Inactivation of *Corynebacterium glutamicum* NCgI0452 and the Role of MgtA in the Biosynthesis of a Novel Mannosylated Glycolipid Involved in Lipomannan Biosynthesis*

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*Mycobacterium tuberculosis* PimB has been demonstrated to catalyze the addition of a mannose residue from GDP-mannose to a monoacylated phosphatidyl-myoo-inositol mannoside (Ac₃PIM₃) to generate Ac₃PIM₄. Herein, we describe the disruption of its probable orthologue Cg-pimB and the chemical analysis of glycolipids and lipoglycans isolated from wild type *Corynebacterium glutamicum* and the *C. glutamicum*:pimB mutant. Following a careful analysis, two related glycolipids, Gl-A and Gl-X, were found in the parent strain, but Gl-X was absent from the mutant. The biosynthesis of Gl-X was restored in the mutant by complementation with either Cg-pimB or Mt-pimB. Subsequent chemical analyses established Gl-X as 1,2-di-D-glucopyranosyluronic acid)-(1→3)-glycerol (ManGlcAGroAc₂) and Gl-A as the precursor, GlcAGroAc₂. In addition, C. glutamicum:pimB was still able to produce Ac₃PIM₄, suggesting that Cg-PimB catalyzes the synthesis of ManGlcAGroAc₂ from GlcAGroAc₂. Isolation of lipoglycans from *C. glutamicum* led to the identification of two related lipoglycans. The larger lipoglycan possessed a lipoarabinomannan-like structure, whereas the smaller lipoglycan was similar to lipomannan (LM). The absence of ManGlcAGroAc₂ in *C. glutamicum*:pimB led to a severe reduction in LM. These results suggested that ManGlcAGroAc₂ was further extended to an LM-like molecule. Complementation of *C. glutamicum*:pimB with Cg-pimB and Mt-pimB led to the restoration of LM biosynthesis. As a result, Cg-PimB, which we have assigned as MgtA, is now clearly defined as a GDP-mannose-dependent α-mannosyltransferase from our *in vitro* analyses and is involved in the biosynthesis of ManGlcAGroAc₂.

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The *Corynebacteriaceae* represent a distinct and unusual group within Gram-positive bacteria, with the most prominent members being the human pathogens *Mycobacterium tuberculosis* and *Mycobacterium leprae*. In addition, the human pathogen *Corynebacterium diphtheriae* is the causal agent of diphtheria, and serious economic losses occur from the infection of animals by corynebacteria, such as *Corynebacterium pseudotuberculosis* and *Corynebacterium matruchotii*.

Furthermore, nonpathogenic bacteria belong to this taxon, such as *Corynebacterium glutamicum*, which is used in the industrial production of amino acids (4, 5).

A common feature to all these bacteria is that they possess an unusual cell wall matrix composed of mycolic acids, arabinogalactan, and peptidoglycan that is often referred to as the mycolyl-arabinogalactan-peptidoglycan complex (6–13). In addition, they also possess a similar array of cell wall-associated glycolipids, such as phosphatidyl-myoo-inositol (PI)*mannosides (PIMs) and lipoglycans, termed lipomannan (LM) and lipoarabinomannan (LAM) (12, 14–18).

Four major PIMs, mono- and diacyl dimannosides (Ac₂PIM₃ and Ac₃PIM₄) and mono- and diacyl hexamannosides (Ac₃PIM₄ and Ac₄PIM₅), usually accumulate (12) with intermediates occurring in low abundance. Furthermore, the characteristic mycobacterial lipoglycans, LAM and LM, are both multiglycosylated versions of PIMs. We initially proposed the biosynthetic pathway PI → PIM → LM → LAM (15), which is now largely supported by biochemical and genetic evidence (19–22). PimA catalyzes the addition of Manp provided by GDP-mannose to the 2-position of the myoo-inositol of PI to form PIM₁ (21), whereas PimB might be responsible for the addition of a second Manp to the 6-position to yield Ac₃PIM₃.
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TABLE 1

