Identification of a novel $\alpha(1\rightarrow6)$ mannosyltransferase MptB from Corynebacterium glutamicum by deletion of a conserved gene, NCgl1505, affords a lipomannan- and lipoarabinomannan-deficient mutant

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Summary

Mycobacterium tuberculosis and Corynebacterium glutamicum share a similar cell wall structure and orthologous enzymes involved in cell wall assembly. Herein, we have studied C. glutamicum NCgl1505, the orthologue of putative glycosyltransferases Rv1459c from M. tuberculosis and MSMEG3120 from Mycobacterium smegmatis. Deletion of NCgl1505 resulted in the absence of lipomannan (Cg-LM-A), lipoarabinomannan (Cg-LAM) and a multi-mannosylated polymer (Cg-LM-B) based on a 1,2-di-O-C16/C18:1-(\(\alpha\)-D-glucopyranosyluronic acid)-(1\(\rightarrow\)3)-glycerol (GlcAGroAc2) anchor, while syntheses of triacylated-phosphatidyl-myoinositol dimannoside (Ac1PIM2) and Man1GlcAGroAc2 were still abundant in whole cells. Cell-free incubation of C. glutamicum membranes with GDP-[14C]Man established that C. glutamicum synthesized a novel \(\alpha(1\rightarrow6)\)-linked linear form of Cg-LM-A and Cg-LM-B from Ac1PIM2 and Man1GlcAGroAc2 respectively. Furthermore, deletion of NCgl1505 also led to the absence of in vitro synthesized linear Cg-LM-A and Cg-LM-B, demonstrating that NCgl1505 was involved in core \(\alpha(1\rightarrow6)\) mannan biosynthesis of Cg-LM-A and Cg-LM-B, extending Ac1PI[^14C]M2 and[^14C]Man1GlcAGroAc2 primers respectively. Use of the acceptor \(\alpha\)-D-Manp-(1\(\rightarrow\)6)-\(\alpha\)-D-Manp-O-C8 in an in vitro cell-free assay confirmed NCgl1505 as an \(\alpha(1\rightarrow6)\) mannosyltransferase, now termed MptB. While Rv1459c and MSMEG3120 demonstrated similar in vitro \(\alpha(1\rightarrow6)\) mannosyltransferase activity, deletion of the Rv1459c homologue in M. smegmatis did not result in loss of mycobacterial LM/LAM, indicating a functional redundancy for this enzyme in mycobacteria.

Introduction

The taxon Corynebacterinae belongs to the Actinomycetes family which includes human pathogens, such as Mycobacterium tuberculosis, Mycobacterium leprae and Corynebacterium diphtheriae, the causal agents of tuberculosis, leprosy and diphtheria respectively (Coyle and Lipsky, 1990; Bloom and Murray, 1992). Some animal pathogens, for instance, Corynebacterium pseudotuberculosis and Corynebacterium matruchotii (Coyle and Lipsky, 1990; Funke et al., 1997; Stackebrandt et al., 1997), also belong to the Corynebacterinaeae. In addition, the family member Corynebacterium glutamicum is widely used for the industrial production of amino acids (Eggeling and Bott, 2005). These bacilli share a unique cell wall ultra-structure that is composed of a mycolyl-arabinogalactan-peptidoglycan (mAGP) complex (Daffe et al., 1990; McNeil et al., 1990; 1991; Besra et al., 1995; Brennan, 2003; Dover et al., 2004). The esterified mycolates of the mAGP complex are considered to be packed side by side and are intercalated by lipids and glycolipids. This combined lipid structure gives rise to an asymmetric bilayer critical for the survival of these organisms (Minnikin et al., 2002).
In addition to the mAGP complex, other glycolipids, such as phosphatidylyl-myo-inositol (PI) mannosides (PIMs) and lipoglycans, termed lipomannan (LM) and lipoarabinomannan (LAM), are also found in this outer leaflet (Hill and Ballou, 1966; Brennan and Ballou, 1967; Brennan and Nikaido, 1995; Besra et al., 1997; Morita et al., 2004). However, LM and LAM possess important physiological functions, and play key roles in the modulation of the host response during infection (Schlesinger et al., 1994; Chatterjee and Khoo, 1998; Nigou et al., 2002; Maeda et al., 2003). The modulation of the immune response by LAM has been attributed to its terminal-capping motif (Nigou et al., 2002; 2003). Different permutations of LAM capping have been found in Mycobacterium strains, including ManLAM (Chatterjee et al., 1993; Khoo et al., 1995), PILAM (Gilleron et al., 1997) and (non-capped) LAM (Guerardel et al., 2002). Slow-growing mycobacteria, such as M. tuberculosis and M. leprae, produce ManLAM, which enables them to infect macrophages and dendritic cells (Schlesinger et al., 1994; Tascon et al., 2000). ManLAM inhibits the production of proinflammatory cytokines, such as IL-12 and TNF-α and inhibits phagosomal maturation (Knutson et al., 1998; Nigou et al., 2002; Fratti et al., 2003), while PILAM from the non-pathogenic fast-growing Mycobacterium smegmatis strain induces the proliferation of these cytokines (Adams et al., 1993; Gilleron et al., 1997).

The current model of lipoglycan biosynthesis follows a linear pathway, PI→PIM→LM→LAM (Besra and Brennan, 1997) (Fig. 1). PI is glycosylated by an α-mannopyranosyl (Manp) residue catalysed by PimA (Rv2610c), which transfers Manp from GDP-mannose to the 2-position of PI to form PIM1 (Kordulakova et al., 2002). PIM1 is further glycosylated by PimB (Rv0557), which may occur before, or after acylation of PIM1, by Rv2611c (Kordulakova et al., 2003) and results in the formation of Ac1PIM2 (Schaeffer et al., 1999). However, recently, this second mannosylation step in the biosynthesis of Ac1PIM2 has now been shown to be catalysed by PimB′ (Rv2188c, NCgl2106), while PimB (Rv0557, NCgl0452), now termed MgtA, is involved in synthesizing a novel mannosylated glycolipid, 1,2-di-O-C10-C18:1-(α-D-mannopyranosyl)-(1→4)-(α-D-glucopyranosyluronic acid)-(1→3)-glycerol (ManpGlcAGroAc2) (Tatituri et al., 2007; Lea-Smith et al., 2008; Mishra et al., 2008). The analysis of deletion mutants of NCgl0452 and NCgl2106 established that this glycolipid is further modified to produce a multi-mannosylated derivative, Man₁₂₋₁₅GlcAGroAc₂ (Cg-LM-B) which is coincident on SDS-PAGE with PI-based Cg-LM, which is now termed Cg-LM-A (Tatituri et al., 2007; Lea-Smith et al., 2008; Mishra et al., 2008). Previous studies have shown that RvD2-ORF1 from M. tuberculosis CDC1551, designated as PimC, catalysed further α-mannosylation of Ac₃PIM₄ resulting in Ac₄PIM₅ (Kremer et al., 2002). Recently, PimE (Rv1159) has been shown to be involved in higher PIM biosynthesis and directly in the biosynthesis of Ac₄PIM₅ (Morita et al., 2006), however, the enzyme responsible for the synthesis of Ac₄PIM₄ from Ac₃PIM₃ remains to be identified.

