

# Lipoarabinomannan biosynthesis in *Corynebacterineae*: the interplay of two $\alpha(1\rightarrow2)$ -mannopyranosyltransferases MptC and MptD in mannan branching

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## Summary

Lipomannan (LM) and lipoarabinomannan (LAM) are key *Corynebacterineae* glycoconjugates that are integral components of the mycobacterial cell wall, and are potent immunomodulators during infection. LAM is a complex heteropolysaccharide synthesized by an array of essential glycosyltransferase family C (GT-C) members, which represent potential drug targets. Herein, we have identified and characterized two open reading frames from *Corynebacterium glutamicum* that encode for putative GT-Cs. Deletion of *NCgl2100* and *NCgl2097* in *C. glutamicum* demonstrated their role in the biosynthesis of the branching  $\alpha(1\rightarrow2)$ -Manp residues found in LM and LAM. In addition, utilizing a chemically defined nonasaccharide acceptor, azidoethyl 6-O-benzyl- $\alpha$ -D-mannopyranosyl-(1 $\rightarrow$ 6)-[ $\alpha$ -D-mannopyranosyl-(1 $\rightarrow$ 6)]-7-D-mannopyranoside, and the glycosyl donor C<sub>50</sub>-polyprenol-phosphate-[<sup>14</sup>C]-

mannose with membranes prepared from different *C. glutamicum* mutant strains, we have shown that both *NCgl2100* and *NCgl2097* encode for novel  $\alpha(1\rightarrow2)$ -mannopyranosyltransferases, which we have termed MptC and MptD respectively. Complementation studies and *in vitro* assays also identified Rv2181 as a homologue of Cg-MptC in *Mycobacterium tuberculosis*. Finally, we investigated the ability of LM and LAM from *C. glutamicum*, and *C. glutamicum* $\Delta$ mptC and *C. glutamicum* $\Delta$ mptD mutants, to activate Toll-like receptor 2. Overall, our study enhances our understanding of complex lipoglycan biosynthesis in *Corynebacterineae* and sheds further light on the structural and functional relationship of these classes of polysaccharides.

## Introduction

The *Corynebacterineae*, a sub-order of the Actinobacteria, represent an unusual group within Gram-positive bacteria, with a distinctive cell wall architecture. Prominent members are the human pathogens, *Corynebacterium diphtheriae*, *Mycobacterium tuberculosis* and *Mycobacterium leprae* (Bloom and Murray, 1992). Typically, the cell walls of *Corynebacterineae* contain mycolic acids (m), arabinogalactan (AG) and peptidoglycan (P), which are covalently linked to each other to form the mycolyl-arabinogalactan-peptidoglycan (mAGP) complex (Daffe *et al.*, 1990; McNeil *et al.*, 1990; 1991; Besra *et al.*, 1995; 1997; Brennan, 2003; Dover *et al.*, 2004). In addition, specialized glycopospholipids, phosphatidyl-myoinositol mannosides (PIMs) and lipoglycans, lipomannan (LM) and lipoarabinomannan (LAM), are found in the outer leaflet of mAGP (Hill and Ballou, 1966; Brennan and Ballou, 1967; 1968; Brennan and Nikaido, 1995; Besra *et al.*, 1997; Morita *et al.*, 2004). The non-pathogenic bacillus, *Corynebacterium glutamicum*, also belongs to this class of bacteria, and is widely used in the industrial production of amino acids (Eggeling and Bott, 2005). Use of this easily cultivatable bacterium, together with the fact that the cell walls of *Corynebacterineae* share identical basic structures and building blocks, have contributed significantly to decipher the biosynthesis of their complex cell walls. For instance, in both *C. glutamicum* and *M.*

Accepted 16 March, 2011. \*For correspondence. E-mail g.besra@bham.ac.uk; Tel. (+44) (0)121 415 8125; Fax (+44) (0)121 414 5925. Re-use of this article is permitted in accordance with the Terms and Conditions set out at [http://wileyonlinelibrary.com/onlineopen/OnlineOpen\\_Terms](http://wileyonlinelibrary.com/onlineopen/OnlineOpen_Terms)

*tuberculosis* the orthologous acyl-CoA carboxylase genes (Gande *et al.*, 2007) together with Pks (Gande *et al.*, 2004), are key to mycolic acid biosynthesis, and the glycosyltransferases AftA (Alderwick *et al.*, 2006a), and AftB (Seidel *et al.*, 2007a) introduce specific arabinofuranose (Araf) residues into AG, and AftC in both AG and LAM of *Mycobacterium smegmatis* (Birch *et al.*, 2008; 2010). A speciation-specific difference is the presence of the glycosyltransferase RptA in *C. glutamicum* that introduces decorating rhamnose residues into AG (Birch *et al.*, 2009), whereas a structurally very similar protein introduces galactosamine residues into AG of *M. tuberculosis* (Skovierova *et al.*, 2010).

A particular interesting group of cell wall components are the lipoglycans LM and LAM that are based on PIMs. They play poorly defined roles in *Corynebacterineae* but mycobacterial LAM has been implicated in many of the key aspects of the pathogenesis of tuberculosis and leprosy, such as induction of phagocytosis, phagosomal alteration and acquired T cell-mediated immunity (Briken *et al.*, 2004). Mycobacterial LAM has attached to the PIM base, an elongated  $\alpha(1\rightarrow6)$  linear,  $\alpha(1\rightarrow2)$  branched mannan 'core', of approximately 30 mannopyranose (Manp) residues, and linked to its terminus a branched D-arabinan domain. In mycobacteria, this large arabinan domain consists of approximately 70 arabinofuranose (Araf) residues and is assembled through several distinct motifs (Chatterjee *et al.*, 1991; Alderwick *et al.*, 2007; Birch *et al.*, 2010), formed via specific  $\alpha(1\rightarrow5)$ ,  $\alpha(1\rightarrow3)$  and  $\beta(1\rightarrow2)$  arabinofuranosyltransferases (Besra *et al.*, 1997; Zhang *et al.*, 2003; Alderwick *et al.*, 2005; Birch *et al.*, 2010), and can be capped to various degrees with either short  $\alpha(1\rightarrow2)$  mannopyranosyl chains, as is the case in *M. tuberculosis* (Chatterjee *et al.*, 1992), or in *M. smegmatis* by inositol phosphate (Khoo *et al.*, 1995), or completely devoid of caps as found in *Mycobacterium chelonae* (Guerardel *et al.*, 2002). There is very limited structural information on LAM in other *Corynebacterineae*, except for *C. glutamicum* (Tatituri *et al.*, 2007a,b). In this organism the core  $\alpha(1\rightarrow6)$  linear  $\alpha(1\rightarrow2)$  branched LM (see Fig. 9), which is structurally akin to mycobacterial LM, is substituted by t-Araf residues to form a modified LAM-like molecule (Tatituri *et al.*, 2007a,b).

The current model of lipoglycan biosynthesis supported by biochemical and genetic studies, follows a linear pathway from PI $\rightarrow$ PIM<sub>2</sub> $\rightarrow$ LM $\rightarrow$ LAM (Besra and Brennan, 1997; Besra *et al.*, 1997). As with AG synthesis selected enzymatic and structural features are in part shared within the *Corynebacterineae*. Glycosylation of phosphatidylmyo-inositol (PI) by different  $\alpha$ -mannopyranosyltransferases and acylation by acyltransferase(s) results in the synthesis of mono- and di-acylated PIMs (Kordulakova *et al.*, 2002; 2003; Kremer *et al.*, 2002; Morita *et al.*, 2006; Lea-Smith *et al.*, 2008; Mishra *et al.*, 2009). Previ-

ous studies have identified the enzymes involved in the early and late stages of LM and LAM biosynthesis in *Corynebacterineae*. For instance, NCgl1505/Rv1459c (MptB) and NCgl2093/Rv2174 (MptA), synthesize the proximal and distal  $\alpha(1\rightarrow6)$  mannan backbone in LM and LAM (Kaur *et al.*, 2007; Mishra *et al.*, 2007; 2008a). However, the enzymatic steps involved in the biosynthesis of mannan branching of LM and LAM, still remain to be studied in detail in *Corynebacterineae* (Kaur *et al.*, 2006; 2008; Sena *et al.*, 2010).

In the current study we have examined two open reading frames from *C. glutamicum*, which encode for putative glycosyltransferase family C (GT-C) members (Liu and Mushegian, 2003). On the basis of mutant studies, chemical and enzymatic data we report that NCgl2100 (Cg-MptC) and its *M. tuberculosis* homologue Rv2181 function as  $\alpha(1\rightarrow2)$ -mannopyranosyltransferases, and NCgl2097 functions as a second  $\alpha(1\rightarrow2)$ -mannopyranosyltransferase (Cg-MptD), both of which are required for complete mannose-branching found in LM and LAM in *Corynebacterineae*.

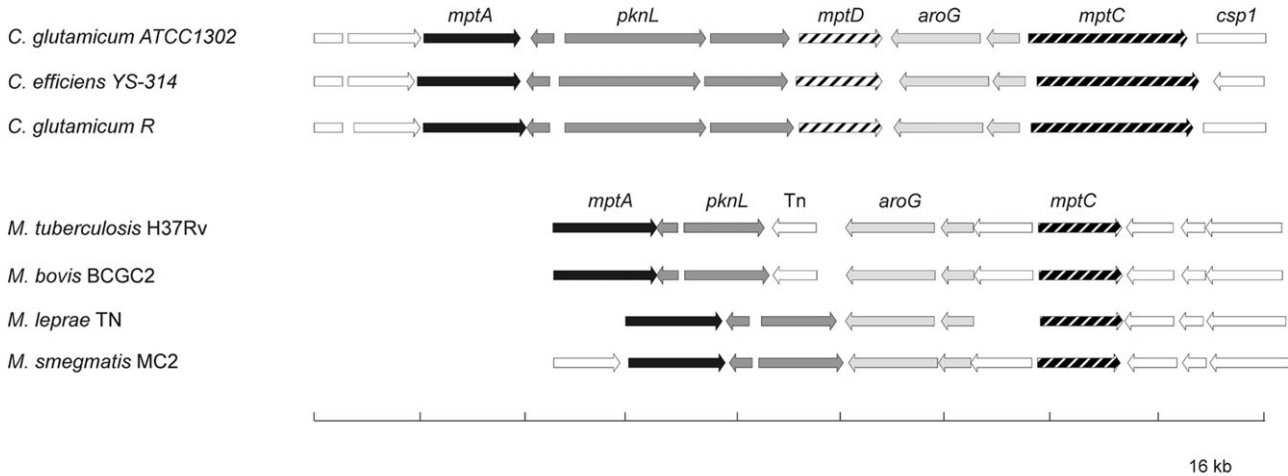
## Results

### Genome locus and structural features of MptC and MptD

We previously identified the  $\alpha(1\rightarrow6)$ -mannopyranosyltransferases, MptA and MptB, which catalyse the transfer of Manp residues from the glycosyl donor C<sub>50</sub>-polyprenol-phosphate mannose, to the distal and proximal ends of the mannan backbone of LM and LAM respectively (Mishra *et al.*, 2007; 2008a). In search of further GT-Cs, which synthesize the elaborated structures of LM and LAM, we interrogated the genome of *C. glutamicum* for additional GT-C family members (Liu and Mushegian, 2003). Located within a 16 kb genomic region containing *mptA*, which has been demonstrated to encode an  $\alpha(1\rightarrow2)$ -mannopyranosyltransferase in *M. tuberculosis* (Kaur *et al.*, 2008), as does its orthologue MSMEG\_4247 in *M. smegmatis* (Kaur *et al.*, 2006; Sena *et al.*, 2010), are two additional GT-Cs (NCgl2100 [Cg-*mptC*], and NCgl2097 [Cg-*mptD*]) (Fig. 1A). In both *Corynebacterium* and *Mycobacterium* additional genes are retained at this locus: a serine/threonine protein kinase (*pknL*), hypothetical proteins (NCgl2099, Rv2179c; NCgl2094, Rv2175c) and a 3-deoxy-7-phosphoheptulonate synthase (*aroG*) (Fig. 1A).

The Cg-*mptC* encoded protein is a long hydrophobic polytopic membrane protein of 812 amino acid (aa) residues. The first half of the protein (1–417 aa), shares 37% identity (55% similarity) with Mt-MptC and 38% identity (57% similarity) to Cg-MptD, whereas the second half (417–812 aa), has no counterpart in various *Mycobacte-*

## A



## B

<b>Cg-MptC</b>	93	LPFTYPPISAALFVP	107	196	GFWIGLAASIKLPTAVFGLYFL	217	327	LLCSPVSWSHHWVWL	341
<b>Cg-MptD</b>	91	LPFIYPPIGAILFAP	105	197	GVLIGLAAAIAKITPLAMLLYFL	218	337	LLISPISWSHHWVWL	351
<b>Mt-PimE</b>	94	LPFTYPPFAAVVFYP	108	201	GLLVGVASGVKLTPAITAVYLV	222	330	LLSPISWTHHWVWL	344
<b>Mt-MptC</b>	78	LPFTYPPPLAAVLFSP	92	190	GLMLGLGIALKLTPAVFLYFL	211	320	LVVSPVSWSHHWVWM	334
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**Fig. 1.** Comparative gene relatedness of  $\alpha(1\rightarrow2)$  and  $\alpha(1\rightarrow6)$  mannopyranosyltransferases.