| Strains and plasmids used in this study | Relevant characteristics | Source |
|----------------------------------------|-------------------------|--------|
| **Strains**                            |                         |        |
| E. coli DH5αmcrr                      | F- endA1 supE44 thi-1 lacI1 recA1 gyrA96 relA1 deoR1 lacZYA-argF | Ref. 26 |
| C. glutamicum                          | Wild type ATCC13032     | ATCC   |
| 13032:pimB                             | Wild type with Cg-pimB inactivated | This work |
| 13032:pimB_pEEx3                      | Inactivation mutant with control plasmid | This work |
| 13032:pimB_pEEx3-Cg-pimB              | Inactivation mutant with Cg-pimB overexpressed | This work |
| 13032:pimB_pEEx3-Mt-pimB              | Inactivation mutant with Mt-pimB overexpressed | This work |
| **Plasmids**                           |                         |        |
| pk19mobsacB                            | Integration vector, Km′ oriV′_E. coli oriT sacB | Ref. 27 |
| pk19mobsacB:pimB                      | Vector for inactivation of Cg-pimB | This work |
| pEEx3                                  | Expression vector, Spec′ | Ref. 28 |
| pEEx3-Cg-pimB                          | Vector for overexpression of Cg-pimB | This work |
| pEEx3-Mt-pimB                          | Vector for overexpression of Mt-pimB | This work |

EXPERIMENTAL PROCEDURES

Strains and Culture Conditions—The strains and plasmids used are given in Table 1 (26–28). C. glutamicum ATCC 13032 (wild type) and Escherichia coli DH5αmcrr were grown in Luria-Bertani broth (LB; Difco) at 30 and 37 °C, respectively. For C. glutamicum, kanamycin was used at a concentration of 25 μg/ml, and spectinomycin was used at a concentration of 250 μg/ml where appropriate. The minimal medium used for C. glutamicum was CGXII, and mutants were selected on LBHIS (28). Samples for lipid analyses were prepared by harvesting cells grown either on BHIS for 9 h up to an A600 nm of 7–8 or on CGXII for 17 h up to A600 nm of 47–51. Cells were harvested by centrifugation, followed by saline washing and freeze drying. M. tuberculosis H37Rv DNA was obtained from Dr. J. T. Belisle and the NIH Tuberculosis Research Materials and Vaccine Testing Contract at Colorado State University. All other chemicals were reagent grade or better and obtained from Sigma.

Construction of Plasmids—The inactivation vector pk19mobsacB::pimB was made by amplification of a 359-bp internal fragment of C. glutamicum pimB using the primer pair p557up and p557low (Table 2). After purification and treatment with polynucleotide kinase, the fragment was ligated with Smal-cleaved pk19mobsacB. To construct pEEx3-Cg-pimB, chromosomal DNA of C. glutamicum together with primers p557up3 and p557low3 and KOD DNA polymerase were used to amplify Cg-pimB. The resulting 1232-bp fragment was cloned into the Smal site of pUC18, from which the fragment was resolated by digestion with Scal/EcoRI to ligate it with SmaI/EcoRI-cleaved pEEx3. Similarly, Mt-pimB was amplified using primers ppimbMtex and ppimbMtrev using M. tuberculosis chromosomal DNA. The resulting 1153-bp fragment was cloned into pUC18, subsequently excised as a SmaI/EcoRI fragment, and ligated with SmaI/EcoRI-cleaved pEEx3. All cloned fragments were verified by nucleotide sequencing.

Construction of Strains—Cells of C. glutamicum were made competent as described (28) and transformed by electroporation with pk19mobsacB::pimB to kanamycin resistance, signifying the integration of the construct into the chromosome. Using the two different primer pairs prsp1/p557up2 and puni/p557low2, respectively, the correct disruption of Cg-pimB integration was verified. Competent cells of one disruption mutant were chosen and transformed by electroporation with either pEEx3-Cg-pimB or pEEx3-Mt-pimB to spectinomycin resistance, and the plasmid integrity of the recombinant clones was confirmed in plasmid preparations.