The point at which lipoglycan biosynthesis continues probably occurs after Ac₄PIM₅ and Man₁₂₋₁₅GlcAGroAc₂ in C. glutamicum (Gibson et al., 2003; Tatituri et al., 2007; Mishra et al., 2008), where a transition occurs from glycosyltransferases which utilize nucleotide sugars (i.e. GDP-Man) as substrate to glycosyltransferases which utilize polypropyl-phosphate sugars (i.e. polypropyl-phosphomannomannose, PPM) and which belong to the GT-C superfamily, and are membrane-bound (Liu and Mush-egian, 2003). Recently, we (Mishra et al., 2007) and others (Kaur et al., 2007) reported a novel α-mannosyltransferase, MptA (Rv2174), involved in the
latter stages of Ms-LM/LAM, Cg-LAM, Cg-LM-A and Cg-LM-B biosynthesis in Corynebacterineae. The core mannann backbone is further glycosylated by Rv2181 and results in the synthesis of α(1→2)-Manp-linked branches, characteristic of the mannann backbone in LM and LAM (Kaur et al., 2006). The mature LM is then elaborated with arabinose by the essential arabinofuranosyltransferase EmbC (G.S. Besra, unpubl. res.) to form LAM (Berg et al., 2005). Recently, a novel mannosyltransferase, Rv1635c (and MT1671), has been shown to add terminal Manp residues to the mature LAM in M. tuberculosis to form ManLAM (Dinadayala et al., 2006; Appelmelk et al., 2007). However, the enzyme involved in the early stages of linear LM/LAM mannann core biosynthesis through an α(1→6) mannosyltransferase prior to MptA remains to be identified (Fig. 1).

In this study, we have examined the function of C. glutamicum NCgl1505, and its orthologous genes Rv1459c of M. tuberculosis and MSMEG3120 of M. smegmatis encoding a putative GT-C glycosyltransferase. The NCgl1505 gene and its orthologues based on the results described below have been designated as mptB, as an acronym for mannopyranosyltransferase B. Null mutants of C. glutamicum together with in vitro cell-free assays established that NCgl1505 is a key α(1→6) mannosyltransferase involved in the initiation of core mannannbiosynthesis of Cg-LM-A and Cg-LM-B from Corynebacterineae extending Ac1PIM2 and ManGlcAGroAc2 respectively. In addition, the M. tuberculosis orthologue Rv1459c and M. smegmatis MSMEG3120 demonstrated α(1→6) mannosyltransferase activity in a membrane-based in vitro assay when utilizing a C. glutamicum ΔmptBΔmptA double mutant complemented with either plasmid-encoded Rv1459c or MSMEG3120. Finally, using a M. smegmatis null mutant of MSMEG3120, we also demonstrate that the mycobacterial orthologue of NCgl1505 is functionally redundant.

Results

Genome locus and structural features of Rv1459c/NCgl1505

Glycosyltransferases belonging to the GT-C superfamly have been shown by us (Alderwick et al., 2005; Alderwick et al., 2006b; Mishra et al., 2007; Seidel et al., 2007) and others (Dinadayala et al., 2006; Kaur et al., 2006; 2007; Morita et al., 2006) to play important roles in the biosynthesis of the cell wall heteropolysaccharides arabinogalactan (AG), LM-A, LM-B and LAM in Corynebacterineae. Our attention was recently drawn to a putative glycosyltransferase encoded by M. tuberculosis Rv1459c and C. glutamicum NCgl1505, which are members of the GT-C family of glycosyltransferases.

Orthologues of these genes are present in all Mycobacterium and Corynebacterium species as well as the sequenced Nocardia farcinica IFM 10152 and Rhodococcus sp. RHA1 strains (Fig. 2A). In addition, this gene is retained in M. leprae, supporting the hypothesis that NCgl1505 encodes for a protein possessing a vital function inherent to this group of bacteria.

The glycosyltransferase encoded by NCgl1505 is a polytopic membrane protein, which is comprised of 558 amino acid (aa) residues, and is predicted to encode 15 hydrophobic segments (HSs) (Fig. 2B). Rv1459c constitutes 591 aa, with the additional length mostly due to an extended loop between HSs 7 and 8. This loop extension is not present in Mycobacterium paratuberculosis or M. smegmatis. It contains a number of repeated Pro and Arg residues, and similarly highly charged repeat sequences are found in loop regions of other transporters, without having a specific function (Eng et al., 1998; Vrijic et al., 1999). The sequence identity of the orthologues NCgl1505 and Rv1459c is 37% (52% similarity) and can therefore be considered very high. The strongest conserved regions are found in loops connecting HSs and adjacent regions with intermediate hydrophobicity, like those between HSs 3–4, HSs 7–8 and HSs 13–14 (Fig. 2B). Within the highest conserved regions; five of the six fully conserved acidic Asp and Glu residues are located, given as D and E in Fig. 2B, which are known to play important roles as general bases and nucleophiles in enzyme catalysis. They are also retained in the MptB orthologue in N. farcinica IFM 10152 and Rhodococcus sp. RHA1 are therefore likely to be involved in catalysis, or in interactions with the sugar donor or acceptor (Liu and Mushegian, 2003). Interestingly, among the glycosyltransferases of M. tuberculosis and C. glutamicum previously identified (Alderwick et al., 2006a; Dinadayala et al., 2006; Kaur et al., 2006; Morita et al., 2006; Seidel et al., 2007), NCgl1505 and Rv1459c possess the highest identities to the recently identified mannosyltransferase MptA (Kaur et al., 2007; Mishra et al., 2007) and, based on the results described below, the NCgl1505 gene and its orthologues have been designated as MptB.

Construction and growth of C. glutamicum ΔmptB and complemented strains

In order to delete mptB in C. glutamicum, the non-replicative plasmid pK19mobsacBΔmptB was constructed carrying sequences adjacent to Cg-mptB. Using this vector, C. glutamicum was transformed to kanamycin resistance, indicating integration of the vector into the genome by homologous recombination (Fig. 2C). The sacB gene enables for selection of loss of vector in a second homologous recombination event, which can
result either in the original wild-type genomic organization or in clones deleted of Cg-mptB. Ninety clones exhibiting the desired phenotype of vector loss (kanamycin-sensitive, sucrose-resistant) were analysed by PCR, but only one single colony was found to have Cg-mptB excised, whereas the others resulted in a wild-type genotype. The low number of recombinant knockouts indicates that the loss of Cg-mptB is apparently a disadvantage for cell viability, similar to that of previously observed mutants with altered mycolate (Gande et al., 2004) or arabinogalactan biosynthesis (Alderwick et al., 2006b). The resulting clone was subsequently termed C. glutamicum DmptB and confirmed by PCR with different primer pairs to have Cg-mptB deleted, whereas controls with C. glutamicum wild type resulted in the expected larger amplification product (Fig. 2C).