A. The genomic region in selected *Corynebacteriaceae* containing the GT-C mannopyranosyltransferases MptA, MptC and MptD.

B. Partial sequence alignment of the strongly related  $\alpha(1\rightarrow2)$  mannopyranosyltransferases Cg-MptC, Cg-MptD, Mt-PimE and Mt-MptC illustrating conserved residues.

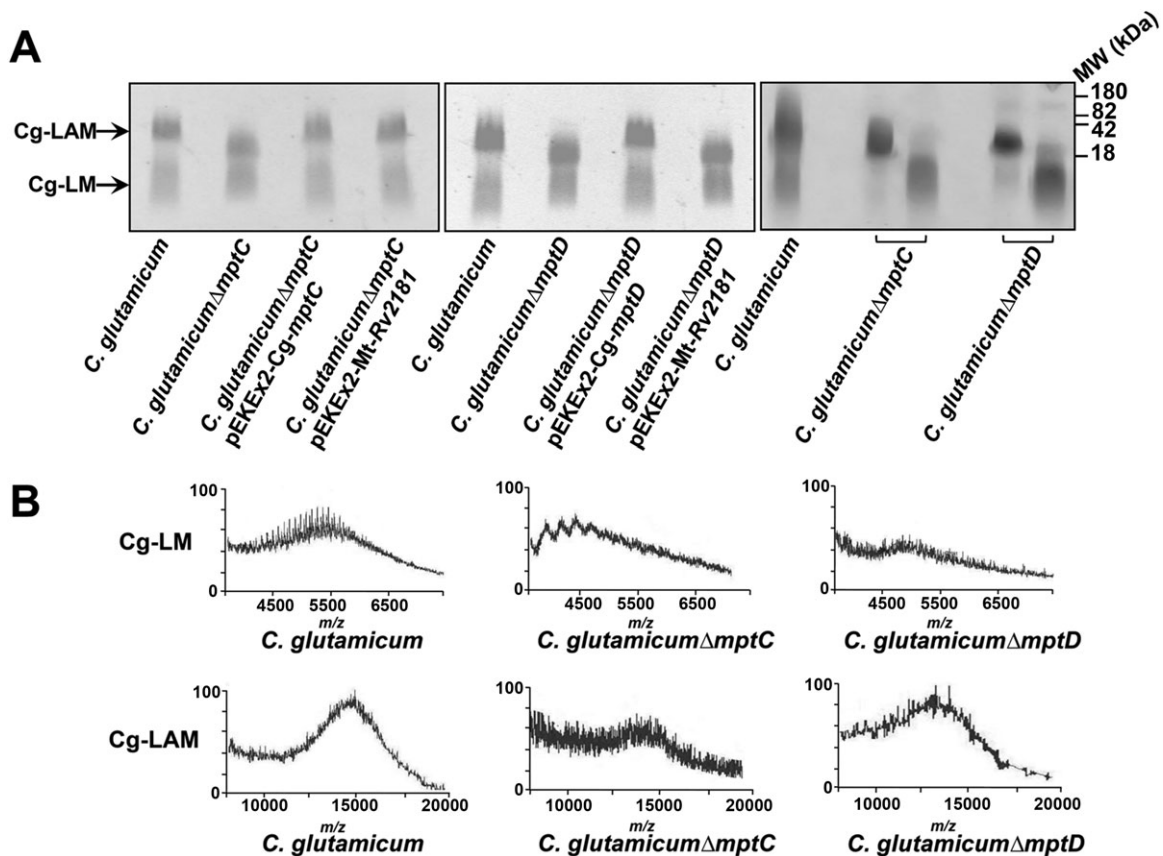
*rium* species. The protein appears to be a fusion of two membrane proteins, functional as an  $\alpha(1\rightarrow2)$ -mannopyranosyltransferase as described below, and termed in this study Cg-MptC. Cg-MptD is a hydrophobic polytopic membrane protein of 436 amino acid residues with 11 transmembrane helices (TMH). After TMH-1 and TMH-7, larger loop regions are present probably localized in the periplasm. Cg-MptC, Cg-MptD and Mt-MptC, together with Mt-PimE, an  $\alpha(1\rightarrow2)$ -mannopyranosyltransferase demonstrated to be involved in PIM<sub>6</sub> synthesis (Morita *et al.*, 2006), share a similar size, degree of hydrophobicity and regions of high identity (Fig. 1B), suggesting closely related functions.

#### Construction of deletion mutants and growth phenotype

The construct pK19mobsacB $\Delta$ Cg-*mptC* was made containing 12 nucleotides (nt) of the 3' end of *NCgl2100* together with the genomic upstream sequences, and 36 nt of the 5' end together with genomic downstream sequences (Table S1). This non-replicative vector was used to transform *C. glutamicum* to kanamycin-resistance (Kan<sup>r</sup>) indicating chromosomal integration. Sucrose-resistant (Suc<sup>r</sup>) clones were selected in a second round of positive selection, indicating loss of the vector-encoded *sacB* function (Schäfer *et al.*, 1994).

From 12 Kan<sup>s</sup> and Suc<sup>r</sup> clones analysed via PCR, seven had lost *NCgl2100*, whereas in the remaining five the genomic wild type situation was restored. One clone with deleted *NCgl2100* was selected and will be referred to as *C. glutamicum* $\Delta$ *mptC* (Fig. S1). In an analogous manner, pK19mobsacB $\Delta$ Cg-*mptD* was constructed and used to select from the wild type of *C. glutamicum* by double cross-over events as described above for deletion of *Cg-mptD*, yielding *C. glutamicum* $\Delta$ *mptD* (Fig. S1). Starting from *C. glutamicum* $\Delta$ *mptC* and using pK19mobsacB $\Delta$ Cg-*mptD* the double deletion mutant *C. glutamicum* $\Delta$ *mptC* $\Delta$ *mptD* was also constructed (Fig. S1).

The mutants were inoculated into liquid BHI and CGXII medium and their growth patterns studied. There was no statistical difference observed in three independent cultivations for each of the strains. The growth rates in CGXII were  $0.39 \pm 0.02$  h<sup>-1</sup> for wild-type *C. glutamicum*, *C. glutamicum* $\Delta$ *mptC* and *C. glutamicum* $\Delta$ *mptD*, and  $0.37 \pm 0.02$  h<sup>-1</sup> for *C. glutamicum* $\Delta$ *mptC* $\Delta$ *mptD* (data not shown). The growth rates on BHI medium were  $0.66 \pm 0.07$  h<sup>-1</sup> for all three strains and illustrate that the genes are not essential for growth as observed for other GT-Cs involved in mAGP synthesis like, *Cg-emb*, *Cg-aftA* and *Cg-aftB* (Alderwick *et al.*, 2005; 2006a; Seidel *et al.*, 2007a).



**Fig. 2.** Lipoglycan profiles of wild-type and mutant strains of *C. glutamicum*.

A. The left-hand and middle panels illustrate lipoglycans extracted from wild-type, mutant and complemented strains analysed using SDS-PAGE and visualized using a Pro-Q emerald glycoprotein stain (Invitrogen). The right-hand panel illustrates purified Cg-LAM and Cg-LM from *C. glutamicum* $\Delta$ *mptC* and *C. glutamicum* $\Delta$ *mptD*, respectively, in comparison with wild-type *C. glutamicum* lipoglycans. Lipoglycan profiles are represented with standard molecular weight markers of glycoproteins of 180, 82, 42 and 18 kDa.

B. MALDI-TOF-MS spectra of Cg-LM and Cg-LAM from different strains of *C. glutamicum*. MALDI-TOF-MS spectra were acquired in the linear negative mode with delayed extraction using 2,5-dihydrobenzoic acid as a matrix.

#### Purification and general characteristic features of lipoglycans from *C. glutamicum* $\Delta$ *mptC* and *C. glutamicum* $\Delta$ *mptD*

Lipoglycans from wild-type *C. glutamicum*, *C. glutamicum* $\Delta$ *mptC* and *C. glutamicum* $\Delta$ *mptD* with complemented strains *C. glutamicum* $\Delta$ *mptC* pEKEx2-Cg-*mptC*, *C. glutamicum* $\Delta$ *mptC* pEKEx2-Rv2181, *C. glutamicum* $\Delta$ *mptD* pEKEx2-Cg-*mptD* and *C. glutamicum* $\Delta$ *mptD* pEKEx2-Rv2181, were extracted from delipidated cells by ethanol/water extraction followed by hot-phenol water treatment and enzymatic degradation. The crude lipoglycan extract was subjected to hydrophobic interaction and gel permeation chromatography allowing the separation of Cg-LM (Cg-LM-A and Cg-LM-B) and Cg-LAM (Tatituri *et al.*, 2007b; Lea-Smith *et al.*, 2008; Mishra *et al.*, 2008b). Because the mannan backbone structures of Cg-LM-A and Cg-LM-B are similar as revealed through previous matrix-assisted laser desorption/ionization time-

of-flight mass spectrometry (MALDI-TOF-MS), and gas chromatography-mass spectrometry (GC-MS) analyses of partially methylated alditol acetates (Mishra *et al.*, 2008b), subsequent analyses focused on the Cg-LM as a mixture. The extracted lipoglycans were examined for their size and mobility on 15% SDS-PAGE (Fig. 2A). The crude extracts from wild-type *C. glutamicum* showed the presence of Cg-LM and Cg-LAM (Tatituri *et al.*, 2007a; Lea-Smith *et al.*, 2008; Mishra *et al.*, 2008b) (Fig. 2A), while the crude lipoglycans from mutant strains, *C. glutamicum* $\Delta$ *mptC* and *C. glutamicum* $\Delta$ *mptD*, showed subtle differences in their migration on SDS-PAGE (Fig. 2A). Purified Cg-LAM from *C. glutamicum* $\Delta$ *mptC* and *C. glutamicum* $\Delta$ *mptD* (Fig. 2A) migrated faster on SDS-PAGE in comparison with those from wild-type *C. glutamicum*, indicating that these lipoglycans were smaller in size with low molecular weights. In addition, fractionation using gel permeation chromatography also revealed a degree of heterogeneity in Cg-LM and



Cg-LAM, as evident when fractions were pooled. Complementation of *C. glutamicum* $\Delta$ *mptC* and *C. glutamicum* $\Delta$ *mptD* by pEKEx2-Cg-*mptC* and pEKEx2-Cg-*mptD*, respectively, restored the wild-type phenotype, as illustrated by the slower and co-migration on SDS-PAGE in comparison with wild-type *C. glutamicum* (Fig. 2A). In contrast, cross-complementation experiments, *C. glutamicum* $\Delta$ *mptC* by pEKEx2-Cg-*mptD*, and *C. glutamicum* $\Delta$ *mptD* by pEKEx2-Cg-*mptC*, failed to restore a wild-type phenotype and reversion to a slower migration of products on SDS-PAGE (Fig. S2), as observed in our previous experiments described above (Fig. 2A).

The molecular weights of the lipoglycans were investigated by MALDI-TOF-MS. The Cg-LAM from wild-type *C. glutamicum* exhibited a broad unresolved peak centred at *m/z* 15 000 (approximately 85 Manp/Araf glycosyl residues), and Cg-LM at *m/z* 5500 (approximately 25 Manp residues), indicating a molecular weight of approximately 15 and 5.5 kDa for the major molecular species of these lipoglycans (Fig. 2B). Interestingly, Cg-LAM from *C. glutamicum* $\Delta$ *mptC* (Fig. 2A) peaked at *m/z* 13 800 (approximately 78 Manp/Araf glycosyl residues), indicating a decrease of around 1.2 kDa for the Cg-LAM isolated from *C. glutamicum* $\Delta$ *mptC* as compared with wild-type Cg-LAM (Fig. 2B). In addition, Cg-LM from *C. glutamicum* $\Delta$ *mptC* (Fig. 2A) exhibited a broad unresolved peak centred at *m/z* 4400 (approximately 20 Manp glycosyl residues), indicating a molecular weight of approximately 4.4 kDa, a decrease of 1.1 kDa in comparison with wild-type *C. glutamicum* (Fig. 2B). Similarly, the lipoglycans, Cg-LM and Cg-LAM from *C. glutamicum* $\Delta$ *mptD* (Fig. 2A) are smaller and centred around *m/z* 13 000 (approximately 73 Manp/Araf glycosyl residues), and 4700 (approximately 20 Manp glycosyl residues) respectively (Fig. 2B). The differences in size of Cg-LAM and Cg-LM from the mutant strains, suggest that there is a difference in a common component of both mutant strains, which also reflects the earlier observation on SDS-PAGE (Fig. 2A).