Lipid Extraction and Analysis—Polar lipids and apolar lipids were initially extracted from 6 g of dry C. glutamicum cells according to the procedures of Dobson et al. (29) by stirring in 220 ml of methanolic saline (20 ml of 0.3% NaCl and 200 ml of CH3OH) and 220 ml of petroleum ether for 2 h. The cells were centrifuged at 3000 rpm for 5 min. The resulting biphasic solu-
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The crude lipoglycan extract was resuspended in buffer A (50 mM ammonium acetate and 15% propan-1-ol) and subjected to octyl-Sepharose CL-4B hydrophobic interaction chromatography (2.5 × 50 cm) as previously reported (33). The column was initially washed with 4 column volumes of buffer A to ensure removal of neutral glycan, followed by buffer B (50 mM ammonium acetate and 50% propan-1-ol). The eluate was collected and concentrated to ~1 mL and precipitated using 5 mL of C₄H₈O₂H, and the sample was dried using a Savant SpeedVac. The freeze-dried sample containing the retained material from the hydrophobic interaction column was then resuspended in buffer C (0.2 mM NaCl, 0.25% sodium deoxycholate (w/v), 1 mM EDTA, and 10 mM Tris-HCl, pH 8) to a final concentration of 200 mg/mL. The sample was gently mixed and left to incubate for 48 h at room temperature. The sample was then loaded onto a 200 mL Sephacryl S-200 column previously equilibrated with buffer C. The sample was eluted with 400 mL of buffer C at a flow rate of 3 mL/h, collecting 1.5-ml fractions. The fractions were monitored by SDS-PAGE using either a silver stain utilizing periodic acid and silver nitrate (34) or a Pro-Q emerald glycoprotein stain (Invitrogen), and individual fractions were pooled and dialyzed extensively against buffer D (10 mM Tris-HCl, pH 8, 0.2 mM NaCl, 1 mM EDTA) for 72 h with frequent changes of buffer. The samples were further dialyzed against deionized water for 48 h with frequent changes of water, lyophilized, and stored at −20°C.

Glycosyl Compositional and Linkage Analysis—Glycosyl compositional analysis was performed by either routine gas chromatography (GC) or capillary electrophoresis analysis as described previously (35). Glycosyl linkage analyses were performed as described previously (36). Briefly, per-O-methylated samples were hydrolyzed using 500 µL of 2 M trifluoroacetic acid.
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at 110 °C for 2 h, reduced using 350 μl of a 10 mg/ml solution of NaBD₄ (1 m aqueous NH₄OH/C₂H₅OH, 1:1, v/v), and per-O-acetylated using 300 μl of acetic anhydride for 1 h at 110 °C. The resulting alditol acetates were solubilized in cyclohexane before analysis by GC and gas chromatography/mass spectrometry (GC/MS) (37).

GC analysis was performed using a Thermoquest Trace GC 2000 equipped with a flame ionization detector. Samples were separated using a temperature program as follows. Injector temperature was set at 50 °C, held for 1 min, and then increased to 110 °C at 20 °C/min. The oven was held at 110 °C and then ramped to 290 °C at 8 °C/min and held for 5 min to ensure that all of the products had eluted from the column. All of the data were collected and analyzed using Xcaliber (version 1.2) software.

Gl-A, Gl-X, and Ac₁PIM₂ Matrix-assisted Laser Desorption Ionization-Time of Flight-Mass Spectrometry (MALDI-TOF-MS) Analyses—Analyses of PIMs were carried out on a Voyager DE-STR MALDI-TOF instrument (PerSeptive Biosystems, Framingham, MA) using the reflectron mode of detection as previously described (38). PIMs were analyzed by the instrument operating at 20 kV in the negative ion mode, using an extraction delay time set at 200 ns. Typically, spectra from 100–250 laser shots were summed to obtain the final spectrum. All of the samples were prepared for MALDI-TOF-MS analyses using the on-probe sample clean-up procedure with cation exchange resin. The 2-(4-hydroxyphenylazo)-benzoic acid matrix was used at a concentration of 10 mg/ml in C₆H₄OH/H₂O (1:1, v/v). Typically, 0.5 μl of PIM sample (10 μg) in a CHCl₃/CH₃OH/H₂O solution and 0.5 μl of the matrix solution, containing 5–10 cation exchange beads, were deposited on the target, mixed with a micropipette, and dried under a gentle stream of warm air.

Analyses of the Gl-A and Gl-X were carried out on a 4700 Proteomics Analyzer (with TOF-TOF optics; Voyager DE-STR; Applied Biosystems, Framingham, MA) using the reflectron mode. Ionization was effected by irradiation with a Nd:YAG laser (355 nm) operating by pulses of 500 ps with a frequency of 200 Hz. Gl-A and Gl-X were analyzed in the positive ion mode. Ionization was effected by irradiation with a Nd:YAG laser (355 nm) operating by pulses of 500 ps with a frequency of 200 Hz. Gl-A and Gl-X were analyzed in the positive ion mode.