In liquid culture, growth of C. glutamicum DmptB was very poor. Only when rich brain heart infusion (BHI) medium was used was a growth rate of 0.13 h⁻¹ obtained (Fig. 2D) in comparison with wild-type C. glutamicum growth rate of 0.31 h⁻¹ (Mishra et al., 2007) and, on the same medium supplemented with 500 mM sorbitol (BHIS), the growth rate was 0.51 h⁻¹, which is still lower than that of the wild type on this medium (0.70 h⁻¹). C. glutamicum DmptB was transformed with pVWEx-Cg-mptB and the resultant complemented strain exhibited a growth rate of 0.66 h⁻¹, almost superimposable to that of the wild type in BHIS medium.
Polar lipid analysis of C. glutamicum and C. glutamicumΔmptB

Lyophilized cells were extracted using petroleum-ether and methanolic saline to initially recover apolar lipids. Further processing of the methanolic extract afforded the polar lipid fraction which was examined by two-dimensional thin-layer chromatography (2D-TLC). In both the wild-type C. glutamicum and C. glutamicumΔmptB, Ac1PIM2 and Man1GlcAGroAc2 (Tatituri et al., 2007) were visualized either by α-naphthol/sulphuric acid (specific for sugars), 5% ethanolic molybdophosphoric acid (general lipid stain) (Fig. S1) or Dittmer and Lester reagent (specific for phospholipids). In both C. glutamicum and C. glutamicumΔmptB, no products could be observed which correspond to higher PIMs (i.e. Ac1PIM3 through to Ac1PIM6) or higher mannose variants of Man1GlcAGroAc2 (Tatituri et al., 2007; Mishra et al., 2008). The presence of only Ac1PIM2 and Man1GlcAGroAc2, and the inability to synthesize Cg-LAM, Cg-LM-A and Cg-LM-B by C. glutamicumΔmptB (as shown below) demonstrated that MptB is involved in the early steps of α1(→6) mannan core biosynthesis by extending the substrates Ac1PIM2 and Man1GlcAGroAc2.

Analysis of lipoglycans from C. glutamicum, C. glutamicumΔmptB and C. glutamicumΔmptB pVWEx-Cg-mptB

Lipoglycans were extracted by refluxing delipidated cells in ethanol, followed by hot-phenol extraction, protease digestion and dialysis to remove impurities. The extracted lipoglycans were examined initially on 15% SDS-PAGE (Fig. 3A). Extracts from wild-type C. glutamicum showed the presence of Cg-LAM, Cg-LM-A and Cg-LM-B with the latter product based on previous results comigrating with Cg-LM-A (Tatituri et al., 2007; Mishra et al., 2008), while all of these lipoglycans were absent from C. glutamicumΔmptB. Complementation of C. glutamicumΔmptB by transformation with plasmid pVWEx-Cg-mptB restored the wild-type phenotype (Fig. 3A). In addition, transformation of C. glutamicumΔmptB with plasmid pVWEx-Cg-mptA failed to restore the wild-type phenotype (data not shown).

Construction and growth of C. glutamicumΔmptAΔmptB and complemented strains

As a result of the similarity of MptB with MptA, we wanted to exclude any possible interferences and constructed a
strain of C. glutamicum deficient in mptB and mptA. For this purpose, C. glutamicumΔmptB was transformed with plasmid pK19mobsacBΔmptA (Mishra et al., 2007) and processed as described in Experimental procedures to afford the double mutant, C. glutamicumΔmptBΔmptA. Analysis of this strain showed that its growth characteristics were very similar to C. glutamicumΔmptB (data not shown). For further analysis, C. glutamicumΔmptAΔmptB was transformed with plasmid-encoded Cg-mptB, Cg-mptA, Mt-mptB and Ms-mptB.

Analysis of lipoglycans from C. glutamicumΔmptBΔmptA, C. glutamicumΔmptBΔmptA pVWEx-Cg-mptB and C. glutamicumΔmptBΔmptA pVWEx-Cg-mptA

In addition to MptB, C. glutamicum possesses the known α(1→6) mannosyltransferase MptA, which is involved in the later stages of core mannan biosynthesis (Mishra et al., 2007) and, as a result, we wanted to study the in situ specificity of these glycosyltransferases. For this purpose, lipoglycans were extracted from C. glutamicumΔmptBΔmptA, and from the same strain carrying either pVWEx-Cg-mptB or pVWEx-Cg-mptA and analyzed by 15% SDS-PAGE (Fig. 3B). Extracts from C. glutamicumΔmptBΔmptA indicated that, as expected, no lipoglycans were present, whereas the presence of pVWEx-Cg-mptB resulted in formation of a truncated (Cg-t) version of Cg-LM-A and Cg-LM-B (Mishra et al., 2007; 2008). However, lipoglycan extracts from C. glutamicumΔmptBΔmptA carrying pVWEx-Cg-mptA were identical to that of C. glutamicumΔmptBΔmptA, indicating that MptA fails to substitute for MptB in the double mutant. As pVWEx-Cg-mptA results in functional MptA (Mishra et al., 2007), this result shows that MptA is unable to substitute in vivo for MptB. Therefore, both MptA and MptB are distinct and MptB is involved in the initial steps of Cg-LAM, Cg-LM-A and Cg-LM-B biosynthesis, prior to MptA. Furthermore, analysis of C. glutamicumΔmptBΔmptA carrying either pVWEx-Mt-mptB or pVWEx-Ms-mptB resulted in a complete lack of lipoglycan biosynthesis (data not shown), indicating that Mt-MptB and Ms-MptB do not function in vivo as the initial α(1→6) mannosyltransferase probably because of an inability to extend Ac1PI[M2 and Man1GlcAGroAc2 by mannose residues as shown below through in vitro chase experiments.

In vitro incorporation of radiolabelled Man from GDP-[14C]Man into membrane lipids utilizing C. glutamicum, C. glutamicumΔmptB and complemented strains

Incorporation of [14C]Man from GDP-[14C]Man into CHCl3/CH3OH (2:1) and CHCl3/CH3OH/H2O (10:10:3)-soluble lipids was examined using membrane/cell envelope extracts prepared from C. glutamicum as described previously utilizing mycobacterial membrane/cell envelope fractions (Besra et al., 1997). TLC autoradiography (Fig. 4A, lane 1) of the CHCl3/CH3OH (2:1)-soluble lipids synthesized by wild-type C. glutamicum membrane/cell envelope extracts contained as expected β-D-mannopyranosyl-1-monophosphoryl-decaprenol ([C50-PP][14C]M), [14C]Man1GlcAGroAc2 and Ac1PI[14C]M2. The identity of the three labelled lipids was established by: (i) base treatment, i.e. degradation of Ac1PI[14C]M2 and [14C]Man1GlcAGroAc2 (Fig. 4A, lane 2), (ii) addition of amphomycin, which specifically chelates polypropenyl phosphates in the presence of Ca2+ and thus inhibiting the transfer of Man from GDP-Man to polypropenyl carriers (Fig. 4A, lane 3) and (iii) in comparison with known standards (Tatituri et al., 2007). As expected from the analysis of whole cells, C. glutamicumΔmptB synthesized comparable levels of all three radiolabelled lipids using membrane/cell envelope extracts prepared from C. glutamicumΔmptB (Fig. 4A, lane 4).

The above reaction mixtures were then further processed as described in the Experimental procedures section to provide the CHCl3/CH3OH/H2O (10:10:3)-soluble lipids initially using membrane/cell envelope extracts prepared from C. glutamicum to provide [14C]mannooligosaccharides (Fig. 4B, no. 1), which were further characterized by a series of degradation experiments. The [14C]mannooligosaccharides were sensitive to acetylation (see Fig. 4B, no. 3), thus establishing a core α(1→6)-linear mannan backbone within the CHCl3/CH3OH/H2O (10:10:3)-soluble lipids. In separate experiments, the addition of amphomycin to block C50-PP[14C]M synthesis also inhibited the synthesis of the α(1→6)-linear mannan lipids, demonstrating that the synthesis of these CHCl3/CH3OH/H2O (10:10:3)-soluble lipids is PPM-dependent (Fig. 4B, no. 2) and similar to the previously characterized in vitro synthesized mycobacterial products (Besra et al., 1997). SDS-polyacrylamide gel electrophoresis and subsequent autoradiography of the dried gels demonstrated that the CHCl3/CH3OH/H2O (10:10:3)-soluble lipids (Fig. 4B, no. 1) had slightly reduced mobility, indicating that they were smaller in size (Fig. 4B, left-panel inset), presumably because of their lack of α(1→2) branching characteristic of Cg-LM-A and Cg-LM-B (Tatituri et al., 2007). As expected, synthesis of CHCl3/CH3OH/H2O (10:10:3)-soluble lipids using membranes from C. glutamicumΔmptB was completely abolished (Fig. 4B, no. 4). Furthermore, complementation with pVWEx-Cg-mptB restored synthesis of CHCl3/CH3OH/H2O (10:10:3)-soluble lipids (Fig. 4B, no. 5).
Incorporation of [14C]Man from GDP-[14C]Man into corynebacterial membrane/cell envelope lipids.