#### Glycosyl composition of purified lipoglycans from *C. glutamicum* $\Delta$ *mptC* and *C. glutamicum* $\Delta$ *mptD*

Purified lipoglycans from wild-type *C. glutamicum*, *C. glutamicum* $\Delta$ *mptC* and *C. glutamicum* $\Delta$ *mptD* were analysed as alditol acetates and their glycosyl composition was determined by gas chromatography (GC) (Fig. S3). GC analysis of alditol acetates prepared from wild-type Cg-LAM revealed a molar ratio of Araf : Manp of 0.47:1.0. The mutant Cg-LAM from *C. glutamicum* $\Delta$ *mptC* and *C. glutamicum* $\Delta$ *mptD* yielded a significant reduction in Manp content concomitant with a relative increase in the amount of Araf. The *C. glutamicum* $\Delta$ *mptC* yielded a mutant Cg-LAM with an Araf : Manp ratio of 0.59:1.0, while

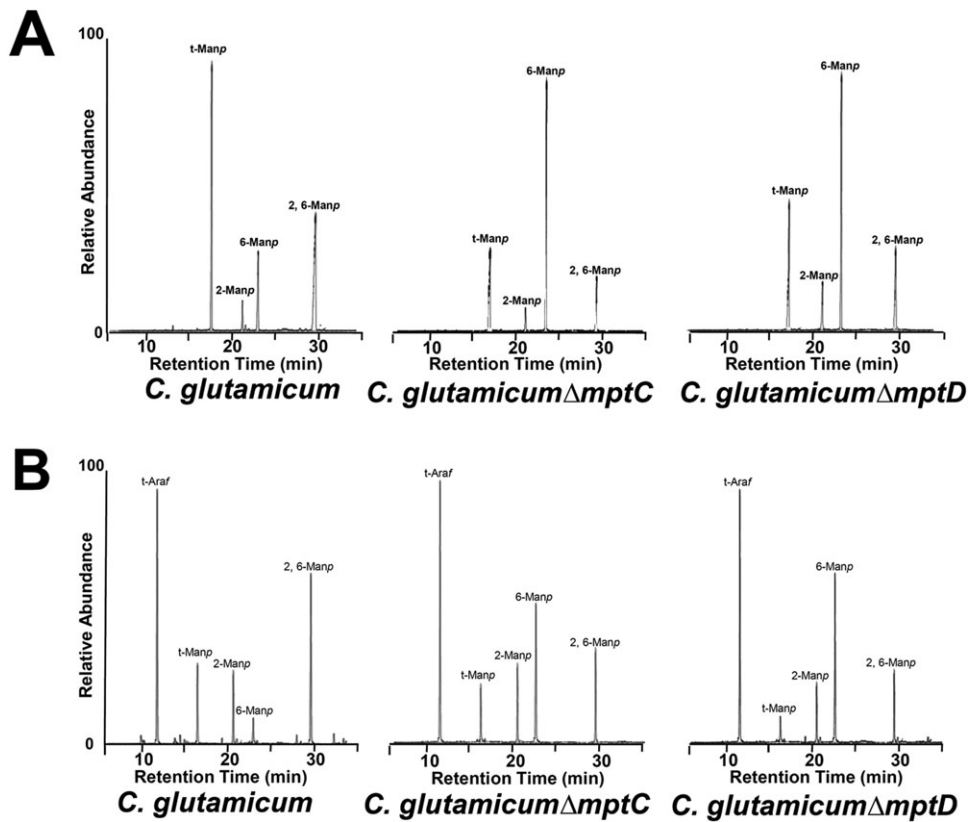
Cg-LAM from *C. glutamicum* $\Delta$ *mptD*, yielded an Araf : Manp ratio of 0.7:1.0. The data suggest that both Cg-MptC and Cg-MptD are involved in the synthesis of the mannan portion of Cg-LAM together with Cg-MptA and Cg-MptB (Mishra *et al.*, 2007; 2008a).

#### Glycosyl linkage of purified lipoglycans from *C. glutamicum* $\Delta$ *mptC* and *C. glutamicum* $\Delta$ *mptD*

The glycosyl linkages present in the lipoglycans from wild-type and mutant strains of *C. glutamicum* were analysed by GC-MS of per-*O*-methylated alditol acetate derivatives prepared from purified lipoglycans. Cg-LM from *C. glutamicum* indicated a normal profile of glycosidic linkages corresponding to t-Manp (49%), 6-Manp (15%), 2-Manp (6%) and 2,6-Manp (30%) (Fig. 3A). However, the relative ratios of different linkages in Cg-LM from *C. glutamicum* $\Delta$ *mptC* [t-Manp (23%), 6-Manp (58%), 2-Manp (6%) and 2,6-Manp (13%)] and *C. glutamicum* $\Delta$ *mptD* [t-Manp (25%), 6-Manp (45%), 2-Manp (8%) and 2,6-Manp (22%)] indicated subtle changes in the mannan domain in Cg-LM from these strains consistent with the earlier MALDI-TOF-MS data indicating a reduction in size (Fig. 2B). The relative abundance of t-Manp and 2,6-Manp were reduced with a concomitant increase in the abundance of 6-Manp in Cg-LM from these strains (Fig. 3A). Similarly, per-*O*-methylated alditol acetate derivatives of Cg-LAM from these strains showed a net reduction in relative abundance of t-Manp and 2,6-Manp and increase in 6-Manp (Fig. 3B). For instance, Cg-LAM from *C. glutamicum* indicated a normal profile of glycosidic linkages corresponding to t-Araf (40%), t-Manp (14%), 6-Manp (6%), 2-Manp (11%) and 2,6-Manp (29%) (Fig. 3A). Whereas the relative ratios of different linkages in Cg-LAM from *C. glutamicum* $\Delta$ *mptC* were t-Araf (40%), t-Manp (6%), 6-Manp (30%), 2-Manp (11%) and 2,6-Manp (13%) and *C. glutamicum* $\Delta$ *mptD* were t-Araf (40%), t-Manp (8%), 6-Manp (26%), 2-Manp (12%) and 2,6-Manp (14%). The complementation of *C. glutamicum* $\Delta$ *mptC* and *C. glutamicum* $\Delta$ *mptD* with pEKEx2-Cg-*mptC* and pEKEx2-Cg-*mptD*, respectively, restored the wild-type phenotype in these strains (data not shown). Altogether, the data together suggest that Cg-LM and Cg-LAM from *C. glutamicum* $\Delta$ *mptC* and *C. glutamicum* $\Delta$ *mptD* have reduced 2,6-Manp branching residues indicating that MptC and MptD are involved in the synthesis of  $\alpha(1\rightarrow2)$ -Manp residues in Cg-LM and Cg-LAM in *C. glutamicum*.

#### Characterization of Cg-LAM from *C. glutamicum* $\Delta$ *mptC* $\Delta$ *mptD*

Because the data in Fig. 3A,B suggest that deletion of *mptC* or *mptD* results in only partial removal of 2,6-Manp branching residues, we constructed a *C. glutamicum* $\Delta$ -



**Fig. 3.** Glycosyl linkage analysis of Cg-LM and Cg-LAM from different strains of *C. glutamicum*.

A. Cg-LM per-*O*-methylated samples were hydrolysed using 2 M TFA, reduced and per-*O*-acetylated. The resulting partially per-*O*-methylated, per-*O*-acetylated alditol acetates were analysed using GC-MS.

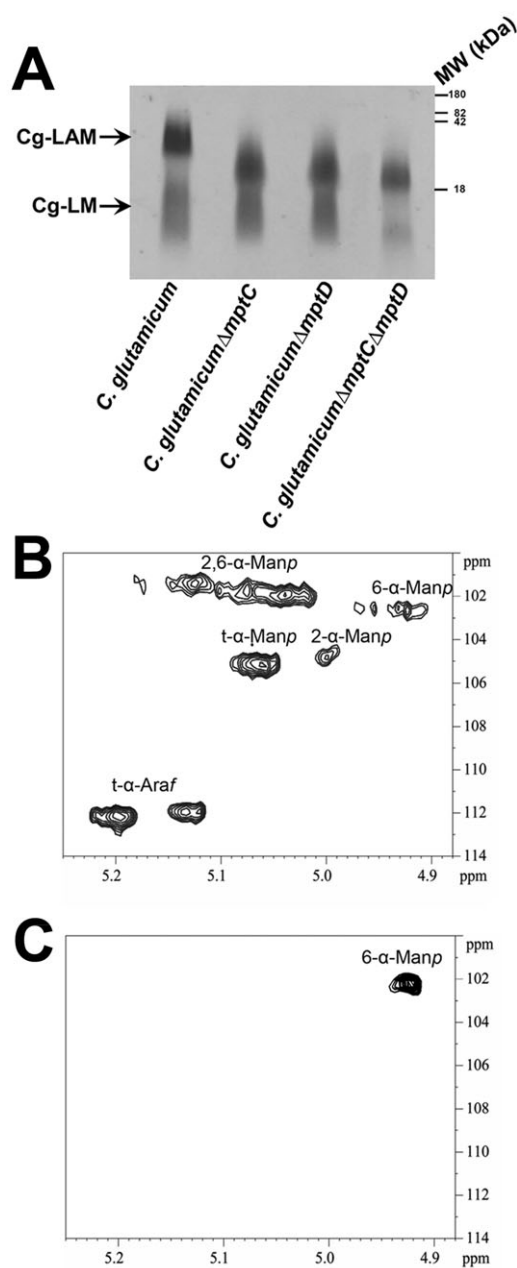
B. Cg-LAM per-*O*-methylated samples were hydrolysed using 2 M TFA, reduced and per-*O*-acetylated. The resulting partially per-*O*-methylated, per-*O*-acetylated alditol acetates were analysed using GC-MS. The partially per-*O*-methylated, per-*O*-acetylated alditol acetates were identified based on their characteristic alditol cleavage acetal profiles: t-Araf (*m/z* 118, 161), t-Manp (*m/z* 118, 102, 145, 161, 162, 205), 2-Manp (*m/z* 129, 130, 161, 190), 6-Manp (*m/z* 118, 129, 162, 189, 233) and 2,6-Manp (*m/z* 129, 130, 189, 190).

*mptCΔmptD* double mutant strain (as described earlier). The extracted lipoglycans were examined for their size and mobility on 15% SDS-PAGE (Fig. 4A). The crude lipoglycan extracts of *C. glutamicumΔmptCΔmptD* in comparison with wild-type *C. glutamicum*, *C. glutamicumΔmptC* and *C. glutamicumΔmptD*, migrated faster on SDS-PAGE, indicating that these lipoglycans were smaller in size (Fig. 4A). The purified Cg-LAM from *C. glutamicumΔmptCΔmptD* was analysed by  $^1\text{H}$ - $^{13}\text{C}$ -NMR HMQC (Fig. 4C) and compared with our previously reported  $^1\text{H}$ - $^{13}\text{C}$ -NMR HMQC (Fig. 4B) assignment of anomeric resonances of Cg-LAM from *C. glutamicum* (Tatituri *et al.*, 2007b). Briefly, correlations at  $\delta_{\text{H1C1}}$  5.20/112.2 and 5.13/112.0 were attributed to t- $\alpha$ -Araf units; 5.06/105.2 to t- $\alpha$ -Manp units; 5.12/101.4, 5.07/101.7, and 5.04/101.9 to 2,6- $\alpha$ -Manp units; 4.93/102.6 to 6- $\alpha$ -Manp units; and 5.00/104.9 to 2- $\alpha$ -Manp units, which were consistent with our above glycosyl linkage data for Cg-LAM from *C. glutamicum* (Fig. 2B). In contrast, the  $^1\text{H}$ - $^{13}\text{C}$ -NMR HMQC (Fig. 4C) of Cg-LAM from *C. glutamicumΔmptCΔmptD* was dominated by a single anomeric resonance at  $\delta_{\text{H1C1}}$

4.92/102.5 and assigned to 6- $\alpha$ -Manp units, consistent with a complete removal of 2,6- $\alpha$ -Manp branching residues. GC-MS of per-*O*-methylated alditol acetate derivatives from Cg-LAM purified from *C. glutamicumΔmptCΔmptD* confirmed these results and the identification of 2,3,4-tri-*O*-CH<sub>3</sub>-1,5,6-tri-*O*-COCH<sub>3</sub>-mannitol with the characteristic fragment alditol cleavage ions *m/z* 102, 118, 129, 162, 189 and 233 (Fig. S4).