RESULTS

Genomic Organization of the pimB Locus—Mt-pimB has been shown to encode an α-mannosyltransferase potentially involved in Ac₁PIM₂ biosynthesis (19), and this gene is predicted to be essential in M. tuberculosis (41). However, the biosynthesis of PIMs remains unaffected upon disruption of Mt-pimB, suggesting either a degree of redundancy or that Mt-pimB performed another function in M. tuberculosis. Mt-pimB lies within a cluster of genes involved in menaquinone biosynthesis (Fig. 1A). Directly upstream is a small open reading frame of unknown function and menD, and directly down-

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**FIGURE 1.** Comparison of the *pimB* locus within the *Corynebacteriaceae* and inactivation of *Cg-pimB*. A, the locus consists in *M. tuberculosis* of *Mt-pimB* with adjacent genes presumably involved in the biosynthesis of menaquinone and a bromoperoxidase (*bpoC*). The locus in other *Mycobacterium* species and *Corynebacterium* species is largely syntenic. *par*, paratuberculosis. B, construction of *C. glutamicum* 13032::*pimB*. Shown is *Cg-pimB* with its adjacent genes NGCl0451 and NGCl0453 and the strategy to disrupt *Cg-pimB* using vector pK19mobsacB*pimB*-int. This vector carries a 359-bp internal fragment of *Cg-pimB* of 1221 bp in size, thereby enabling homologous recombination with the wild type genome to generate *C. glutamicum*-pimB. The arrow pairs marked P1 (prsp1/p557up2) and P2 (puni/p557low2) locate the primers used for the PCR analysis to confirm the proper integration of vector pK19mobsacB*pimB*-int in *C. glutamicum* 13032::*pimB* and its absence in wild type *C. glutamicum*. The results of the PCR analysis are shown on the right, where P1 and P2 mark the result obtained with primers P1 and P2, respectively. Samples were applied pairwise with the amplificate obtained in wild type *C. glutamicum* and *C. glutamicum*-pimB. This vector carries a 359-bp internal fragment of *Cg-pimB* of 1221 bp in size, thereby enabling homologous recombination with the wild type genome to generate *C. glutamicum*-pimB. The arrow pairs marked P1 (prsp1/p557up2) and P2 (puni/p557low2) locate the primers used for the PCR analysis to confirm the proper integration of vector pK19mobsacB*pimB*-int in *C. glutamicum* 13032::*pimB* and its absence in wild type *C. glutamicum*. The results of the PCR analysis are shown on the right, where P1 and P2 mark the result obtained with primers P1 and P2, respectively. Samples were applied pairwise with the amplificate obtained from *C. glutamicum* 13032::*pimB* applied in the left lane, exhibiting the expected PCR products of 442 and 603 bp, respectively, whereas these were absent in wild type *C. glutamicum* (WT). St marks the standard, and the arrowheads are located at 0.5, 1, and 1.5 kb, respectively.

Stream is menH. The entire locus consisting of seven genes is syntenic in all sequenced *Mycobacterium* species and, in part, also in *Corynebacterium*, both genera belonging to the taxon *Corynebacteriaceae* (Fig. 1A). The putative orthologue of *Mt-pimB* from *C. glutamicum*, *Cg-pimB* (NCgl0452), shares 49% sequence identity with the *M. tuberculosis* gene, and, since *C. glutamicum* can be regarded as a model organism of this taxon due to its archetypical genomic organization as manifested in a low number of gene duplications, its structural simplicity, and ease of handling (42), we decided to study the function of *Mt-pimB* in more detail using *C. glutamicum*.

**Construction and Growth of the C. glutamicum 13032::pimB Disruption Mutant**—*C. glutamicum* ATCC13032 was transformed to kanamycin resistance with plasmid pK19mobsacB::*pimB* (Fig. 1B) using the method we have previously reported (43, 44). Fourteen colonies were obtained from 1 µg of plasmid DNA. The clones were analyzed via PCR, and all were found to have the vector integrated chromosomally, thus demonstrating disruption of the *Cg-pimB* coding sequence. One strain was chosen and termed 13032::*pimB*. This strain displayed no detectable change in phenotype in terms of growth rate or colony morphology (data not shown) and was transformed with either pEKEEx3-3-Cg-pimB or pEKEEx3-Mt-pimB as well as the unmodified vector, pEKEEx3, as a control for further studies.