A. TLC autoradiography of labelled CHCl₃/CH₃OH (2:1)-soluble lipids, C₅₀-PP[¹⁴C]M, [¹⁴C]Man1GlcAGroAc₂ and Ac₁PI[¹⁴C]M₂ using GDP-[¹⁴C]Man and membrane/cell envelope extracts from C. glutamicum and C. glutamicum ΔmptB. Membrane/cell envelope fractions were incubated with GDP-[¹⁴C]Man in a total volume of 100 µl for 60 min in either the absence or presence of amphotomycin (10 µg) and Ca²⁺ ions per reaction mixture pre-incubated with membranes for 15 min. Enzymatically synthesized products C₅₀-PP[¹⁴C]M, [¹⁴C]ManGlcAGroAc₂ and Ac₁PI[¹⁴C]M₂ were isolated as described in Experimental procedures to provide washed CHCl₃/CH₃OH (2:1)-soluble lipids and also subjected to base treatment. Aliquots (10%) were taken for scintillation counting and the remaining products subjected to TLC/autoradiography using CHCl₃/CH₃OH/NH₄OH/H₂O (65:25:0.4:3.6, v/v/v/v). C. glutamicum CHCl₃/CH₃OH (2:1)-soluble lipids (lane 1), base treatment of CHCl₃/CH₃OH (2:1)-soluble lipids (lane 2), amphotomycin treatment (lane 3) and C. glutamicum ΔmptB CHCl₃/CH₃OH (2:1)-soluble lipids (lane 4).

B. Characterization of CHCl₃/CH₃OH/H₂O (10:10:3)-soluble lipids as α(1→6)-linear mannooligosaccharides. The insoluble pellet from the above reaction mixtures following extraction with CHCl₃/CH₃OH (2:1) was sequentially washed with 0.9% NaCl in 50% CH₃OH, 50% CH₃OH and CH₃OH, prior to extraction with CHCl₃/CH₃OH/H₂O (10:10:3) and an aliquot (10%) taken for scintillation counting and the remaining product analysed by SDS-PAGE/autoradiography (left-panel inset). C. glutamicum (no. 1), amphotomycin treatment (no. 2) and acetolysis treatment of CHCl₃/CH₃OH/H₂O (10:10:3)-soluble lipids (no. 3), C. glutamicum ΔmptB (no. 4) and C. glutamicum ΔmptB pVWE-Cg-mptB (no. 5) as described in the Experimental procedures.

C and D. Incorporation of in vitro in situ Ac₁PI[¹⁴C]M₂ and [¹⁴C]Man,GlcAGroAc₂ into α(1→6)-linear mannooligosaccharides with either C. glutamicum, C. glutamicumΔmptB or C. glutamicumΔmptB pVWE-Cg-mptB membrane preparations. Membranes were initially pre-treated with amphotomycin, labelled using GDP-[¹⁴C]Man, re-harvested by centrifugation and extensively washed with buffer. At t = 0 min, an aliquot of membranes (20%) was processed as described in the Experimental procedures for CHCl₃/CH₃OH (2:1)-soluble lipids and analysed by TLC/autoradiography using CHCl₃/CH₃OH/NaOH/H₂O (65:25:0.4:3.6, v/v/v/v) (C) and CHCl₃/CH₃OH/H₂O (10:10:3)-soluble lipids by SDS-PAGE/autoradiography (D). The carefully washed [¹⁴C]-labelled membranes were re-incubated for a further 60 min following the addition of 0.5 mg cold C₅₀-PPM (Gurcha et al., 2002). At t = 60 min, an equivalent membrane aliquot as based on t = 0 was again analysed for CHCl₃/CH₃OH (2:1) and CHCl₃/CH₃OH/H₂O (10:10:3)-soluble lipids as described above (C and D).
Amphotericin-treated wild-type *C. glutamicum* membrane/cell envelope extracts were initially pulsed with GDP-[^14]C]Man during a short incubation period (15 min) which was shown earlier to inhibit the synthesis of the CHCl3/CH3OH/H2O (10:10:3)-soluble lipids but, instead of extracting with CHCl3/CH3OH (2:1), the[^14]C]Man-labelled membranes were re-harvested by ultracentrifugation at 100,000 g, carefully washed and re-centrifuged twice using cold buffer, to remove unused GDP[^14]C]Man. An aliquot of the[^14]C]Man-labelled membranes were extracted with CHCl3/CH3OH (2:1) and contained as expected solely Ac[P[^14]C]M2 (3329 c.p.m.) and[^14]C]Man,GlcAGroAc2 (2530 c.p.m.) as determined by TLC autoradiography and phosphorimaging (Fig. 4C). The CHCl3/CH3OH/H2O (10:10:3)-soluble lipids at t = 0 gave 226 c.p.m. The[^14]C]Man-labelled membranes were then further incubated for 60 min following the addition of excess exogenous cold C50-PPM (Gurcha et al., 2002) prior to the standard extraction method to provide CHCl3/CH3OH (2:1) and CHCl3/CH3OH/H2O (10:10:3)-soluble lipids. The t = 60 chase time revealed a loss of radioactivity from both Ac[P[^14]C]M2 (1709 c.p.m.) and[^14]C]Man,GlcAGroAc2 (5474 c.p.m.) to 240 c.p.m. The[^14]C]Man-labelled primers Ac1PI[^14]C]M2 and[^14]C]Man,GlcAGroAc2 remained as expected solely Ac1PI[^14]C]M2 (3329 c.p.m.) and[^14]C]Man,GlcAGroAc2 (2530 c.p.m.) as determined by TLC autoradiography with the corresponding products of[^14]C]mannoooligosaccharide lipids (2895 c.p.m.) (Fig. 4D). The *in vitro in situ* chase experiment demonstrated that the α(1→6)-[^14]C]mannooligosaccharide lipids were produced from both Ac[P[^14]C]M2 and[^14]C]Man,GlcAGroAc2. Similar experiments repeated with *C. glutamicum ΔmptB in situ* prepared[^14]C]-labelled membranes as above resulted in comparable products at t = 0 and t = 60 for CHCl3/CH3OH (2:1)-soluble lipids [Ac[P[^14]C]M2 (t = 0, 3345 c.p.m.; t = 60, 2968 c.p.m.) and[^14]C]Man,GlcAGroAc2 (t = 0, 5840 c.p.m.; t = 60, 5025 c.p.m.)] and a lack of the synthesis of α(1→6)-[^14]C]mannooligosaccharide lipids (240 c.p.m.) from the elongation primers Ac[P[^14]C]M2 and[^14]C]Man,GlcAGroAc2 following the ‘chase period’ (Fig. 4C and D). Complementation of *C. glutamicum ΔmptB* by transformation with plasmid pVWEx-Cg-mptB resulted in Ac[P[^14]C]M2 (t = 0, 3229 c.p.m.; t = 60, 1725 c.p.m.) and[^14]C]Man,GlcAGroAc2 (t = 0, 5367 c.p.m.; t = 60, 2550 c.p.m.) and *in vitro in situ* synthesis of α(1→6)-[^14]C]mannooligosaccharide lipids (2471 c.p.m.) to levels comparable to wild type *C. glutamicum* (Fig. 4C and D). The data clearly demonstrate that Cg-MptB functions *in vivo* and *in vitro* as the initial α-mannosyltransferase, which extends Cg1PIM2 and Man1GlcAGroAc2. However, under the same *in vitro in situ* chase conditions, *C. glutamicum ΔmptB pVWEx-Mt-mptB* (or pVWEx-Ms-mptB) failed to elongate the primers Ac[P[^14]C]M2 and[^14]C]Man,GlcAGroAc2 and restore synthesis of the α(1→6)-[^14]C]mannooligosaccharides (data not shown). In addition, experiments conducted with *C. glutamicum ΔmptB pVWEx-Mt-mptB* and *C. glutamicum ΔmptA pVWEx-Ms-mptB* and the addition of the exogenous primer Ac[P[^14]C]M2 isolated from a *M. bovis* BCG PimE mutant also failed to restore the synthesis of the α(1→6)-[^14]C]mannooligosaccharides (data not shown).