#### *In vitro* $\alpha(1\rightarrow2)$ -mannopyranosyltransferase assay utilizing a novel blocked nonasaccharide acceptor

For characterization of  $\alpha(1\rightarrow2)$ -mannopyranosyltransferase activities of Cg-MptC and Cg-MptD a nonasaccharide acceptor, azidoethyl 6-*O*-benzyl- $\alpha$ -D-mannopyranosyl-(1 $\rightarrow$ 6)-[ $\alpha$ -D-mannopyranosyl-(1 $\rightarrow$ 6)]-D-mannopyranoside, Acc-Man<sub>9</sub> (Fig. 5A, compound **1**) was chemically synthesized (Fig. 5A,B). The synthesis of the target nonamannopyranoside **1** was planned starting from the previously synthesized **2** (Aqueel *et al.*, 2008) via synthesis of oligosaccharide **3** (Fig. 5A). For the synthesis

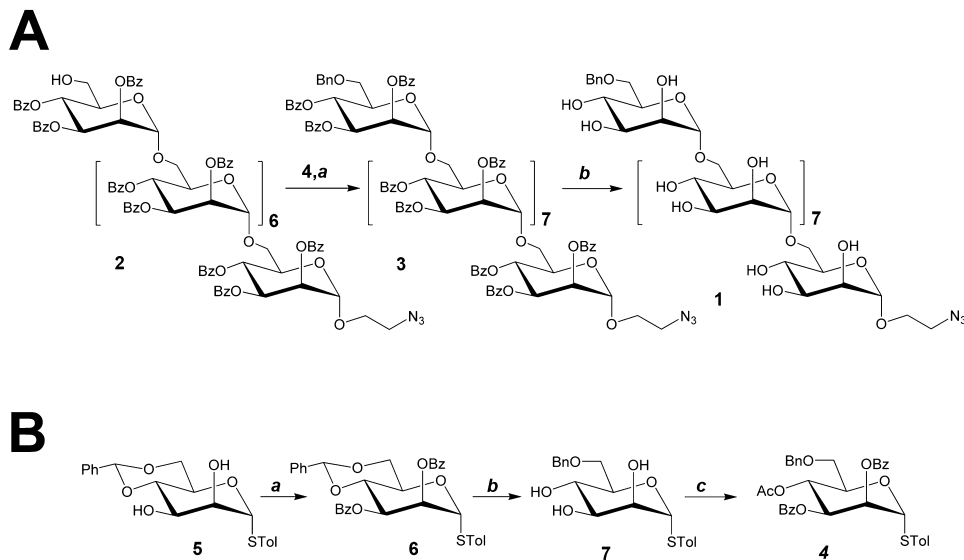


**Fig. 4.** Lipoglycan analysis of *C. glutamicum* $\Delta$ mptC $\Delta$ mptD. A. Lipoglycans were extracted from wild-type and mutant strains and were analysed using SDS-PAGE and visualized using a Pro-Q emerald glycoprotein stain (Invitrogen). Lipoglycan profiles are represented with standard molecular weight markers of glycoproteins of 180, 82, 42 and 18 kDa. B. 2D  $^1\text{H}$ - $^{13}\text{C}$ -NMR HMQC characterization of Cg-LAM from *C. glutamicum* as described in Tatituri *et al.* (2007b). C. 2D  $^1\text{H}$ - $^{13}\text{C}$ -NMR HMQC characterization of Cg-LAM from *C. glutamicum* $\Delta$ mptC $\Delta$ mptD. NMR spectra of Cg-LAMs were recorded in  $\text{D}_2\text{O}$  at 313 K.

of **3**, donor thioglycoside **4** was synthesized starting from the reported 5,6-*O*-benzylidene protected thioglycoside **5** (Cumpstey *et al.*, 2004) (Fig. 5B). In a straight forward reaction sequence, thioglycoside **5** was treated with

benzoyl chloride in pyridine to access the thioglycoside **6**, which on regioselective ring opening with  $\text{Et}_3\text{SiH}$  gave 6-OBn thioglycoside **7**. The  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectral studies confirmed the structure of **7**. An acetate protecting group was installed at the 4-OH position in thioglycoside **7** by reacting with acetic anhydride in pyridine resulting in the thioglycoside donor **4**. An acetate group was specifically chosen to easily characterize the reaction product during coupling of **4** and **2** by NMR. The donor thioglycoside **4** and **2** were coupled in the presence of NIS and triflic acid at  $-20^\circ\text{C}$ , resulting in nonasaccharide **3** after chromatographic purification. The observed singlet signal in the  $^1\text{H}$  NMR spectrum of **3** at  $\delta$  1.88 ppm was assigned to the three protons of the acetyl protecting group, and the resonances at  $\delta$  4.48 and 4.28 ppm (doublets,  $J = 11.9$  Hz), for one proton each to the benzyl protecting group, supporting the structure of **3**. The  $^{13}\text{C}$  NMR spectrum further confirmed the structure of **3**, with the presence of signals at 73.45 and 20.81 ppm, which were assigned to the  $\text{CH}_2$  of the benzyl and  $\text{CH}_3$  of the acetyl carbons respectively. The ester protecting groups in oligosaccharide **3** were removed in an overnight reaction using sodium methoxide in  $\text{CH}_3\text{OH}$ . The reaction was neutralized using  $\text{H}^+$  Amberlite resin and the reaction product purified using Bio-Beads SM-4 using a  $\text{CH}_3\text{OH}$ - $\text{H}_2\text{O}$  mobile phase and 0–60% gradient resulting in the desired nonmannoside **1**. The  $^1\text{H}$  NMR spectrum of **1** showed the presence of benzyl signals at  $\delta$  4.64 and 4.61 ppm (doublet,  $J = 11.7$  Hz). In the  $^{13}\text{C}$  NMR spectrum, the benzyl carbon was observed at  $\delta$  73.32 ppm and  $\text{CH}_2\text{N}_3$  at  $\delta$  51.79 ppm. Finally, the structure of **1** was further supported by HR-MS, which showed a  $[\text{M} + \text{Na}]^+$  peak at 1685.55.

The structural features of the acceptor allowed its specific isolation and purification of *in vitro* reaction products synthesized by distinct  $\alpha(1\rightarrow2)$ -mannopyranosyltransferases using a strong anion exchange cartridge, thus removing from the *in vitro* assay unused  $\text{C}_{50}$ -polyprenol-phospho- $^{14}\text{C}$ -mannose (PP- $^{14}\text{C}$ -M) and other *in vitro* synthesized products, such as higher  $^{14}\text{C}$ -PIMs,  $^{14}\text{C}$ -LM and  $^{14}\text{C}$ -LAM. In addition, the non-reducing sugar of **1** (which we have abbreviated as Acc-Man<sub>9</sub>) is blocked at the *O*-6 position by a benzyl ether, thus preventing further elongation by endogenous activities through MptA and MptB via  $\alpha(1\rightarrow6)$ -mannopyranosyltransferases. Overall, the utilization of Acc-Man<sub>9</sub> and the *in vitro* assay provides a novel route to evaluate distinct  $\alpha(1\rightarrow2)$ -mannopyranosyltransferases in *Corynebacterineae* (Fig. 6A). The *in vitro* transfer of  $^{14}\text{C}$ -Manp from PP- $^{14}\text{C}$ -M onto the Acc-Man<sub>9</sub> acceptor was examined using membrane preparations from wild-type and mutant strains of *C. glutamicum* (Fig. 6B). In this improved assay, membrane preparations from wild-type *C. glutamicum* transferred  $3072 \pm 132$  cpm of  $^{14}\text{C}$ -Manp



**Fig. 5.** Chemical synthesis of the nonasaccharide acceptor azidoethyl 6-*O*-benzyl- $\alpha$ -D-mannopyranosyl-(1 $\rightarrow$ 6)-[ $\alpha$ -D-mannopyranosyl-(1 $\rightarrow$ 6)]<sub>7</sub>-D-mannopyranoside (1) and key intermediate 4. Reagents and conditions. A. (a) NIS, triflic acid, CH<sub>2</sub>Cl<sub>2</sub>, -20°C–0°C, 1 h, 82%; (b) Sodium methoxide, CHCl<sub>3</sub>-CH<sub>3</sub>OH (1:2), overnight, 35%; B. (a) BzCl, pyridine, room temperature, 4 h, 94%; (b) Et<sub>3</sub>SiH, triflic acid, CH<sub>2</sub>Cl<sub>2</sub>, -78°C, 1 h, 80%; (c) acetic anhydride, pyridine, room temperature, 5 h, 94%.

from PP-[<sup>14</sup>C]M onto the synthetic Acc-Man<sub>9</sub> via an  $\alpha$ (1 $\rightarrow$ 2)-mannopyranosyltransferase (Fig. 6B and inset). Assays repeated in the absence of Acc-Man<sub>9</sub> afforded background activity 102  $\pm$  12 cpm, demonstrating the efficiency of the assay (Fig. 6B). Membrane preparations from both *C. glutamicum* $\Delta$ mptC and *C. glutamicum* $\Delta$ mptD used in above assays afforded  $\alpha$ (1 $\rightarrow$ 2)-mannopyranosyltransferase activities, but in each case the relative level of activity in comparison with wild-type *C. glutamicum* membranes was reduced (Fig. 6B).

The analysis of [<sup>14</sup>C] reaction products was simplified further by utilizing membrane preparations from the branching deficient double mutant, *C. glutamicum* $\Delta$ mptC $\Delta$ mptD, lacking Cg-MptC and Cg-MptD. The *C. glutamicum* $\Delta$ mptC $\Delta$ mptD mutant possessed background (149  $\pm$  15 cpm) incorporation of [<sup>14</sup>C]-Manp (Fig. 6B), demonstrating the complete absence of  $\alpha$ (1 $\rightarrow$ 2)-mannopyranosyltransferase activity in this double mutant. Therefore, the double mutant, *C. glutamicum* $\Delta$ mptC $\Delta$ mptD was used to analyse the enzymatic activities of Cg-MptC and Cg-MptD. Cg-mptC and Cg-mptD were cloned into pEKEx2 and introduced into *C. glutamicum* $\Delta$ mptC $\Delta$ mptD resulting in *C. glutamicum* $\Delta$ mptC $\Delta$ mptD pEKEx2-Cg-mptC and *C. glutamicum* $\Delta$ mptC $\Delta$ mptD pEKEx2-Cg-mptD. *C. glutamicum* $\Delta$ mptC $\Delta$ mptD pEKEx2-Cg-mptC resulted in 1570  $\pm$  83 cpm of Acc-Man<sub>9</sub>-[<sup>14</sup>C]-Man<sub>x</sub>, while *C. glutamicum* $\Delta$ mptC $\Delta$ mptD pEKEx2-Cg-mptD resulted in 953  $\pm$  24 cpm of Acc-Man<sub>9</sub>-[<sup>14</sup>C]-Man<sub>x</sub>.

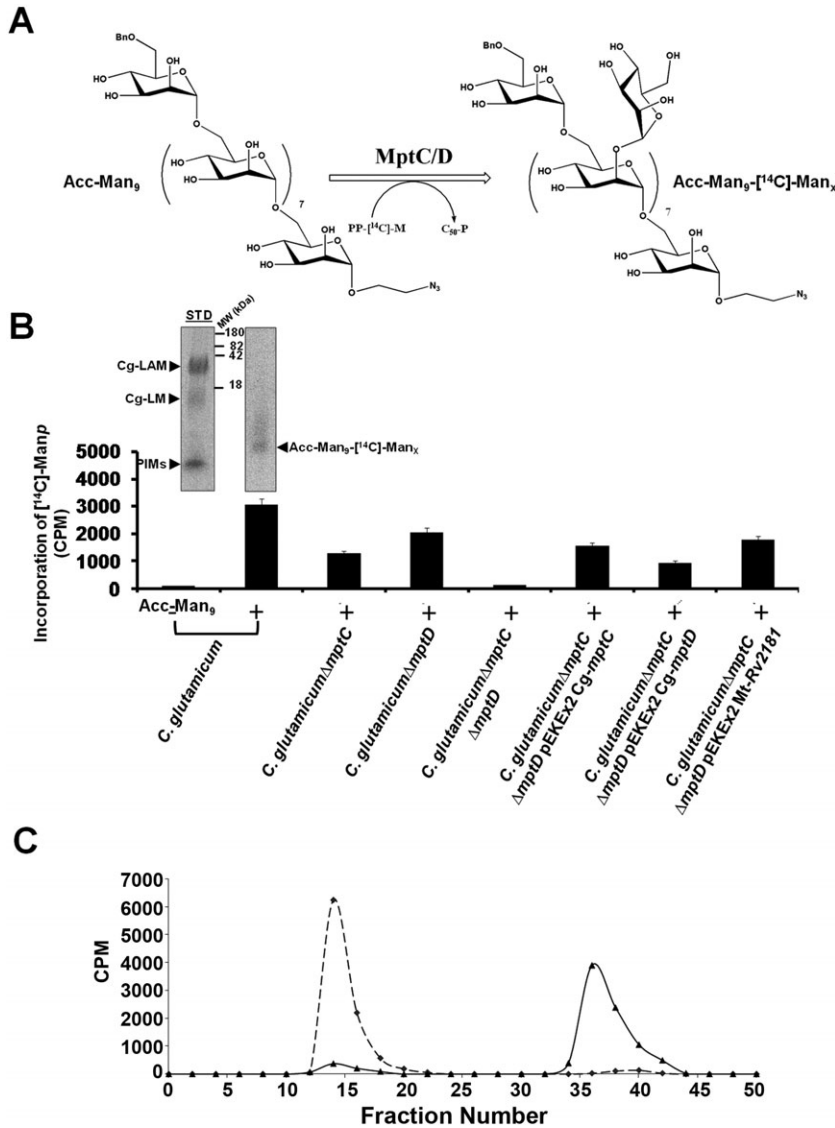
#### Acc-Man<sub>9</sub>-[<sup>14</sup>C]-Man<sub>x</sub> selective cleavage by an $\alpha$ (1 $\rightarrow$ 2)-D-mannosidase

The extracted Acc-Man<sub>9</sub>-[<sup>14</sup>C]-Man<sub>x</sub> reaction product from the above assays performed with *C. glutamicum* membranes (Fig. 6B) were dried and subsequently treated with a previously characterized  $\alpha$ (1 $\rightarrow$ 2)-D-mannosidase from *Trichoderma reesei* (Maras *et al.*, 2000) as described (Ichishima *et al.*, 1981; Yokoyama and Ballou, 1989) to determine the  $\alpha$ (1 $\rightarrow$ 2) addition of Manp. The digested mixtures were fractionated on a gel filtration column. The control Acc-Man<sub>9</sub>-[<sup>14</sup>C]-Man<sub>x</sub> reaction product, before  $\alpha$ (1 $\rightarrow$ 2)-D-mannosidase treatment eluted from the Bio-Gel P-2 column at fractions 13–16, and this completely enzymatically digested product (measured through the release of [<sup>14</sup>C]-Manp), was retained and eluted in later fractions 35–42 with a [<sup>14</sup>C]-mannose standard (Fig. 6C; Cooper *et al.*, 2002). The results demonstrate that the addition of [<sup>14</sup>C]-Manp to Acc-Man<sub>9</sub> is via an  $\alpha$ (1 $\rightarrow$ 2)-mannopyranosyltransferase, and combined with the data from Fig. 6B and the use of the *C. glutamicum* $\Delta$ mptC $\Delta$ mptD mutant strain, has shown that MptC and MptD are solely responsible for this  $\alpha$ (1 $\rightarrow$ 2)-mannopyranosyltransferase activity.

