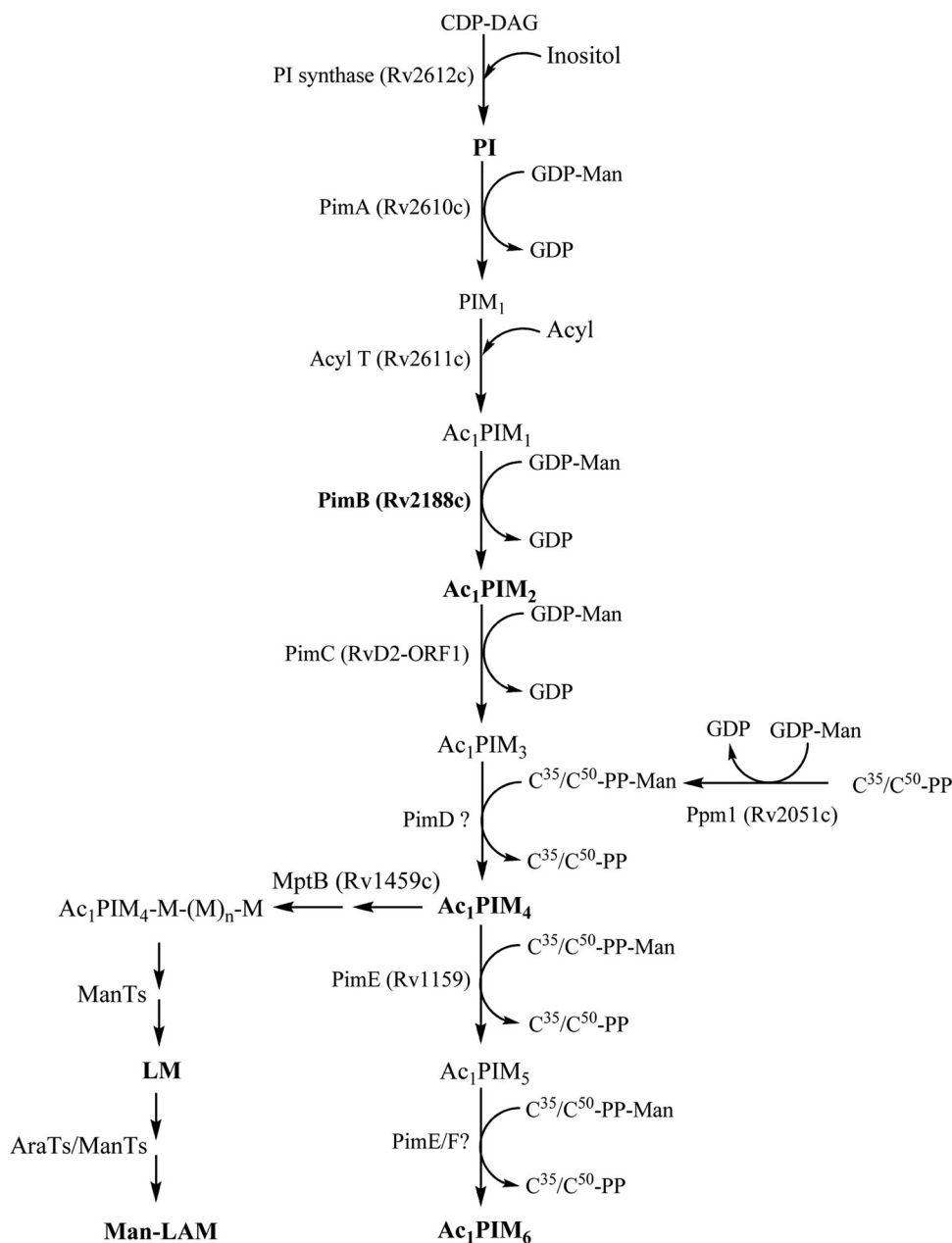


FIG. 6. Mannolipid synthesis using recombinant PimB'_{Cg}. (A) Recombinant PimB'_{Cg} was purified using Ni²⁺-affinity chromatography and purity determined on a 12% SDS-PAGE gel. (B) TLC-autoradiography of synthesized mannolipids, using GDP[¹⁴C]Man and lipid extracts from *C. glutamicum* ΔpimB' ΔmgtA (lane 1) and purified Ac₁PIM₁ (lane 2) with purified PimB'_{Cg}. Enzymatically synthesized products were isolated and subjected to TLC/autoradiography using CHCl₃/CH₃OH/NH₄OH/H₂O (65:25:0.4:3.6, vol/vol/vol/vol).

While PimB'_{Cg} (Rv2188c ortholog) was expressed as a soluble protein (Fig. 6A) and shown to be active in an in vitro assay (see below), MgtA_{Cg} resulted in an inactive protein (data not shown). The activity of purified PimB'_{Cg} was initially determined in a well-established in vitro assay utilizing GDP[¹⁴C]Man and purified polar lipid extracts from *C. glutamicum* ΔpimB' ΔmgtA, which possess Ac₁PIM₁ and GlcAGroAc₂. The resulting products from the assay involving PimB'_{Cg} showed a high substrate specificity of the enzyme toward Ac₁PIM₁ and a relaxed specificity toward GlcAGroAc₂ (Fig. 6B, lane 1). In addition, the assay performed with highly purified Ac₁PIM₁ (Fig. 6B, lane 2) resulted in the formation of Ac₁PI[¹⁴C]M₂. Altogether, the data support the findings from the previous section and the redundant features of these enzymes in vitro (22).

It has been previously shown by us and others that a high degree of functional redundancy exists in a number of biosynthetic pathways in mycobacteria, e.g., MptB (17), PimC (12), and EmbA and EmbB (3) in PIM/LM/LAM and arabinogalactan biosynthesis and the antigen 85 complex in mycolic acid biosynthesis (20). The generation of the *C. glutamicum* ΔpimB' ΔmgtA mutant has clearly enabled the assignment of the precise function of the mycobacterial glycosyltransferases Rv0557 and Rv2188c. It is surprising that a glucuronosyl diacylglycerol-based lipid or lipoglycan has not been identified in mycobacteria or a potential role for Rv0557 in *M. tuberculosis*. However, it is possible that Rv0557 might supplement for the "loss of function" of Rv2188c, as suggested by our in vitro mannolipid studies, due to the essentiality of PIM biosynthesis (3). The identification of a precise role for Rv0557 in *M. tuberculosis* will require the generation of a conditional mutant in *M. tuberculosis* devoid of Rv0557/Rv2188c or novel methods to fractionate polar lipids and lipoglycans from *M. tuberculosis* in search of such glycolipids. A revised biosynthetic

FIG. 7. Revised PIM and LAM biosynthetic pathway in *M. tuberculosis*.

pathway for PIM synthesis that takes into account the findings of the current study is presented in Fig. 7. On the basis of the biochemical studies, we have assigned the functions of Rv2188c as an $\text{Ac}_1\text{PIM}_1\text{:}\alpha\text{-D-mannose-}\alpha\text{-(1}\rightarrow\text{6)-phosphatidyl-}myo\text{-inositol-mannosyltransferase}$ (originally termed PimB'_{Mt}) and Rv0557 as a $\text{GlcAGroAc}_2\text{:}\alpha\text{-D-mannose-}\alpha\text{-(1}\rightarrow\text{4)-}\alpha\text{-D-glucopyranosyl-uronicacid-mannosyltransferase}$ (originally termed PimB_{Mt}), which we have reassigned as PimB_{Mt} and MgtA_{Mt} , respectively, in *M. tuberculosis*.

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