In *vitro analysis of α(1→6) mannosyltransferase activity using* *C. glutamicum ΔmptB, C. glutamicum ΔmptB ΔmptA and complemented strains*

Initial attempts to develop an *in vitro* assay using either purified recombinant-expressed MptB, or *Escherichia coli* membranes harbouring the protein, have thus far proved unsuccessful. Alternatively, we assessed the capacity of membrane preparations from *C. glutamicum* and its recombinant strains to catalyse α(1→6) mannosyltransferase activity in a previously defined acceptor assay utilizing the neoglycolipid acceptor α-D-Manp-(1→6)-α-D-Manp-O-C6 and C50-PP[^14]C]M as a sugar donor (Brown et al., 2001) (Fig. 5A). The TLC autoradiography of products from *in vitro* assays when assayed with wild-type *C. glutamicum* resulted in the formation of product X, a trisaccharide α-D-[^14]C]Manp-(1→6)-α-D-Manp-(1→6)-α-D-Manp-O-C6, and product Y, a tetrasaccharide α-D-[^14]C]Manp-(1→6)-α-D-Manp-(1→6)-α-D-Manp-O-C6 (Fig. 5B). These products comigrated on TLC autoradiography with the corresponding products from *in vitro* in *C. glutamicum*ΔmptB (89 217 ± 4269 c.p.m.) in comparison with wild-type *C. glutamicum* (92 325 ± 5017 c.p.m.) (Fig. 5B). This reduction in activity corresponded to the residual α(1→6) mannosyltransferase activity observed in *C. glutamicum ΔmptA* (2053 ± 604 c.p.m.) (Fig. 5B) (Mishra et al., 2007). These results suggested the presence of two α(1→6) mannosyltransferase activities utilizing this neoglycolipid acceptor, catalysed by MptA and MptB, with the former more efficiently utilizing the neoglycolipid acceptor as a substrate. Assays containing membrane preparations from *C. glutamicum ΔmptB ΔmptA* showed no product formation on TLC, indicating a complete abrogation of both α(1→6) manno-
ferase activities from C. glutamicum (Fig. 5B). Analysis of the double mutant with pVWEx-Cg-mptB revealed a significant but weak band (2682 ± 940 c.p.m.) corresponding to product X on TLC analysis; however, when complemented with pVWEx-Cg-mptA, a similar phenotype to that of C. glutamicum ΔmptB could be observed (80 614 ± 4135 c.p.m. for X), although at a slower transfer rate. The data confirmed that NCGL1505 is an α(1→6) mannopyranosyltransferase; however, the specific α(1→6) mannopyranosyltransferase activity is much lower in comparison with MptA, under the assay conditions utilizing the neoglycolipid acceptor.

In vitro and mutational analysis of the mycobacterial MptB

To study the function of the mycobacterial MptB, we transformed the C. glutamicum ΔmptBΔmptA double mutant with a plasmid containing either M. tuberculosis Rv1459c (pVWEx-Mt-mptB) or M. smegmatis MSMEG3120
Membrane preparations of these strains restored in vitro \( \alpha(1\rightarrow6) \) mannopyranosyltransferase activity (Fig. 5D) by formation of the trisaccharide product \( X \) (Mt-MptB, 3159 c.p.m. and Ms-MptB, 2949 c.p.m.) to a similar level to that of the isogenic strain with pVWEx-Cg-mptB (Fig. 5B), showing that the \( M. \) tuberculosis and \( M. \) smegmatis gene could restore activity in an in vitro cell-free assay with the \( C. \) glutamicum double mutant. We then generated a null mutant of \( M. \) smegmatis mc\(^{\text{c155}} \) MSMEG3120 (homologue of \( \text{Rv1459c} \)) using specialized transduction (Fig. 6A), and analysed total lipids and lipoglycans in the mutant strain. Surprisingly, the mutant strain \( \Delta \)MSMEG3120 had a total lipid profile identical to the parental wild-type strain \( M. \) smegmatis mc\(^{\text{c155}} \) (TLC system designed to separate PIMs and other phospholipids is shown in Fig. 6B) and also synthesized LM and LAM (Fig. 6C). These results suggested that MSMEG3120, unlike its corynebacterial counterpart, was redundant and it was likely that another \( \alpha \)-mannosyltransferase compensated for the loss of its function in the \( \Delta \)MSMEG3120 mutant.

**Discussion**

Over the past decade, much research has been carried out on the mechanisms and genetics of mycobacterial cell
wall carbohydrate biosynthesis, particularly the formation of the essential AG (Daffe et al., 1993; Besra et al., 1995; Belanger et al., 1996; Kremer et al., 2001; Alderwick et al., 2005; 2006a,b; Berg et al., 2007; Seidel et al., 2007) and the immunomodulatory heteropolysaccharides LM and LAM (Schaeffer et al., 1999; Kordulakova et al., 2002; Kremer et al., 2002; Zhang et al., 2003; Dinadayala et al., 2006; Kaur et al., 2006; 2007; Mishra et al., 2007). An archetypal biosynthetic pathway is now emerging for the formation of these important macromolecules, which predominantly include enzymes from the GT-A, B and C superfamily of glycosyltransferases (Liu and Mushegian, 2003) (Fig. 1). PimA, PimB, PimB' and PimC, all of which are GT-A/B glycosyltransferases, have been shown to be involved in PIM biosynthesis, which serves as a substrate for LM/LAM extension and maturation (Schaeffer et al., 1999; Kordulakova et al., 2002; Kremer et al., 2002; Lea-Smith et al., 2008; Mishra et al., 2008). We and others recently identified the GT-C glycosyltransferase MptA as an α(1→6) mannosyltransferase involved in intermediate LM biosynthesis, specifically in distal α(1→6) core LM formation (Kaur et al., 2007; Mishra et al., 2007). Apart from a core α(1→6) mannan backbone, α(1→2) mannose residues punctuate LM, and the GT-C glycosyltransferase Rv2181 has been identified to be responsible for some, if not all, of these branched mannose residues (Kaur et al., 2006). At some point, LM is further glycosylated by other GT-C glycosyltransferases, such as EmbC for the biosynthesis of LAM (Zhang et al., 2003) and then mannosyl-capped (Dinadayala et al., 2006; Appelmelk et al., 2007).