#### Functional identification of the mycobacterial homologue of Cg-MptC

As described above (Fig. 1), Cg-MptC showed highest similarity of 55% to Rv2181 of *M. tuberculosis*, while





**Fig. 6.** *In vitro*  $\alpha(1\rightarrow2)$ -mannopyranosyltransferase assay utilizing a nonasaccharide acceptor.

A. Reaction scheme illustrating *in vitro* assays performed with the chemically synthesized nonasaccharide acceptor, azidoethyl 6-*O*-benzyl- $\alpha$ -D-mannopyranosyl-(1 $\rightarrow$ 6)-[ $\alpha$ -D-mannopyranosyl-(1 $\rightarrow$ 6)]- $\beta$ -D-mannopyranoside,  $\text{Acc-Man}_9$ , and either MptC or MptD, resulting in the synthesis of  $\text{Acc-Man}_9\text{-[}^{14}\text{C]-Man}_x$ .

B. The reactions were set up with and without  $\text{Acc-Man}_9$  (+ or -),  $\text{PP-[}^{14}\text{C]-M}$ , and membrane preparations from different strains of *C. glutamicum* as described in the *Experimental procedures*.

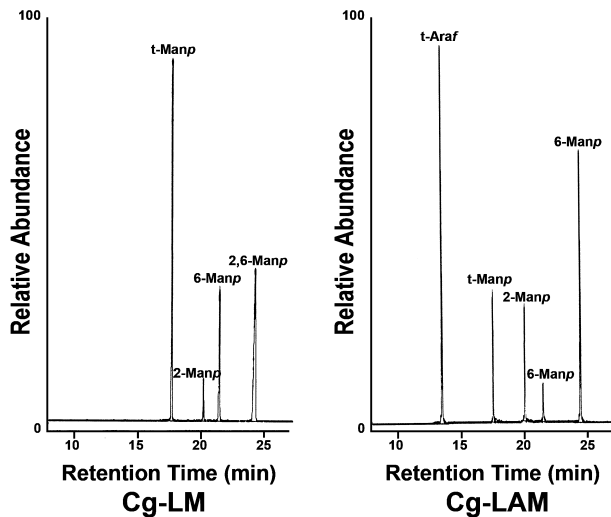
C. The  $\text{Acc-Man}_9\text{-[}^{14}\text{C]-Man}_x$  product from the use of *C. glutamicum* membranes was dried and subsequently incubated with an  $\alpha(1\rightarrow2)$ -mannosidase. The reaction mixture was then fractionated on a Bio-Gel P-2 gel filtration column. The control  $\text{Acc-Man}_9\text{-[}^{14}\text{C]-Man}_x$  (dotted line) product before  $\alpha(1\rightarrow2)$ -D-mannosidase treatment eluted from the Bio-Gel P-2 column at fractions 11–13, and the digested product, released  $^{14}\text{C}$ -Man (solid line), was retained and eluted in later fractions 33–39, co-eluting with a  $^{14}\text{C}$ -mannose standard (Cooper *et al.*, 2002).

*Cg*-MptD showed weaker, but significant similarity of 47.9% to Rv2181. To assay for a complementary function of Rv2181 in *C. glutamicum*, plasmid pEKEx2-Mt-Rv2181 was constructed. Upon introduction into *C. glutamicum* $\Delta\text{mptC}$ , the wild-type phenotype of *C. glutamicum* was restored (Fig. 2A) illustrating that Mt-MptC is functional and utilizes the corynebacterial substrates. In addition, Rv2181 was very specific for *C. glutamicum* $\Delta\text{mptC}$  as complementation of the mutant phenotype in *C. glutamicum* $\Delta\text{mptD}$  was not obtained (Fig. 2A). The complementation of *C. glutamicum* $\Delta\text{mptC}$  with pEKEx2-Mt-Rv2181 also restored the wild-type glycosyl linkage phenotype in this strain (Fig. 7, and see Fig. 3A and B). Furthermore, use of membrane preparations from *C. glutamicum* $\Delta\text{mptC}$  $\Delta\text{mptD}$  pEKEx2-Mt-Rv2181 and the synthetic  $\text{Acc-Man}_9$  (Fig. 6A) resulted in the incorporation of  $1811 \pm 87$  cpm of  $^{14}\text{C}$ -Manp into the  $^{14}\text{C}$ -mannan

product (Fig. 6B). Altogether, these data strongly suggest that Rv2181 is a homologue of *Cg*-mptC and both encode for  $\alpha(1\rightarrow2)$ -mannopyranosyltransferase activities, involved in the synthesis of the  $\alpha(1\rightarrow2)$ -Manp branches of LM and LAM in *Corynebacterineae*.

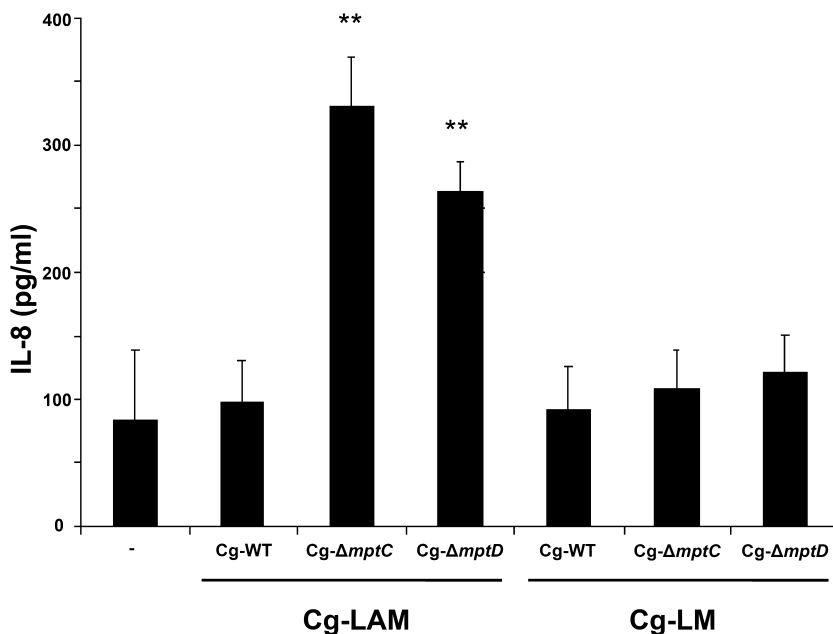
#### Activation of Toll-like receptor 2 (TLR2) by wild-type and mutant *Cg*-LM and LAM

Both LM and LAM are bioactive molecules thought to be involved in modulating the host immune response (Briken *et al.*, 2004). Bacterial lipoglycans are recognized by a variety of host immune receptors including TLR2. In fact, mycobacterial LMs are well known to be potent activators of this latter receptor hence providing them strong pro-inflammatory activity (Vignat *et al.*, 2003; Quesniaux *et al.*, 2004; Gilleron *et al.*, 2006). On the other hand,



**Fig. 7.** Characterization of the mycobacterial homologue Rv2181 of Cg-MptC. Lipoglycans were extracted from *C. glutamicum* $\Delta$ mptC pEKEx2-Mt-Rv2181, and the resulting Cg-LM and Cg-LAM products purified, per-*O*-methylated, and hydrolysed using 2 M TFA, reduced and per-*O*-acetylated. The resulting partially per-*O*-methylated, per-*O*-acetylated alditol acetates were analysed by GC-MS and products identified based on their characteristic alditol cleavage acetal profiles: t-Araf (*m/z* 118, 161), t-Manp (*m/z* 118, 102, 145, 161, 162, 205), 2-Manp (*m/z* 129, 130, 161, 190), 6-Manp (*m/z* 118, 129, 162, 189, 233) and 2,6-Manp (*m/z* 129, 130, 189, 190).

LAMs are only poorly active; a feature attributed to the presence of the arabinan domain that masks the 'bioactive' mannan core (Vignal *et al.*, 2003; Nigou *et al.*, 2008; Birch *et al.*, 2010). To investigate the ability of Cg-LM and Cg-LAM to activate TLR2 to assess possible consequences of Cg-*mptC* and Cg-*mptD* inactivation in this



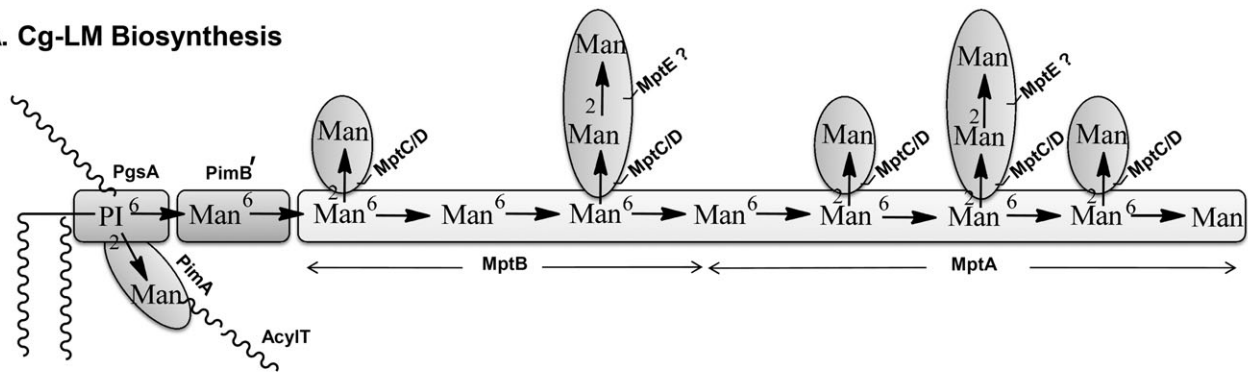
**Fig. 8.** TLR-2 stimulation by wild-type and mutant Cg-LM and Cg-LAM. HEK293 cells transfected with TLR2 were stimulated with 50  $\mu$ g ml<sup>-1</sup> of wild-type (WT) or mutant ( $\Delta$ mptC/ $\Delta$ mptD) Cg-LAM or Cg-LM. Unstimulated cells (-) served as a control. After 24 h of stimulation (37°C), supernatants were harvested and analysed for IL-8 content using ELISA. The results represent the mean IL-8 production  $\pm$  SD from three independent experiments (each experiment was performed in triplicate). Double asterisks indicate highly significant ( $P < 0.001$ ) differences as compared to the unstimulated control cells.

process, purified LM and LAM from *C. glutamicum* $\Delta$ mptC and *C. glutamicum* $\Delta$ mptD, were used to stimulate HEK293 cells expressing TLR2. To prevent interference of co-purified lipopeptides, the glycolipids were first pre-treated with hydrogen peroxide (Morr *et al.*, 2002; Geurtsen *et al.*, 2009; Birch *et al.*, 2010). As shown in Fig. 8, wild-type Cg-LAM was unable to activate TLR2. This result is consistent with an earlier study in which it was shown that LAMs substituted with single arabinosyl residues were unable to induce the production of tumour necrosis factor- $\alpha$  in human THP-1 monocytes (Nigou *et al.*, 2008). Interestingly, LAMs isolated from the *C. glutamicum* $\Delta$ mptC and *C. glutamicum* $\Delta$ mptD mutant strains demonstrated an increased activity as evidenced by the enhanced TLR2-dependent IL-8 production by HEK293 cells (Fig. 8). This suggests that partial removal of  $\alpha(1\rightarrow2)$ -Manp units, increases the ability of Cg-LAM to activate TLR2. In contrast to mycobacterial LMs, wild-type Cg-LM did not show any activity towards TLR2. Furthermore, mutation of Cg-*mptC* and Cg-*mptD* did not alter this behaviour and the mutant LMs remained inactive (Fig. 8). These results demonstrate that wild-type Cg-LAM and Cg-LM, are both TLR2 inactive. Furthermore, partial removal of  $\alpha(1\rightarrow2)$ -Manp branching may promote TLR2 activation, but this is only the case for Cg-LAM.