**Chromatographic Analysis of Polar Lipids**—Polar lipids were extracted from wild type *C. glutamicum*, and glycolipid profiles were recorded by two-dimensional TLC (Fig. 2). The faster
migrating lipids were confirmed as trehalose monoglycero-
cololate and glucose monoglicero-cololate (data not shown). Interestingly, in the pattern shown in Fig. 2A, not all of the remaining glycolipids gave a positive response with the Ditt-
mer-Lester lipid phosphate reagent. The predominant lipid phosphate spot, which was also carbohydrate-positive, corre-
sponded to Ac1PIM2, and was confirmed by negative ion mode MALDI-MS analyses due to the characteristic ions at m/z 1398 (M − H)− and in positive mode at m/z 1444 (M − H + 2Na)− (data not shown). The phosphorus-free glycolipid, indicated as Gl-X (Fig. 2A) was unusual in terms of chromatographic mobility and staining properties. Analysis of the C. glutamicum 13032:pimB disruption mutant revealed that synthesis of Ac1PIM2 was intact; however, it failed to produce Gl-X (Fig. 2B). Transformation with pEKEx3-Mt-pimB complemented the mutant phenotype and restored Gl-X biosynthesis (Fig. 2C).

To further clarify the structure of Gl-X and the role of Cg-
pimB, the crude polar lipid extract was fractionated using anion exchange chromatography on DEAE-cellulose using a stepwise gradient of increasing ammonium acetate concentration in CHCl3/CH3OH ranging from 1 mM to 500 mM. It was antici-
pated that since Gl-X was phosphorus-negative, this would provide a convenient purification protocol allowing the neutral glycolipid to elute from the column while retaining the contaminating phospholipids. Surprisingly, Gl-X was also retained on the DEAE column and was eluted with 15 mM ammonium acetate in CHCl3/CH3OH (2:1), just before Ac1PIM2, suggesting that it possesses an acidic group. Pooled fractions containing Gl-X were purified further by prepar-
tive TLC and analyzed by MALDI-TOF-MS, 1H,13C two-di-
dimensional COSY, and two-dimensional HSQC NMR.

Chemical Composition of Gl-X—Initial glycosyl composi-
tional analysis using alditol acetates determined the presence of mannone by GC (data not shown). 1H and 13C NMR spectra recorded in d6-Me2SO were in agreement with a diglycosyl diacylated glycerol. Indeed, the 1H,13C HSQC NMR spectrum showed two anomeric resonances at δH/C1/C2 4.98/100.3 (I1) and 4.57/99.0 (I2). The diacylated glycerol unit was identified by two-dimensional 1H,1H COSY NMR spectrum (Fig. 3A) from its deshielded H-2 (III2) proton resonance at 5.09 ppm that correlated with H-1, H-1′, H-3, and H3′ resonances at 4.15 (III1), 4.31 (III1′), 3.45 (III3), and 3.65 (III3′) ppm, respectively. Glycerol carbons resonated at 62.2 (C-1), 69.4 (C-2), and 64.8 (C-3) ppm in agreement with the literature (39).

Gl-X was subsequently analyzed both in negative and posi-
tive mode MALDI-TOF-MS. Spectra were only obtained in positive mode, revealing a molecular ion at m/z 977 (Fig. 4A). Positive ion MALDI-TOF CID-MS/MS spectrum of the cation-
ized sodiated precursor ion (M − H + 2Na)+ of Gl-X revealed ions at m/z 721 and 695, corresponding to the loss of C16 and C18:1 fatty acids, respectively (Fig. 4B), which were also later confirmed by fatty acid methyl ester analysis by GC/MS (data not shown).

However, a simple Man-Man-GroAc2 (containing no inosi-
tol) and both mannone units interconnected did not coincide with the deduced molecular weight (m/z 977), suggesting that we were not dealing with a simple Man-Man type structure but a Man-Y-GroAc2 with Y possibly carrying an acidic function, explaining the retention of Gl-X on DEAE-cellulose. Indeed, the molecular ion at m/z 977 is in agreement with a Man-Y-
Gro-C16/C18:1 structure, where Y represents a hexosyl uronic acid in the (M − H + 2Na)+ form. MALDI-TOF/TOF analysis of the per-O-methylated Gl-X glycan observed at m/z 579 (M + Na)+ gave data rich in informative fragment ions (Fig. 4C). In particular, the data are indicative of Y being consistent with a hexuronic acid (Fig. 4C, inset). These assignments were sub-
stantiated by MS/MS analysis of the per-O-deuteriomethylated glycan observed at m/z 606 (M + Na)+. Furthermore, mild hydrolysis of the per-O-deuteriomethylated glycan with meth-
anoic HCl resulted in a shift of 3 mass units to m/z 603, consis-
tent with methyl exchange on the carboxylic group of hexu-
ronic acid.