In this study, we have characterized the role of a putative glycosyltransferase (NCgl1505) belonging to the GT-C superfamily of glycosyltransferases (Liu and Mushegian, 2003) by virtue of genomic deletion in C. glutamicum. We present MptB as a PPM-dependent α(1→6) mannosyltransferase, involved in early stages of proximal α(1→6) core Cg-LM-A and Cg-LM-B biosynthesis in C. glutamicum (Fig. 7).

Our initial in vivo and in vitro studies of PIM and Man1GlcAGroAc2 biosynthesis in C. glutamicum ΔmptB highlighted no apparent change in lipid profiles, compared with those from wild-type C. glutamicum (Figs S1 and 4A). It is reasonable to conclude from the data that MptB is not involved in either early PIM or Man1GlcAGroAc2...
biosynthesis. This was not surprising as these early biosynthetic steps are completely unique to enzymes belonging to the GT-A/B glycosyltransferase family, which utilize GDP-mannose as a substrate (Liu and Mushegian, 2003). Assays utilizing membrane preparations from *C. glutamicum* and *C. glutamicum*ΔmptB indicated that there was no further accumulation of higher mannosylated versions of PIMs and Man₄GlcAGroAc₂. The lack of higher mannosylated versions in *C. glutamicum* suggests that the next committed step in lipoglycan biosynthesis stems from Ac₁PIM₂ and Man₁GlcAGroAc₂ and that this is catalysed by Cg-MptB.

As a result of absence of MptB, *C. glutamicum*ΔmptB is unable to synthesize Cg-LAM, Cg-LM-A and Cg-LM-B *in vivo*, which is in contrast to our earlier studies on MptA, where a truncated Cg-LM-A and Cg-LM-B species was synthesized (Mishra et al., 2007). In *C. glutamicum*, we now also present *in vitro* evidence that Ac₆PIM₂ and Man₄GlcAGroAc₂ are acceptors for Cg-MptB, the first GT-α-mannosyltransferase committed to Cg-LM-A and Cg-LM-B biosynthesis. This is supported by *in vitro* *in situ* chase experiments elongating the Ac₆PIM₁[¹⁴C]M₂ and [¹⁴C]Man₄GlcAGroAc₂ primers by the sugar donor C₅₀-PPM. These crucial observations, together with the presence of Ac₆PIM₂ and Man₄GlcAGroAc₂, completely support our hypothesis that Cg-MptB mannosylates Ac₆PIM₂ and Man₄GlcAGroAc₂. Our previous experiments on glycosyltransferase activities in membranes prepared from *C. glutamicum*ΔmptA identified a residual α(1→6) mannosyltransferase activity (Mishra et al., 2007). This α-mannosyltransferase activity can now be attributed to the presence of MptB as, upon its deletion in *C. glutamicum*, a partial depletion in α(1→6) mannosyltransferase activity is observed and a complete loss of activity is found upon deletion of both Cg-mptA and Cg-mptB. These data together with the *in vivo* analyses identify MptB as a *bona fide* α(1→6) mannosyltransferase. Interestingly, α(1→6) mannan extension is more complex in *Mycobacterium* based on the evidence that Mt-MptB and M. smegmatis mptB are acceptors for Cg-MptB mannosyltransferase activity and suggest a slightly different substrate specificity of the MptB orthologues of *M. tuberculosis* and *M. smegmatis*. Although, clearly α(1→6) mannosyltransferase(s) based on *in vitro* data, studies are currently underway exploring heterologous protein expression systems for Mt-MptB and Ms-MptB in combination with a variety of substrates in a revised *in vitro* assay format.

Given the high degree of homology between the *C. glutamicum* and mycobacterial orthologues of MptB and the similar organization of neighbouring genes in the two genera, we expected deletion of *M. smegmatis* mptB (MSMEG3120) to have the same effect as that in *C. glutamicum*. However, surprisingly, the *M. smegmatis* mptB mutant still synthesised LM and LAM, indicating that another, yet unidentified, α-mannosyltransferase could substitute for MptB in the mutant *M. smegmatis* strain. It has been previously shown that a high degree of functional redundancy exists in key enzymes involved in mycobacterial cell wall assembly, for instance, PimB/PimB′ and MgtA (Schaeffer et al., 1999; Tatituri et al., 2007; Lea-Smith et al., 2008; Mishra et al., 2008), PimC (Kremer et al., 2002), and EmbA and EmbB (Berg et al., 2007) in PIM/LM/LAM and AG biosynthesis, and the antigen 85 complex in mycolic acid biosynthesis (Puech et al., 2002). In this particular case, the *C. glutamicum* mutant study enabled the assignment of function to the GT-C glycosyltransferase NCg1505, which would have otherwise not been possible if similar studies would have concentrated solely on mycobacterial species.

Interestingly, the mechanism of how Ac₆PIM₂ traverses the cytoplasmic membrane remains poorly understood. Bioinformatic inspection of the locus surrounding MptB has highlighted two possible candidates for potential flippases. Downstream of the putative glycosyltransferase Rv1459c, three conserved genes are located in all *Corynebacterinaeae* and the expression of the four-gene locus in *C. glutamicum* is translationally coupled (Wang et al., 2006). This presents strong evidence for a functional coupling of the putative glycosyltransferase Rv1459c with Rv1458c, Rv1457c and Rv1456c. The latter genes encode for two ABC transporter integral membrane proteins, with Rv1458c encoding for an ATP-dependent binding protein. Applying structure prediction comparisions and hidden Markov models (Soding et al., 2005), Rv1458c exhibits remote structural similarities to sugar-binding proteins of ABC carriers, such as the sugar-binding protein of *Pyrococcus horikoshii* or the maltose/maltodextrin-binding protein MALK of *E. coli* (Lu et al., 2005). Rv1457c encodes a permease component of an ABC-2-type transporter, characteristically involved in catalysing the export of drugs and carbohydrates (Reizer et al., 1992). As transmembrane channels of ABC-2-type transporters are either homo- or heterooligomers and Rv1456c has features of a transporter protein, it is plausible to suggest that the membrane channel coupled to the glycosyltransferase might be a heterooligomer made up of Rv1457c and Rv1456c. In a previous study, Wang et al. (2006) proposed that one or more of the proteins encoded by the orthologues of Rv1456c-Rv1459c gene locus in *C. matruchotii* was involved in mycolic acid transport. A transposon mutant with an insertion in the cluster had an altered mycolic acid profile. However, in light of the evidence described in this work, this change in mycolylation may be an indirect effect as a result of the loss of Cg-LAM and Cg-LM-A/B. Further examination of this gene locus is required for characterization of potential roles in mycolic acid and glycolipid transport across the membrane bilayer.
Experimental procedures

Bacterial strains and growth conditions

Corynebacterium glutamicum ATCC 13032 (referred to the remainder of the text as C. glutamicum) and E. coli DH5αmcrr were grown in Luria–Bertani broth (Difco) at 30°C and 37°C respectively. The recombinant strains generated in this study were grown on rich BHI medium (Difco), and the salt medium CGXII used for C. glutamicum as described (Eggeling and Bott, 2005). Kanamycin and ampicillin were used at a concentration of 50 μg ml⁻¹. Samples for lipid analysis were prepared by harvesting cells at an OD of 10–15, followed by a saline wash and freeze drying. The remainder of the text as C. glutamicum

Construction of plasmids and strains

The genes analysed were the orthologues of Rv1459c and NCgl1505 from M. tuberculosis and C. glutamicum, respectively, termed mptB. The vectors made were pVWEx-Mt-mptB, pVWEx-Ms-mptB, pVWEx-Cg-mptB, pET-Mt-mptB, pET-Cg-mptB and pK19mobscAB. To construct the deletion vector pK19mobscAB\_Mt\_mptB, cross-over PCR was applied with primer pairs AB (A, CGTATATGCTTTTATATTGCTTG; B, GAAATTGCTTGCTGTTGAC) and CD (C, TTTTAAATTTTAATTTTTATGATGTGATG; D, CAATCTGCTTTCTTGACATCTTCATTACAT). All primers used were PCR-amplified from DNA obtained from the NIH Tuberculosis Research Materials and Vaccine Testing Contract at Colorado State University. All other chemicals were of reagent grade and obtained from Sigma-Aldrich.