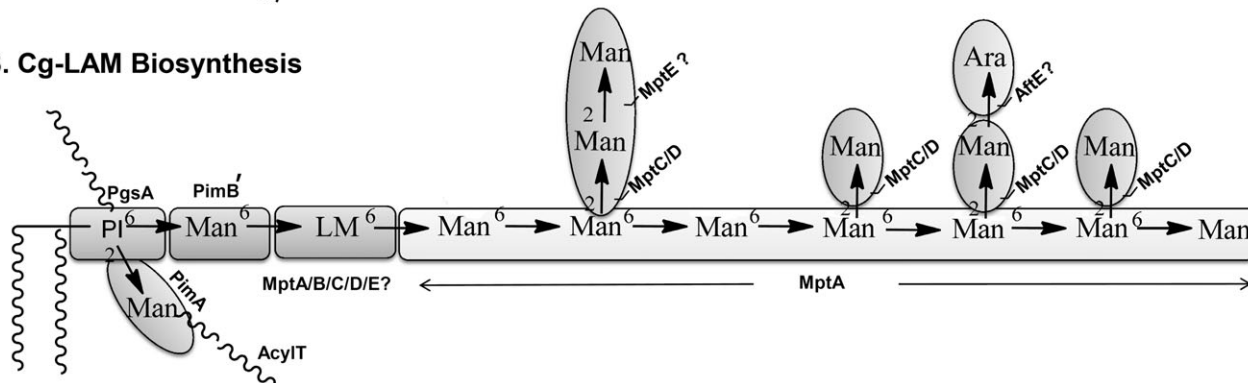
## Discussion

Apart from sharing a similar cell wall architecture, *C. glutamicum* possesses similar genetic loci responsible for cell wall biosynthesis like *M. tuberculosis*. As a model, it has proven extremely useful in the study of essential genetic elements of *M. tuberculosis* (Alderwick *et al.*,

### A. Cg-LM Biosynthesis



### B. Cg-LAM Biosynthesis



**Fig. 9.** Biosynthetic pathway of LM (A) and LAM (B) biogenesis in *Corynebacterineae*. Cg-LM biosynthesis initially involves the formation of the  $\alpha(1\rightarrow6)$  mannan backbone by MptB, followed by  $\alpha(1\rightarrow2)$ -Manp branching by MptC (or MptD). This biosynthetic precursor would then be the substrate for further  $\alpha(1\rightarrow6)$ -Manp elongation by MptA, followed by  $\alpha(1\rightarrow2)$ -Manp branching by MptD (or MptC) to afford initially Cg-LM (A). Further extension by MptA along with additional GT-C glycosyltransferases further modify the  $\alpha(1\rightarrow6)$ -mannan backbone. These GT-Cs include MptC/D and MptE (formation of the dimannoside side-chain), and AftE/MptC/D and formation of AraF-Manp side-chains (B).

2006a,b). In particular, it has enabled us to use it as a suitable model for the identification and functional study of mycobacterial genes involved in mycolic acid (Gande *et al.*, 2004; 2007), arabinogalactan (Alderwick *et al.*, 2005; 2006b; 2007; Seidel *et al.*, 2007a,b; Birch *et al.*, 2008; 2009) and LAM biosynthesis (Gibson *et al.*, 2003; Mishra *et al.*, 2007; 2008a,b; 2009; Tatituri *et al.*, 2007a,b). Previously, we reported the identification of enzymes involved in early and late stages of LM and LAM biosynthesis in *Corynebacterineae* (Mishra *et al.*, 2007; 2008a,b; 2009).

SDS-PAGE and MALDI-TOF-MS analysis of different lipoglycan species, including Cg-LM and Cg-LAM from *C. glutamicum* $\Delta$ mptC and *C. glutamicum* $\Delta$ mptD, suggested that there is a difference in a similar component of all these species. Two-dimensional thin-layer chromatography (2D-TLC) analysis supplemented with negative MALDI-TOF-MS of extracted glycolipids from mutants failed to show any differences in the PIM precursors (data not shown), and the presence of MptA and MptB in these mutants, negates the possibility of a change in the  $\alpha(1\rightarrow6)$  mannan backbone of these lipoglycans, leaving the only possibility of a change in  $\alpha(1\rightarrow2)$ -Manp branching, suggesting that MptC and MptD are involved in syn-

thesis of these  $\alpha(1\rightarrow2)$ -Manp units in these lipoglycans. In addition, glycosyl linkage analysis revealed a reduction in the relative abundance of t-Manp and 2,6-Manp with a concomitant increase in the abundance of 6-Manp in Cg-LM and Cg-LAM from these strains. The cumulative chemical analysis of *C. glutamicum* $\Delta$ mptC and *C. glutamicum* $\Delta$ mptD agrees with the view that Cg-MptC and Cg-MptD are involved in synthesis of  $\alpha(1\rightarrow2)$ -Manp units in Cg-LM and Cg-LAM in *C. glutamicum*. This view was strengthened by the analysis of a *C. glutamicum* $\Delta$ mptC $\Delta$ mptD double mutant and the specificity of MptC and MptD in cross-complementation studies (Fig. S2). In summary, the data clarified the occurrence of residual t- $\alpha$ -Manp,  $\alpha(1\rightarrow2)$ -Manp and t- $\alpha$ -Araf units in the Cg-MptC and Cg-MptD mutants, as the singular mutants only partially removed 2,6-Manp branching. The absence of these units in the *C. glutamicum* $\Delta$ mptC $\Delta$ mptD mutant provides evidence that these units are present as t- $\alpha$ -Manp-( $\alpha(1\rightarrow2)$ -Manp and t- $\alpha$ -Araf-( $\alpha(1\rightarrow2)$ -Manp motifs (Fig. 9). Furthermore, specific *in vitro* assays utilizing a chemically synthesized nonasaccharide acceptor demonstrate that both Cg-mptC and Cg-mptD encode for  $\alpha(1\rightarrow2)$ -mannopyranosyltransferases, responsible for the synthesis of the  $\alpha(1\rightarrow2)$ -Manp units on the mannan backbone of

LM and LAM. From the earlier studies with Rv2181 (Kaur *et al.*, 2008), and the fact that Cg-MptC and Rv2181 are homologues, it is tempting to speculate that Cg-MptC adds  $\alpha(1\rightarrow2)$ -Manp residues to the proximal backbone of Cg-LM, or one could imply that Rv2181 *in vitro* has MptC function. A plasmid encoding Rv2181 was used to transform the *C. glutamicum* mutants, and Rv2181 was able to restore the wild-type phenotype in only the *C. glutamicum* $\Delta$ mptC mutant, and possessed  $\alpha(1\rightarrow2)$ -mannopyranosyltransferase activity in the defined *in vitro* acceptor assay reported in this study.

Whereas the LM/LAM backbone is potentially rather similar within the *Corynebacterineae*, as indicated also from the relatedness of the  $\alpha(1\rightarrow6)$ -mannopyranosyltransferases (Fig. 1), it is clear from structural, immunological and genetic evidence, that the more elaborated structures and decorations of these glycolipids are variable. Known structural variations include large arabinan domains in *M. tuberculosis* (Chatterjee *et al.*, 1991; McNeil *et al.*, 1994), Man-capping of arabinan domains in *M. tuberculosis* (Chatterjee *et al.*, 1992) and their PI capping in *M. smegmatis* (Khoo *et al.*, 1995). Accordingly, also immunological properties of LM/LAM are different (Briken *et al.*, 2004). This variability is naturally reflected at the genetic level. An example is the capping GT-C transferase of *M. tuberculosis* encoded by Rv1635c (Appelmeik *et al.*, 2008), which is not present in *C. glutamicum*. The fact that MptC in *C. glutamicum* is a fusion protein carrying a second domain with unknown function, also illustrates that mature LM and LAM glycolipids are species-specific and accordingly the corresponding biosynthesis genes more prone to rapid evolution. Duplication or fusion of genes may indicate that the types of enzymes involved in the later steps of LM and LAM synthesis are highly dynamic and of evolutionary change within the *Corynebacterineae* (Xie *et al.*, 2003).

A key question in lipoglycan biosynthesis is the division of LM and LAM abundance in the cell wall. The separation of LM and LAM in *C. glutamicum*, biosynthetically probably occurs after the initial formation of the  $\alpha(1\rightarrow6)$  mannan backbone by MptB, followed by  $\alpha(1\rightarrow2)$ -Manp branching by MptC (or MptD). The product of these two enzymes would then be the preferred substrate for further  $\alpha(1\rightarrow6)$ -Manp elongation by MptA, followed by  $\alpha(1\rightarrow2)$ -Manp branching by MptD (or MptC) (Fig. 9). Additional GT-C glycosyltransferases remain to be characterized involved in further modification of the  $\alpha(1\rightarrow6)$ -mannan backbone. These include how the dimanoside (via MptE?) and Araf-Manp side-chains are synthesized (AftE?) and are summarized in Fig. 9.

Both LAM and LM are immunomodulatory molecules (Briken *et al.*, 2004). Whereas LAM has an important role during intracellular survival, for instance by preventing phagosome-lysosome fusion (Fratti *et al.*, 2003), LM has

strong pro-inflammatory activity through its ability to activate TLR2 (Vignal *et al.*, 2003; Quesniaux *et al.*, 2004; Gilleron *et al.*, 2006). Previous research has demonstrated that the pro-inflammatory activity of LM is at least partly based upon its acylation status (Gilleron *et al.*, 2006; Doz *et al.*, 2007). PI-based LAM and LM species can differ in their degree of acylation, each carrying between one and four acyl chains. Investigation of the immune activating properties of differentially acylated LM species demonstrated that LM should at least be tri-acylated to be pro-inflammatory, with species carrying less acyl chains exhibiting immunosuppressive activity in a mechanism independent of TLR2 (Doz *et al.*, 2007).

Here we investigated different Cg-LAM and LM species for their ability to activate TLR2. Similar to mycobacterial LAMs, wild-type Cg-LAM was found to be inactive (Fig. 8). This finding is consistent with previous observations that LAM species carrying only t-Araf substitutions, such as LAMs isolated from *Rhodococcus ruber* and *Turicella otitidis*, are largely inactive (Nigou *et al.*, 2008). Comparison of wild-type Cg-LAM to the LAMs isolated from the *C. glutamicum* $\Delta$ mptC and *C. glutamicum* $\Delta$ mptD mutant strains demonstrated that the mutant LAMs had gained the ability to activate TLR2 (Fig. 8). The explanation for this increased activity is currently not known. Interestingly, wild-type Cg-LM, like wild-type Cg-LAM, but unlike mycobacterial LM was found to be completely TLR2-inactive (Fig. 8). The reason for the discrepancy is unclear, but may be related to structural differences in their membrane anchors. In mycobacteria, LAMs and LMs share a common PI anchor, on which further assembly occurs to give mature LM and LAM. However, in *C. glutamicum*, although Cg-LAM contains a normal PI anchor, two different pools of Cg-LM (Cg-LM-A and Cg-LM-B) dominate the majority of lipoglycan composition of the corynebacterial cell wall (Tatituri *et al.*, 2007a; Lea-Smith *et al.*, 2008; Mishra *et al.*, 2008b). The large majority of the Cg-LM species (~95%), Cg-LM-B, contains an anchor based upon a di-acylated  $\alpha$ -D-glucopyranosyluronic acid-(1 $\rightarrow$ 3)-glycerol moiety, and only a minor fraction contains the more classical anchor based on PI, Cg-LM-A (Mishra *et al.*, 2008b). Despite the different lipid anchors, the mannan backbone structures of Cg-LM-A and Cg-LM-B are similar as revealed through GC-MS analysis of partially methylated alditol acetates (Mishra *et al.*, 2008b). This means that the majority of Cg-LM species will contain a di-acylated GlcAGroAc<sub>2</sub> anchor, which is a feature that has been demonstrated to be incompatible with activation of TLR2 (Doz *et al.*, 2007).

Furthermore, a complete investigation of the structural and functional relationship of these lipoglycans during host-pathogen interaction requires the availability of mutants defective in their respective biosynthetic pathways. The availability of complete genome sequences of



several mycobacteria and related actinomycetes, like *C. glutamicum*, and the development of novel tools for genetic manipulation has opened up these possibilities (Cole *et al.*, 1998).

## Experimental procedures

### Bacterial strains, cell culture and growth conditions

*Corynebacterium glutamicum* and *E. coli* DH5 $\alpha$ mc $r$  were grown in LB broth at 30°C and 37°C respectively. For *C. glutamicum* complex medium BHI and minimal medium CGXII was used (Eggeling and Bott, 2005). Kanamycin and ampicillin were used at a concentration of 50  $\mu$ g ml<sup>-1</sup> for selection of recombinants. Samples for lipid analysis were prepared by harvesting cells at an OD of 10–15, followed by a saline wash and freeze drying. HEK293 cells transfected with TLR2 (Flo *et al.*, 2002) were kept in DMEM (Invitrogen) containing 10% FCS, 100 U ml<sup>-1</sup> penicillin, 100  $\mu$ g ml<sup>-1</sup> streptomycin, 0.5 mg ml<sup>-1</sup> G418, 2 mM L-glutamine and 110 mg l<sup>-1</sup> pyruvate.

### Construction of plasmids and strains

To delete *Cg-mptC* and *Cg-mptD* the deletion vectors pK19mobsacB $\Delta$ Cg-*mptC*, and pK19mobsacB $\Delta$ Cg-*mptD* were constructed. In each case cross-over PCR was used with genomic DNA as template and two different PCR's with primer pairs AB and CD (Table S1). The resulting PCR products served as template for primer pairs AD. The PCR product contained 12 nucleotides (nt) of the 3' end of the respective gene together with genomic upstream sequences, and 36 nt of the 5' end together with genomic downstream sequences. The inserts of all plasmids used in this work were confirmed by sequencing. Genes were deleted in by first introducing plasmids prepared from *E. coli* via electroporation into *C. glutamicum* and then selection for sucrose resistance in a procedure as described (Schäfer *et al.*, 1994). Starting from *C. glutamicum* $\Delta$ *mptC* and using pK19mobsacB $\Delta$ Cg-*mptD* the double deletion mutant *C. glutamicum* $\Delta$ *mptC* $\Delta$ *mptD* was made. The starting strain used was the type strain *C. glutamicum* ATCC13032. Chromosomal deletions were confirmed using primer pairs AD, as well as the additional new primer pairs EF, hybridizing outside of the regions used for plasmid constructions.