The linkage of the two different glycosyl units was established from 1H−1H ROESY, T-ROESY NMR, and 13C NMR experiments (data not shown) that revealed that C-4 of the unit II at 78.6 ppm was shifted to low field away from the remaining resonances at 68–74 ppm. H-1 of unit I (I1) at 4.98 ppm showed an intense interresidue NOE with H-4 of unit II (II4) at 3.59 ppm and weaker NOE with H-5 (II5) and H-3 (II3) of unit II at 3.53 and 3.48 ppm, respectively. Taken together, these data indicate that unit I is linked at O-4 of unit II. 1H and 13C reson-
ces of spin system I correlate with a t-Manp unit, with an α-anomeric configuration suggested by the presence of an intense intrasresidue contact between H-1 at 4.98 ppm (I1) and H-2 at 3.64 ppm (I2) and the absence of an intrasresidue H-1/H-3 NOE contact.

Based on the assignment of 1H−1H COSY NMR and proton coupling constants, unit II (sugar Y) was shown to be α-d-glucopyranosyluronic acid as follows. The small J1,3 coupling con-
stant (3.2 Hz) of H-1 at 4.57 ppm (II4) indicated an α-anomeric configuration. The large coupling constant of H-2 (dd) at 3.19 ppm (II1) J1,3 (9.6 Hz), H-4 (t) at 3.59 ppm (II2) J4,5 (9.6 Hz), H-5 (d) at 3.53 ppm (II2) J4,5 (9.6 Hz) correlates with a gluco-config-
uration of sugar Y. The connectivity of H-5 (II2) to only one proton H-4 (II4) in the two-dimensional COSY NMR spectra while a distinct signal for an extra carboxyl group at 171.6 in the 13C NMR spectra is clearly visible indicating that sugar Y is uronic acid. In addition, H-1 of glucuronic acid (II4) at 4.57 ppm showed an intense resonance at 171.6 ppm, demonstrating that unit II is linked at O-3 of the glycerol backbone. The complete assignment of res-
onances of both glycosyl residues is given in Table 3.

Altogether, these data indicate a 1,2-di-O-C16/C18:1-(α-d-
mannopyranosyl)-(1→4)-(α-d-glucopyranosyluronic acid) 
(1→3)-glycerol structure. In addition, and due to the significant 
sequence identity (49%) between Cg-PimB and Mt-PimB and 
their similarity to other GDP-mannose-dependent α-manno-
syltransferases, it is now persuasive to argue that PimB is 
volved in the biosynthesis of Gl-X, which we have solved as ManGlcAAGroAc2 (Fig. 3B).
In Vitro Analysis of Mannosyltransferase Activity—In order to confirm the mannosyltransferase activity and acceptor specificity of both Cg-PimB and Mt-PimB, we prepared membrane fractions from wild type C. glutamicum, the 13032::pimB mutant, and transformants of the latter bearing pEKEx3-Cg-pimB and pEKEx3-Mt-pimB. Analysis of radiolabeled mannosyl lipids, formed after introduction of GDP-[14C]mannose by two-dimensional TLC (Fig. 2, E–H), revealed a clear difference between the profiles derived from the wild type strain and the 13032::pimB mutant. The upper spot of the dominant pair of glycolipids, corresponding to ManGlcAGroAc2, is clearly absent in the mutant profile. Complementation with Cg-pimB in trans, as expected, restored ManGlcAGroAc2 biosynthesis, and, consistent with our extracted lipid profiles, complementation with Mt-pimB was also achieved. In all of the profiles, the second spot corresponding to Ac1PIM2 was evident, the apparent increase in its abundance in the 13032::pimB mutant profile being due to the absence of ManGlcAGroAc2 when equal radioactivity was loaded on the plates. The identities of these two lipids were confirmed by staining of the same TLC plates with the Dittmer-Lester lipid phosphate reagent (data not shown).

Chemical Analysis of C. glutamicum Lipoglycans—We examined extracts of wild type C. glutamicum and the C. glutamicum 13032::pimB disruption mutant for lipoglycans by SDS-PAGE followed by staining for carbohydrates with either silver nitrate or Pro-Q Emerald stain for glycoconjugates. Extracts of wild type C. glutamicum contained two closely migrating lipoglycans (Fig. 5). Interestingly, the lower molecular weight lipoglycan was significantly reduced in the C. glutamicum
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1H and 13C NMR assignment of key resonances of Gl-X

| Residue          | Chemical shifts |
|------------------|-----------------|
|                  | H-1  | C-1  | H-2  | C-2  | H-3  | C-3  | H-4  | C-4  | H-5  | H-5' | H-6/H-6' |
| t-α-Manp (II)    | 4.98  | 3.64  | 3.38  | 3.13  | 3.74  | 3.67/3.17 |
| α-GlcA (II)      | 4.57  | 3.19  | 3.48  | 3.59  | 3.53  |
| Glycerol (III)   | 4.15/4.31 | 5.09  | 3.45/3.65 | 62.2  | 69.4  | 64.8  | 62.1  | 69.4  | 64.8  |

13032::pimB mutant (Fig. 5). A two-step purification protocol was performed to fractionate the lipoglycans from wild type C. glutamicum and the C. glutamicum 13032::pimB mutant, and fractions containing the lipoglycans were monitored by SDS-PAGE stained with either silver nitrate or Pro-Q Emerald staining.