To enable expression of Cg-mptB in C. glutamicum, the primer pair Ms\_for (5′-CGCGTGTCGA CAAGGAGATATAGTATGATGTCGGATCGGCCTGTCG-3′) and Ms\_rev (5′-CCGGAAATTTCTACGGGATTCGAGTGTA GGGTC-3′) was used to amplify Ms-mptB, which was cloned in pUC18 and ligated as an EcoRI/Sall fragment with similar cleaved pEKE2 to generate pEKE2-Ms-mptB.

Lipid extraction and analysis

Polar lipids and apolar lipids were extracted as described previously (Dobson et al., 1985). Briefly, 6 g of dried cells of wild-type, mutant and complemented strains of C. glutamicum or M. smegmatis were mixed thoroughly using the biphasic mixture of methanolic saline (220 ml containing 20 ml of 0.3% NaCl and 200 ml of CH3OH) and petroleum ether (220 ml) for 2 h. The upper petroleum-ether layer containing apolar lipids were separated following centrifugation. The lower methanolic saline extract was further extracted using petroleum ether (220 ml), mixed and centrifuged. The two upper petroleum-ether fractions were combined and dried. Polar lipids were extracted by the addition of CHCl3/CH3OH/0.3% NaCl (260 ml, 9:10:3, v/v/v) added to the lower

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methylated and the filter cake re-extracted twice with CHCl₃/CH₃OH/0.3% NaCl (85 ml, 5:10:4, v/v/v). CHCl₃ (145 ml) and 0.3% NaCl (145 ml) were added to the combined filtrates and stirred for 1 h. The mixture was allowed to settle, and the lower layer containing the polar lipids recovered and dried. The polar lipid extract was examined by 2D-TLC on aluminum-backed plates of silica gel 60 F254 (Merck 5554), using CHCl₃/CH₃OH/H₂O (60:30:6, v/v/v) in the first direction and CHCl₃/CH₃COOH/CH₃OH/H₂O (40:25:3:6, v/v/v/v) in the second direction to separate [¹⁴C]-labelled PIMs. Lipids were visualized by autoradiography by overnight exposure of Kodak X-Omat AR film to the TLC plates to reveal labelled lipids and compared with known standards.

Preparation of enzymatically active membranes and cell envelope fraction

*Mycobacterium smegmatis* and *C. glutamicum* strains used in this study were cultured to the mid-logarithmic growth phase in 1 l BHIS medium supplemented with kanamycin (25 μg ml⁻¹) and IPTG (0.2 mM) where appropriate. Cells were harvested by centrifugation, re-suspended in 20 ml of buffer A (50 mM MOPS pH 7.9, 5 mM β-mercaptoethanol and 5 mM MgCl₂) and lysed immediately by sonication (60 s on, 90 s off for a total of 10 cycles). The lysate was clarified by centrifugation at 27 000 g (4°C, 30 min) and membranes were deposited by centrifugation of the supernatant at 100 000 g (4°C, 90 min). The membranes were re-suspended in buffer A to a final protein concentration of 20 mg ml⁻¹. The 27 000 g pellet was re-suspended in 10 ml of buffer A and 15 ml of Percoll (Pharmacia, Sweden), and centrifuged at 27 000 g for 60 min at 4°C. The particulate, upper diffuse band, containing both cell walls and membranes, was removed, collected by centrifugation, washed three times in buffer A, and finally re-suspended in 1 ml of buffer A. The final concentration of this Percoll-60 cell envelope fraction (P-60) was 20 mg ml⁻¹.

In vitro incorporation of radiolabelled Man from GDP-[¹⁴C]Man into membrane lipids

Initial assays involved incubation of membranes (0.5 mg of protein), P-60 fraction (0.5 mg of protein) in buffer A, containing 1 mM ATP and 0.25 μCi of GDP-[¹⁴C]Man (Amersham Pharmacia Biotech, Uppsala, Sweden, 303 mCi mmol⁻¹) in a final volume of 100 μl incubated at 37°C for 60 min as described (Besra et al., 1997). The reactions were terminated by the addition of CHCl₃/CH₃OH (6 ml, 2:1, v/v), centrifuged and the pellet re-extracted thrice using CHCl₃/CH₃OH (6 ml, 2:1, v/v). The resulting insoluble pellet was sequentially washed three times with 0.9% NaCl in CH₃OH (2 ml), CH₃OH/H₂O (2 ml, 1:1, v/v) and CH₃OH (2 ml) to remove residual GDP-[¹⁴C]Man before extracting three times with CHCl₃/CH₃OH/H₂O (2 ml, 10:10:3, v/v/v) and an aliquot (10%) of the resulting [¹⁴C]-labelled manno-oligosaccharide polymers [α₁(1→6)-linear-LM-A and α₁(1→6/-linear-LM-B] quantified by liquid scintillation counting using 5 ml of EcoSintA (National Diagnostics, Atlanta, GA). The remaining aliquot was analysed by SDS-PAGE/autoradiography. The original combined CHCl₃/CH₃OH (2:1) organic extracts were dried and re-suspended in CHCl₃/CH₃OH/H₂O (4 ml, 10:10:3, v/v/v) followed by the addition of 1.75 ml of CHCl₃ (1.75 ml) and H₂O (0.75 ml). The reaction mixture was vortexed, centrifuged and the upper aqueous phase removed. The organic phase was washed three times with CHCl₃/CH₃OH/H₂O (2 ml, 3:47:48, v/v/v), and the final organic extract dried under a stream of nitrogen to afford C50-PPI[¹⁴C]M, Ac-PPI[¹⁴C]M and [¹⁴C]Man,GlcA,GroAc₂. Alternatively, the combined CHCl₃/
Pre-treatment of membranes with amphomycin and further incorporation of in situ labelled \([^{14}C]\)Man-labelled membrane glycolipids into CHCl₂/CH₃OH/H₂O (10:10:3)-soluble \([^{14}C]\)-labelled mannosoligosaccharide polymers

The lipopetide amphomycin (2 mg) was dissolved in 500 \(\mu\)l of 0.1 M acetic acid, and the solution adjusted to 0.05 M sodium acetate (pH 7.0) with 0.1 M NaOH for a final concentration of 2 mg ml\(^{-1}\) (Gurcha et al., 2002). Membranes/cell envelope (5 mg) in 500 \(\mu\)l of buffer A were pre-incubated with amphomycin (10 \(\mu\)g per 100 \(\mu\)l reaction mixture) at 37°C for 15 min, resulting in inhibition of PPM synthesis, prior to a further short 15 min pulse incubation with 1.25 \(\mu\)Ci of GDP-[\(^{14}C\)]Man (Amersham Pharmica Biotech, Uppsala, Sweden, 303 mCi mmol\(^{-1}\)). A 20% aliquot of the reaction mixture was processed as described above to afford Ac₆P[p][\(^{14}C\)]M₆, [\(^{14}C\)]Man₆GlcAGroAc₂ and CHCl₃/CH₃OH/H₂O (10:10:3)-soluble lipids. The remaining amphomycin-treated membranes/cell envelope containing soluble [\(^{14}C\)]-labelled mannooligosaccharide polymers was subjected to TLC using silica gel plates (5735 silica gel 60F₂₅₄, Merck) developed in CHCl₃/CH₃OH:H₂O:NH₄OH (65:25:3.6:0.5, v/v/v/v) and the products visualized and quantified by phosphorimaging (Kodak K Screen).