To assay for complementation of chromosomal deletions four expression plasmids were used. To construct pEKEx2-*Cg-mptC* primer pairs Ex-2100-RBS-for and Ex-2100-rev were used to amplify the 5' half of *NCgl2100*, the PCR product treated with Sall/EcoRI and ligated with similar treated pEKEx2. To construct pEKEx2-*Cg-mptD* primer pairs Ex-2097-RBS-for and Ex-2097-rev were used to amplify *NCgl2097*, the PCR product treated with Sall/EcoRI and ligated with similar treated pEKEx2. To construct pEKEx2-Mt-*Rv2181*, chromosomal DNA of *M. tuberculosis* served as template, and *Rv2181* was amplified using primer pairs Ex-Rv2181-for and Ex-Rv2181-rev, the former providing the sequence CTGCAG as a ribosome binding site. The amplified product was treated with PstI and EcoRI and ligated with pEKEx2. Plasmids were used to transform the respective

mutants via electroporation to kanamycin resistance, and recombinants confirmed by plasmid preparation.

### Lipid extraction and analysis

Polar and apolar lipids were extracted as described by Dobson *et al.* (1985). Briefly, 6 g of dry *C. glutamicum* cells was treated in 220 ml of methanolic saline (20 ml 0.3% NaCl and 200 ml CH<sub>3</sub>OH) and 220 ml of petroleum ether for 2 h (Dobson *et al.*, 1985). The suspension was centrifuged and the upper layer containing apolar lipids was separated. An additional 220 ml of petroleum ether was added, mixed and centrifuged as described above. The two upper petroleum ether fractions were combined and dried. For polar lipids, 260 ml CHCl<sub>3</sub>/CH<sub>3</sub>OH/0.3% NaCl (9:10:3, v/v/v) was added to the lower aqueous phase and stirred for 4 h. The mixture was filtered and the filter cake re-extracted twice with 85 ml of CHCl<sub>3</sub>/CH<sub>3</sub>OH/0.3% NaCl (5:10:4, v/v/v). Equal amounts of CHCl<sub>3</sub> and 0.3% NaCl (145 ml each) 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 aluminium backed plates of silica gel 60 F<sub>254</sub> (Merck 5554), using CHCl<sub>3</sub>/CH<sub>3</sub>OH/H<sub>2</sub>O (65:25:4, v/v/v) in the first direction and CHCl<sub>3</sub>/CH<sub>3</sub>COOH/CH<sub>3</sub>OH/H<sub>2</sub>O (40:25:3:6, v/v/v/v) in the second direction. The thin-layer chromatographic plates sprayed with the appropriate staining solution to detect the presence of lipids, glycolipids or phospholipids as described (Dobson *et al.*, 1985).

### Extraction and purification of lipoglycans

Lipoglycans were extracted from delipidated cells as previously described (Ludwiczak *et al.*, 2001). Briefly, cells were broken by sonication (MSE Soniprep 150, 12 micron amplitude, 60 s ON, 90 s OFF for 10 cycles, on ice) and the cell debris refluxed five times with 50% C<sub>2</sub>H<sub>5</sub>OH at 68°C, for 12 h intervals. The cell debris was removed by centrifugation and the supernatant containing lipoglycans, neutral glycans and proteins dried. This dried extract was then treated with hot phenol-H<sub>2</sub>O. The aqueous phase was dialysed and dried, followed by extensive treatments with  $\alpha$ -amylase, DNase, RNase chymotrypsin and trypsin. This fraction was dialysed to remove the low MW break-down products formed after the enzyme treatment, thus yielding the crude lipoglycan fraction.

The crude lipoglycan extract was dried and resuspended in buffer A (50 mM ammonium acetate and 15% propan-1-ol) and subjected to Octyl Sepharose CL-4B chromatography (2.5  $\times$  50 cm). The column (2.5  $\times$  50 cm) was washed initially with four column volumes of buffer A to ensure removal of neutral glycans followed by buffer B (50 mM ammonium acetate and 50% propan-1-ol). The eluent was collected and concentrated to approximately 1 ml and precipitated using 5 ml of C<sub>2</sub>H<sub>5</sub>OH. The sample was dried using a Savant Speedvac and then resuspended in buffer C [0.2 M NaCl, 0.25% sodium deoxycholate (w/v), 1 mM EDTA and 10 mM Tris-HCl, pH 8] to a final concentration of 20 mg ml<sup>-1</sup>. The sample was gently mixed and left to incubate for 48 h at room temperature. The sample was then loaded onto a Sephacryl S-200 (2.5 cm  $\times$  50 cm) column previously equilibrated with

buffer C. The sample was eluted with 400 ml of buffer C at a flow rate of 3 ml h<sup>-1</sup>, collecting 1.5 ml fractions. The fractions were monitored by SDS-PAGE using either a silver stain or a Pro-Q emerald glycoprotein stain and individual fractions pooled and dialysed extensively against buffer D (10 mM Tris-HCl, pH 8, 0.2 M NaCl, 1 mM EDTA) for 72 h with frequent changes of buffer. The samples were further dialysed against deionized water for 48 h with frequent changes of water, lyophilized and stored at -20°C.

#### MALDI-TOF-MS and NMR analysis

The matrix used was 2,5-dihydroxybenzoic acid at a concentration of 10 µg µl<sup>-1</sup>, in a mixture of H<sub>2</sub>O/C<sub>2</sub>H<sub>5</sub>OH (1:1, v/v), 0.1% trifluoroacetic acid (TFA). Samples (0.5 µl) at a concentration of 10 µg µl<sup>-1</sup> were mixed with 0.5 µl of the matrix solution. Analyses were performed on a Voyager DE-STR MALDI-TOF instrument (PerSeptive Biosystems, Framingham, MA, USA) using linear mode detection. Mass spectra were recorded in the negative mode using a 300 ns time delay with a grid voltage of 80% of full accelerating voltage (25 kV) and a guide wire voltage of 0.15%. The mass spectra were mass assigned using external calibration. NMR spectra of LAM samples were recorded on a Bruker DMX-500 equipped with a double-resonance (<sup>1</sup>H/<sup>13</sup>C)-BBi z-gradient probe head. All samples were exchanged in D<sub>2</sub>O, with intermediate lyophilization, and then dissolved in 0.7 ml D<sub>2</sub>O and analysed at 313 K. The <sup>1</sup>H and <sup>13</sup>C NMR chemical shifts were referenced relative to internal acetone at 2.225 and 34.00 ppm respectively. All details concerning NMR sequences and the experimental procedures were described previously (Tatituri *et al.*, 2007b).

#### Glycosyl compositional and linkage analysis

Lipoglycans were hydrolysed using 250 µl of 2 M TFA at 120°C for 2 h, reduced with 100 µl of NaBD<sub>4</sub> (10 mg ml<sup>-1</sup> in C<sub>2</sub>H<sub>5</sub>OH:1 M NH<sub>4</sub>OH), and the resultant alditols per-*O*-acetylated using acetic anhydride (100 ml) at 120°C for 1 h, before examination by GC (Tatituri *et al.*, 2007b). GC was performed using a Thermoquest Trace GC 2000 and a DB225 column (Supelco) with samples injected in the splitless mode. The oven was programmed to hold at an isothermal temperature of 275°C for a run time of 15 min. Glycosyl linkage analyses were performed as described previously (Tatituri *et al.*, 2007b). Briefly, lipoglycan samples were per-*O*-methylated using dimethyl sulfinyl carbanion, hydrolysed using 2 M TFA, reduced using NaB<sub>2</sub>H<sub>4</sub>, and per-*O*-acetylated before analysis by GC-MS (Tatituri *et al.*, 2007b). GC-MS was carried out on a Finnigan Polaris GCQ PlusTM using a BPX5 (Supelco) column. The injector temperature was set at 50°C, held for 1 min and then increased to 110°C at 20°C min<sup>-1</sup>. The oven was held at 110°C then ramped to 290°C at 8°C min<sup>-1</sup> and held for 5 min to ensure all the products had eluted from the column. All the data were collected and analysed using Xcaliber (v.1.2) software.

#### General methods for chemical synthesis of Acc-Man<sub>9</sub> (compound 1)

Reactions were performed under a dry argon atmosphere. Whenever necessary, compounds and starting materials

were dried by azeotropic removal of water with toluene under reduced pressure. The reactions were monitored by TLC on pre-coated silica gel (60F<sub>254</sub>) plates (0.25 mm) from E. Merck and visualized using UV light (254 nm) and/or heating after spraying with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> solution (150 g ammonium sulphate, 30 ml H<sub>2</sub>SO<sub>4</sub>, 750 ml H<sub>2</sub>O). Flash column chromatography was carried out on Isco Teledyne CombiFlash Rf200 chromatographic system using pre-packed columns. <sup>1</sup>H and <sup>13</sup>C NMR spectra were recorded at 400 MHz and 100 MHz, respectively, on a Varian 400-MR spectrometer. Coupling constants (*J*) are reported in Hz and chemical shifts ( $\delta$ ) in ppm relative to a residual solvent peak or an internal standard (TMS). The HR-MS spectra were recorded on a Agilent 6140 liquid chromatography mass spectrometry instrument.

#### *p*-Tolyl 2,3-Di-*O*-benzoyl-4,5-*O*-benzylidene-1-thio- $\alpha$ -D-mannopyranoside (6)

Compound 5 (230 mg, 0.61 mmol) was dissolved in 5 ml of anhydrous pyridine, and benzoyl chloride (179 µl, 1.54 mmol) was added dropwise. The reaction mixture was stirred for 4 h at room temperature, poured into ice-water (15 ml) and extracted with CHCl<sub>3</sub> (2 × 10 ml). The combined CHCl<sub>3</sub> layers were washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub> and concentrated. Flash chromatographic purification of the crude product using cyclohexane/ethyl acetate as eluent gave pure 6 (335 mg, 94%) as a colourless solid (mp 66°C). *R*<sub>f</sub> = 0.44 (cyclohexane/ethyl acetate, 2:1). <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz):  $\delta$  8.06 (2H, m, Ar), 7.92 (2H, m, Ar), 7.61 (1H, m, Ar), 7.46 (8H, m, Ar), 7.33 (4H, m, Ar), 7.15 (2H, d, *J* = 6.3 Hz, Ar), 5.95 (1H, dd, *J* = 0.9, 2.7 Hz, H-2), 5.82 (1H, dd, *J* = 2.7, 7.7 Hz, H-3), 5.68 (1H, s, CHPh), 5.59 (1H, d, *J* = 0.9 Hz, H-1), 4.64 (1H, ddd, *J* = 3.9, 6.9, 7.5, Hz, H-5), 4.39 (1H, dd, *J* = 7.2, 7.5 Hz, H-4), 4.33 (1H, dd, *J* = 3.6, 7.8 Hz, H-6a), 3.96 (1H, dd, *J* = 7.5, 7.8 Hz, H-6b), 2.34 (3H, s, CH<sub>3</sub>). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>):  $\delta$  165.40, 165.29 (2 × C = O), 138.52, 138.03, 133.54, 133.11, 133.00, 130.07, 129.98, 129.84, 129.79, 129.57, 129.48, 129.10, 128.59, 128.27, 128.25, 126.19 (Ar), 101.96 (CHPh), 87.31 (C-1), 76.95 (C-4), 72.44 (C-2), 69.22 (C-3), 68.61 (C-6), 65.21 (C-5), 21.17 (CH<sub>3</sub>). HR-MS: *m/z* 605.1604 [M + Na]<sup>+</sup> calculated for C<sub>34</sub>H<sub>30</sub>O<sub>7</sub>Sn, found 605.1611.

#### *p*-Tolyl 2,3-Di-*O*-benzoyl-6-*O*-benzyl-1-thio- $\alpha$ -D-mannopyranoside (7)

To an anhydrous CH<sub>2</sub>Cl<sub>2</sub> (10 ml) solution of compound 6 (300 mg, 0.52 mmol) was added 200 mg of 4 Å molecular sieves under an atmosphere of argon and cooled to -78°C. Et<sub>3</sub>SiH (266 µl, 1.66 mmol) was added dropwise to the cooled reaction mixture followed by addition of triflic acid (115 µl, 1.30 mmol). The reaction mixture was then stirred at -78°C for 1 h. The reaction was quenched by the addition of triethylamine (0.25 ml) and CH<sub>3</sub>OH (0.5 ml), diluted with CH<sub>2</sub>Cl<sub>2</sub> (20 ml), and washed with saturated aqueous NaHCO<sub>3</sub> solution (10 ml), H<sub>2</sub>O (10 ml) and brine (10 ml). The organic layer was dried on Na<sub>2</sub>SO<sub>4</sub>, filtered and the filtrate concentrated to a syrup. Flash chromatographic purification of the crude product using cyclohexane/ethyl acetate as eluent gave pure 7 (241 mg, 80% yield) as colourless foam. *R*<sub>f</sub> = 0.38

(cyclohexane/ethyl acetate, 2:1).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz):  $\delta$  8.03 (2H, m, Ar), 7.94 (2H, m, Ar), 7.58 (1H, m, Ar), 7.52 (1H, m, Ar), 7.43 (3H, m, Ar), 7.34 (8H, m, Ar), 7.08 (2H, d,  $J = 6.0$  Hz, Ar), 5.85 (1H, dd,  $J = 0.9, 2.2$  Hz, H-2), 5.61 (1H, d,  $J = 0.9$  Hz, H-1), 5.57 (1H, dd,  $J = 2.2, 7.2$  Hz, H-3), 4.69 (1H, d,  $J = 9.0$  Hz,  $\text{CH}_2\text{Ph}^a$ ), 4.59 (1H, d,  $J = 9.0$  Hz,  $\text{CH}_2\text{Ph}^b$ ), 4.53 (1H, m, H-5), 4.40 (1H, ddd,  $J = 2.7, 7.2, 7.5$  Hz, H-4), 3.98 (1H, dd,  $J = 3.5, 7.8$  Hz, H-6a), 3.89 (1H, dd,  $J = 2.7, 7.8$  Hz, H-6b), 2.81 (1H, d,  $J = 3.0$  Hz, 4-OH), 2.31 (3H, s,  $\text{CH}_3$ ).  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  166.51, 165.37 ( $2 \times \text{C} = \text{O}$ ), 138.18, 138.04, 133.43, 133.38, 132.68, 129.93, 129.88, 129.85, 129.53, 129.41, 129.32, 128.53, 128.41, 128.39, 127.68, 127.60 (Ar), 86.42 (C-1), 73.76 ( $\text{CH}_2\text{Ph}$ ), 73.18 (C-3), 72.44 (C-5), 72.01 (C-2), 68.99 (C-6), 67.80 (C-4), 21.14 ( $\text{CH}_3$ ). HR-MS:  $m/z$  607.1761 [ $\text{M} + \text{Na}$ ] $^+$  calculated for  $\text{C}_{34}\text{H}_{32}\text{O}_7\text{SNa}$ , found 607.1757.