The larger lipoglycan from wild type C. glutamicum exhibited the basic components of a structure related to mycobacterial LAM and is, henceforth, termed Cg-LAM. GC analysis of the total acid-hydrolyzed Cg-LAM identified arabinose, mannose, and inositol in a ratio of 19:60:1. Per-O-methylation analysis of Cg-LAM indicated the presence of t-Ara, t-Manp, 2-Manp, 6-Manp, and 2,6-Manp (Fig. 6A). Accordingly, the 1H-13C HMQC NMR anomeric region (Fig. 7, A and B) exhibited a pattern of resonances that could be attributed, based on our previous studies with mycobacterial LAMs and LAM-related structures, to these different units (45). Indeed, correlations at δ1H1C1 5.20/112.2 (I1) and 5.13/112.0 (II1) were attributed to two t-Ara units; 5.06/105.2 (III1) to t-Manp units; 5.12/101.4 (IV1), 5.07/101.7 (V1), and 5.04/101.9 (VI1) to 2,6-Manp units; 5.06/105.2 (VII1) to 6-Manp units; and 5.00/104.9 (VIII1) to 2-Manp units. Altogether, these data indicate that Cg-LAM is composed of a PI anchor linked to an α(1→6)Manp backbone substituted at most of the O-2 positions by t-Ara.

In a similar manner, GC analysis of the smaller lipoglycan (Cg-LM) from wild type C. glutamicum contained solely mannose and no trace of inositol. Per-O-methylation analysis of the smaller lipoglycan (Cg-LM) indicated the presence of t-Man, 2-Manp, 6-Manp, and 2,6-Manp (Fig. 6B). Accordingly, the 1H-13C HMQC NMR anomeric region (Fig. 8, A and B) of Cg-LM exhibited correlations at δ1H1C1 5.16/101.4 (I1) and 5.08/105.4 (II1) that were attributed to 2,6-Manp and t-Manp units, respectively. Resonances at δ1H1 4.96, 4.95, and 4.94 (IVa,b,c) that correlated on the two-dimensional 1H-1H HOHAHA (Fig. 8C) of the Cg-LM spectrum with δ1H2 5.03/4.11 (V1) typified the 6-Manp units usually found in the mannan core of mycobacterial lipoglycans and spin system III1 (δ1H1C1 5.03/4.11) characterized 2-Manp units. The 1H-1H HOHAHA (Fig. 8C) spectrum also showed two spin systems with weaker intensity. Resonances at δ 5.25 (Vb2) and 5.29 (Va2) exhibited correlations with proton resonances at δ 4.22 (Vb1), 4.11 (Vb1), 3.93 (Vb3), and 4.16 (Vb1), 4.01 (Vb3), 3.88 (Vb3), respectively. These spin systems were attributed to diacylated glycerol units characterized by deshielded H-2 resonances (5.25 and 5.29 ppm). The NMR data also confirm that Cg-LM also appears to lack inositol and is composed of a diacylglycerol unit linked to an α(1→6)Manp.
backbone substituted at most of the O-2 positions by t-Manp and t-Manp(1→2)-Manp units (Fig. 8D).

Interestingly, in comparison with wild type C. glutamicum, the C. glutamicum 13032::pimB disruption mutant and analysis by SDS-PAGE using Pro-Q Emerald stain for glycoconjugates revealed that the upper lipoglycan appears unaffected (Fig. 5). This was confirmed by glycosyl compositional analysis and per-O-methylation analysis of Cg-pimB-LAM (data not shown). However, the smaller lipoglycan, Cg-pimB-LM, was now barely detectable (Fig. 5). Glycosyl compositional analysis of the residual Cg-pimB-LM now revealed the presence of both mannose and inositol (47:1), and per-O-methylation analysis indicated the presence of t-Manp, 2-Manp, 6-Manp, and 2,6-Manp (data not shown). Furthermore, 1H NMR spectra exhibited an anomeric region with the typical resonances corresponding to these different units (data not shown). The pattern was simpler than that observed for the wild type Cg-LM and corresponded to the profile typically observed for mycobacterial PI-based LM.