Selective cleavage by partial acetylation

[\(^{14}C\)]-mannosylated products were dried and acetylated using 40 \(\mu\)l of pyridine/acetic anhydride (1:1, v/v) for 30 min at 100°C. The products were dried in a Speed Vac and residual acetic acid removed by co-evaporation with toluene (2 \(\times\) 50 \(\mu\)l). The per-O-acetylated products were dissolved in 30 \(\mu\)l of acetic anhydride/acetic acid/sulphuric acid (10:10:1, v/v/v) and acetylation performed for 8 h at 37°C (Brown et al., 1997). The reaction mixture was then quenched by the addition of 10 \(\mu\)l pyridine and 500 \(\mu\)l H₂O. After 1 h, the per-O-acetylated products were recovered by extraction into CHCl₃. The CHCl₃ phase was washed three times with 500 \(\mu\)l of H₂O and dried. The products were then de-O-acetylated using 200 \(\mu\)l of concentrated ammonium hydroxide/methanol (1:1, v/v) for 6 h at 37°C and subsequently dried. The acetylation products derived from \(\alpha\)-D-[\(^{14}C\)]Manp-(1→6)-\(\alpha\)-D-Manp-(1→6)-\(\alpha\)-D-Manp-O-C₆ (stored in C₃H₇OH) and C₆O-[\(^{14}C\)]M (stored in CHCl₃/CH₃OH, 2:1, v/v) were prepared as described. (Gurcha et al., 2002), separated into aliquots into 1.5 ml eppendorf tubes to a final concentration of 2 mM and 0.25 \(\mu\)Ci (0.305 Ci mmol\(^{-1}\)) respectively, and dried under nitrogen. IgePal CA-630 (8 \(\mu\), Sigma Aldrich) was added and the tubes sonicated to re-suspend the lipid-linked components, and the remaining assay components in a final volume of 80 \(\mu\)l were added, which included: 1 mM ATP, 1 mM NADP, and membrane protein (1 mg) from either C. glutamicum, C. glutamicum\(\Delta\)mpTB, C. glutamicum\(\Delta\)mpTA, C. glutamicum\(\Delta\)mpTB pVWEx-Cg-mpTB, C. glutamicum\(\Delta\)mpTB pVWEx-Mt-mpTB and C. glutamicum\(\Delta\)mpTB pVWEx-Ms-mpTB. Assays were incubated at 37°C for 1 h and then quenched by the addition of CHCl₃/CH₃OH (533 \(\mu\)l, 1:1, v/v). The reaction mixtures were then centrifuged at 27 000 g for 15 min at 4°C, the supernatant removed and dried under nitrogen. The residue was re-suspended in C₃H₇OH/H₂O (700 \(\mu\)l, 1:1, v/v) and loaded onto a 1 ml SepPak strong anion exchange cartridge (Supelco) pre-equilibrated with C₃H₇OH/H₂O (1:1, v/v). The column was washed with 2 ml of C₃H₇OH, and the eluate collected, dried and partitioned between the two phases arising from a mixture of n-butanol (3 ml) and water (3 ml). The resulting organic phase was recovered after centrifugation at 3500 g, and the aqueous phase again extracted twice with 3 ml of water-saturated butanol. The pooled extracts were back-washed twice with n-butanol-saturated water (3 ml). The n-butanol fraction was dried and re-suspended in 200 \(\mu\)l of n-butanol. The extracted radiolabelled material was quantified by liquid scintillation counting using 10% of the labelled material and 5 ml of EcoScintA (National Diagnostics, Atlanta, GA). The incorporation of [\(^{14}C\)]Manp was determined by subtracting counts present in control assays (incubations in the absence of acceptor), which were typically less than 100 c.p.m. per assay. The remaining labelled material was subjected to TLC using silica gel plates (5735 silica gel 60F₂₅₄, Merck) developed in CHCl₃/CH₃OH:H₂O:NH₄OH (65:25:3.6:0.5, v/v/v/v) and the products visualized by phosphorimaging (Kodak K Screen).

In vitro analysis of \(\alpha\)(1→6) mannosyltransferase activity

The neoglycolipid acceptors \(\alpha\)-D-Manp-(1→6)-\(\alpha\)-D-Manp-O-C₆ (stored in C₃H₇OH) and C₆O-[\(^{14}C\)]M (stored in CHCl₃/CH₃OH 2:1, v/v) were prepared as described. Gurcha et al., 2002), were separated into aliquots into 1.5 ml eppendorf tubes to a final concentration of 2 mM and 0.25 \(\mu\)Ci (0.305 Ci mmol\(^{-1}\)) respectively, and dried under nitrogen. IgePal CA-630 (8 \(\mu\), Sigma Aldrich) was added and the tubes sonicated to re-suspend the lipid-linked components, and the remaining assay components in a final volume of 80 \(\mu\)l were added, which included: 1 mM ATP, 1 mM NADP, and membrane protein (1 mg) from C. glutamicum, C. glutamicum\(\Delta\)mpTB, C. glutamicum\(\Delta\)mpTA, C. glutamicum\(\Delta\)mpTB pVWEx-Cg-mpTB, C. glutamicum\(\Delta\)mpTB pVWEx-Mt-mpTB and C. glutamicum\(\Delta\)mpTB pVWEx-Ms-mpTB. The per-O-acetylated products were dissolved in 40% propan-1-ol and analysed by TLC using one development with propan-1-ol/acetic acid/H₂O (5:4:1, v/v/v), followed by one development with butan-1-ol/acetic acid/H₂O (5:3:5:1.5, v/v/v/v) and the products visualized by phosphorimaging (Kodak K Screen). The acetylation products derived from the CHCl₃/CH₃OH/H₂O (10:10:3)-soluble [\(^{14}C\)]-labelled mannosoligosaccharide polymers \(\alpha\)(1→6)-linear-LM-A and \(\alpha\)(1→6)-linear-LM-B were re-dissolved in 50 ml Bio-Gel P-2 gel filtration column (30 \(\times\) 1.5 cm; Bio-Rad). Elution from the column was performed using water and 1 ml fractions collected which were subsequently quantified by liquid scintillation counting. The control de-O-acetylated [\(^{14}C\)]-labelled mannosoligosaccharide polymers prior to acetylation eluted form the Bio-Gel P-2 column at fractions 11–13 and degraded.
acetylation products were retained and co-eluted in later fractions 33–39 based on a de-O-acylated PI[14C]Man, [14C]Man,GlcAc2Man,GlcAc2 and [14C]Man standards.

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Supplementary material

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