In summary, these results suggest that Cg-LM is most likely two components, a dominant Cg-LM based on the ManGlcA-GroAc2 and a minor component akin to the characteristic mycobacterial PI-based LM.

**DISCUSSION**

Along with the genus *Mycobacterium*, species of *Corynebacterium* belong to a suprageneric actinomycete taxon termed *Corynebacterianeae*, which also includes *Rhodococcus*, *Nocardia*, and other closely related genera. In this study, we sought to establish the role of Cg-PimB and Mt-PimB and whether *C. glutamicum* possesses both PIMs and lipoglycans reminiscent of *M. tuberculosis* products, suggesting conserved biosynthetic machineries within these two bacteria. In *M. tuberculosis*, Mt-pimB (Rv0557) was shown earlier to encode an α-(1→6)-phosphatidyl-myo-inositol-monomannosyltransferase and to be involved in the formation of Ac₃PIM₂.
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from GDP-mannose and Ac-PIM₁ (19). In this study, we attempted to disrupt Cg-pimB and examine the consequences of PIM and LAM biosynthesis in C. glutamicum.

To our surprise and unrelated to PIMs, the C. glutamicum 13032::pimB disruption mutant was found to possess major differences in cell wall lipids and lipopolysaccharides in comparison with the parental C. glutamicum strain. A novel glycolipid, which we initially termed Gl-X and subsequently characterized as 1,2-di-O-acyl-(α-D-glucopyranosyluronic acid)-(1→3)-glycerol, was restored when complemented with either Cg-PimB or Mt-PimB.

These results raised several interesting questions and possibilities concerning the biochemical role and function of Cg-PimB and Mt-PimB. The immediate question being whether Cg-PimB represents a GDP-mannose-dependent α-mannosyltransferase or adds glucopyranosyluronic acid to a diacylated glycerol precursor. During our purification process of polar lipids, we isolated and characterized in the C. glutamicum 13032::pimB disruption mutant a second phosphorus-free glycolipid, which we termed Gl-A (Fig. 2). Gl-A was subsequently analyzed both in negative and positive mode MALDI-MS. Spectra were only obtained in positive mode, revealing a molecular ion at m/z 815 (M – H + 2Na)⁺ (Fig. 9), which is in agreement with previous reports of a 1,2-di-O-acetyl-(α-D-glucopyranosyluronic acid)-(1→3)-glycerol, which would represent the precursor of ManGlcAGroAc₂ as GlcAGroAc₂ (46). This would suggest that PimB represents a bona fide α-mannosyltransferase, which we have now reassigned as an α-mannosyl-glucopyranosyluronic acid-transferase A (MgtA), to a diacylated glucuronosyl glycerol involved in ManGlcAGroAc₂ biosynthesis, since the synthesis of GlcAGroAc₂ remained unaffected in the C. glutanicum 13032::pimB disruption mutant. This assignment is wholly compatible with our in vitro mannosylation data. Interestingly, we observed no consistent accumulation of Gl-A as might be expected, although the amounts of the lipid were increased in some cultures. Presumably, the regulation of this pathway is complex and will require careful study.

Glycosylated diacylglycerols are commonly found in Gram-positive bacteria as well as in higher plants. It was previously established that these glycosylated diacylglycerols function as precursors/anchors for hyperglycosylated variants, such as the lipomannans, as found in the case of dimannosyl diacylglycerols, except for a few limited cases, such as a diglucosyl diacylglycerol (58). The presence of glycosyl diacylglycerols is commonly found in Mycobacterium and Micrococcus and lipoteichoic acids (47–49). Moreover, related di- and monoacylglycerols containing glucuronosyl residues have been well reported in Pseudomonas spp. (50–52), Bacillus cereus T (53), and halotolerant bacteria (54–57). In relation to Mycobacterium, the data are sparse in regards to the presence of glycosyl diacylglycerols, except for a few limited cases, such as a diglucosyl diacylglycerol (58). The presence of the uronic acid residue in ManGlcAGroAc₂ is interesting due to its rarity with regard to the published literature, especially with reference to Mycobacterium spp. In mycobacteria to date, a glucuronosyl diacylglycerol in Mycobacterium smegmatis (46), uronosyl-containing glycopeptidolipid in Mycobacterium
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