*p*-Tolyl 4-O-Acetyl-2,3-di-O-benzoyl-6-O-benzyl-1-thio- $\alpha$ -*D*-mannopyranoside (4)

Compound **7** (200 mg, 0.34 mmol) was dissolved in 3 ml of dry pyridine, and acetic anhydride (49  $\mu\text{l}$ , 0.51 mmol) was added under an argon atmosphere. The reaction mixture was stirred for 5 h at room temperature, poured into an ice-water mixture (10 ml) and extracted with  $\text{CHCl}_3$  ( $2 \times 15$  ml). The combined  $\text{CHCl}_3$  layers were washed with brine, dried over  $\text{Na}_2\text{SO}_4$  and concentrated. Flash chromatographic purification of the crude product using cyclohexane/ethyl acetate as eluent gave pure **8** (202 mg, 94% yield) as colourless solid (m.p. 93.7°C).  $R_f = 0.45$  (cyclohexane/ethyl acetate, 2:1).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz):  $\delta$  8.02 (2H, m, Ar), 7.91 (2H, m, Ar), 7.53 (2H, m, Ar), 7.45 (2H, m, Ar), 7.38 (2H, m, Ar), 7.32 (7H, m, Ar), 7.07 (2H, d,  $J = 6.0$  Hz, Ar), 5.87 (1H, dd,  $J = 1.5, 2.2$  Hz, H-2), 5.81 (1H, t,  $J = 7.5$  Hz, H-4), 5.65 (1H, dd,  $J = 2.2, 7.3$  Hz, H-3), 5.64 (1H, d,  $J = 1.5$  Hz, H-1), 4.69 (1H, m, H-5), 4.63 (1H, d,  $J = 8.7$  Hz,  $\text{CH}_2\text{Ph}^a$ ), 4.51 (1H, d,  $J = 8.7$  Hz,  $\text{CH}_2\text{Ph}^b$ ), 3.75 (1H, dd,  $J = 3.4, 8.2$  Hz, H-6a), 3.70 (1H, dd,  $J = 2.2, 8.2$  Hz, H-6b), 2.30 (3H, s,  $\text{PhCH}_3$ ), 1.93 (3H, s,  $\text{COCH}_3$ ).  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  169.76 ( $\text{COCH}_3$ ), 165.47, 165.40 ( $2 \times \text{COPh}$ ), 138.28, 137.99, 133.42, 133.34, 132.74, 129.94, 129.91, 129.81, 129.31, 129.20, 129.12, 128.53, 128.46, 128.33, 127.80, 127.60 (Ar), 86.24 (C-1), 73.62 ( $\text{CH}_2\text{Ph}$ ), 71.88 (C-2), 70.95 (C-5), 70.68 (C-3), 68.93 (C-6), 66.85 (C-4), 21.14 ( $\text{PhCH}_3$ ), 20.76 ( $\text{COCH}_3$ ). HR-MS:  $m/z$  649.1867 [ $\text{M} + \text{Na}$ ] $^+$  calculated for  $\text{C}_{36}\text{H}_{34}\text{O}_8\text{SNa}$ , found 649.1852.

1-Azidoethyl 4-O-Acetyl-2,3-di-O-benzoyl-6-O-benzyl- $\alpha$ -*D*-mannopyranosyl-(1 $\rightarrow$ 6)- $\alpha$ -*D*-mannopyranosyl-(1 $\rightarrow$ 6)- $\alpha$ -*D*-mannopyranosyl-(1 $\rightarrow$ 6)-2,3,4-tri-O-benzoyl- $\alpha$ -*D*-mannopyranosyl-2,3,4-tri-O-benzoyl- $\alpha$ -*D*-mannopyranosyl-(1 $\rightarrow$ 6)-2,3,4-tri-O-benzoyl- $\alpha$ -*D*-mannopyranosyl-(1 $\rightarrow$ 6)-2,3,4-tri-O-benzoyl- $\alpha$ -*D*-mannopyranosyl-(1 $\rightarrow$ 6)-2,3,4-tri-O-benzoyl- $\alpha$ -*D*-mannopyranosyl-(1 $\rightarrow$ 6)-2,3,4-tri-O-benzoyl- $\alpha$ -*D*-mannopyranoside (3)

To a solution of thiooctasaccharide **2** (150 mg, 0.04 mmol) in dry  $\text{CH}_2\text{Cl}_2$  (5 ml) were added the donor thioglycoside **4**

(47 mg, 0.08 mmol) and 100 mg of 4 Å molecular sieves. The mixture was cooled to  $-20^\circ\text{C}$  and NIS (11.0 mg, 0.048 mmol) and triflic acid (2  $\mu\text{l}$ , 0.02 mmol) added and the reaction was stirred for 15 min at  $-20^\circ\text{C}$ . The temperature of the reaction mixture was raised to room temperature and after 1 h, 5 ml of saturated aqueous  $\text{NaHCO}_3$  was added. The reaction mixtures were extracted with  $2 \times 10$  ml of  $\text{CHCl}_3$  and the organic layer was washed with saturated aqueous sodium thiosulfate solution (5 ml). The organic layer was dried over  $\text{Na}_2\text{SO}_4$  and concentrated to a syrup. Flash chromatographic purification of the crude product was performed using cyclohexane/ethyl acetate as eluent and gave pure nonasaccharide **3** (169 mg, 82% yield) as colourless solid (m.p.  $202^\circ\text{C}$ ).  $R_f = 0.43$  (cyclohexane/ethyl acetate, 1:1).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz):  $\delta$  8.16 (15H, m, Ar), 8.05 (18H, m, Ar), 7.87 (17H, m, Ar), 7.47 (50H, m, Ar), 7.26 (35H, m, Ar), 6.18 (8H, m), 6.02 (2H, m), 5.98 (7H, m), 5.85 (3H, m), 5.26 (1H, s), 5.22 (1H, d,  $J = 1.2$  Hz), 4.96 (6H, m), 4.48 (1H, d,  $J = 11.9$  Hz,  $\text{CH}_2\text{Ph}^a$ ), 4.57 (1H, dd,  $J = 1.8, 7.5$  Hz), 4.38 (1H, dd,  $J = 2.4, 7.2$  Hz), 4.28 (1H, d,  $J = 11.9$  Hz,  $\text{CH}_2\text{Ph}^b$ ), 4.13 (8H, m), 3.86 (9H, m), 3.66 (1H, dd,  $J = 2.1, 8.4$  Hz), 3.57 (4H, m), 3.37 (7H, m), 1.88 (3H, s,  $\text{CH}_3$ ).  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  166.79 ( $\text{COCH}_3$ ), 165.72, 165.56, 165.54, 165.52, 165.45, 165.37, 165.35, 165.33, 165.25, 165.23, 165.11 ( $\text{COPh}$ ), 133.72, 133.51, 133.44, 133.29, 133.05, 132.96, 130.06, 130.01, 129.86, 129.67, 129.48, 129.42, 129.40, 129.37, 129.28, 129.21, 129.18, 129.15, 129.05, 128.82, 128.78, 128.70, 128.60, 128.51, 128.38, 128.27 (Ar), 98.28, 98.09, 97.95, 97.87 (C-1's), 73.45 ( $\text{OCH}_2\text{Ph}$ ), 70.88, 70.61, 70.53, 70.39, 70.29, 70.25, 70.17, 69.90, 69.71, 69.63, 69.54, 69.47, 69.40, 69.30, 67.19, 67.12, 66.74, 66.41, 66.32, 66.24, 66.03, 65.96, 65.75, 50.48 ( $\text{CH}_2\text{N}_3$ ), 20.81 ( $\text{COCH}_3$ ).

1-Azidoethyl

6-O-Benzyl- $\alpha$ -*D*-mannopyranosyl-(1 $\rightarrow$ 6)-2,3,4-tri-O-benzoyl- $\alpha$ -*D*-mannopyranosyl-(1 $\rightarrow$ 6)- $\alpha$ -*D*-mannopyranosyl-(1 $\rightarrow$ 6)- $\alpha$ -*D*-mannopyranosyl-(1 $\rightarrow$ 6)- $\alpha$ -*D*-mannopyranosyl-(1 $\rightarrow$ 6)- $\alpha$ -*D*-mannopyranosyl-(1 $\rightarrow$ 6)- $\alpha$ -*D*-mannopyranoside (1)

To an ice-cooled solution of compound **3** (150 mg, 0.03 mmol) in dry  $\text{CH}_2\text{Cl}_2$ - $\text{CH}_3\text{OH}$  (1:2, 10 ml) was added a sodium methoxide solution in  $\text{CH}_3\text{OH}$  (25% w/v, 2.0 ml). The reaction mixture was allowed to stir overnight at room temperature and  $\text{H}^+$  Amberlite resin (200 mg) was added. It was filtered, solvent was evaporated and the residue was washed with  $\text{CHCl}_3$  ( $3 \times 10$  ml). An aqueous solution (2 ml) crude nonasaccharide was passed through a small column of Bio-Beads SM-4 (15 g, 20–50 mesh) and eluted with 0–60%  $\text{CH}_3\text{OH}$ - $\text{H}_2\text{O}$ . The aqueous solution that remained after brief evaporation under vacuum was frozen and lyophilized to a colourless foam (19 mg, 35%).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz):  $\delta$  7.68 (1H, m, Ar), 7.38 (4H, m, Ar), 4.89 (1H, s), 4.64 (1H, d,  $J = 11.6$  Hz,  $\text{OCH}_2\text{Ph}$ ), 4.61 (1H, d,  $J = 11.7$  Hz,  $\text{OCH}_2\text{Ph}$ ), 3.99–3.60 (63H, m), 2.68 (2H, m,  $\text{CH}_2\text{N}_3$ ).  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  138.04, 128.39, 128.37, 127.01 (Ar), 101.77, 101.04, 100.96, 100.72, 100.55 (C-1's), 74.48, 74.37, 73.32 ( $\text{OCH}_2\text{Ph}$ ), 73.18, 72.86, 72.77, 72.62, 72.56, 72.42, 72.04, 70.97, 68.84, 68.71, 68.56, 67.86, 67.45, 67.31, 67.00,



51.79 (CH<sub>2</sub>N<sub>3</sub>). HR-MS: m/z 1658.5548 [M + Na]<sup>+</sup> calculated for C<sub>63</sub>H<sub>101</sub>N<sub>3</sub>O<sub>46</sub>Na, found 1658.5503.

#### In vitro $\alpha(1\rightarrow2)$ -mannopyranosyltransferase assay utilizing a novel nonasaccharide acceptor, Acc-Man<sub>9</sub>

*Corynebacterium glutamicum*, *C. glutamicum*ΔmptC, *C. glutamicum*ΔmptD, complemented strains, and a double mutant devoid of Manp branching activities (MptC and MptD) were used to characterize the enzymatic activity of Cg-MptC and Cg-MptD, and Mt-Rv2181. Membranes from all strains of *C. glutamicum* were prepared as described previously (Mishra et al., 2008a) and resuspended in 100 mM sodium acetate (pH 6.0), containing 5 mM β-mercaptoethanol and 7 mM MgCl<sub>2</sub> (Buffer E) to a final concentration of 10–15 mg ml<sup>-1</sup>. The chemically synthesized nonasaccharide acceptor, azidoethyl 6-O-benzyl-α-D-mannopyranosyl-(1→6)-[α-D-mannopyranosyl-(1→6)]<sub>7</sub>-D-mannopyranoside (Acc-Man<sub>9</sub>), and PP-[<sup>14</sup>C]-M [Gurcha et al., 2002; stored in CHCl<sub>3</sub>/CH<sub>3</sub>OH, 2:1, v/v], were aliquoted into 1.5 ml Eppendorf tubes to a final concentration of 2 mM and 250 000 cpm (0.305 Ci mmol<sup>-1</sup>), respectively, and dried under nitrogen. IgePal CA-630 (8 μl, Sigma Aldrich) was added and the tubes sonicated to resuspend the lipid linked components, and 1 mM ATP, 1 mM NADP, 5 mM MnCl<sub>2</sub>, and membrane protein (1 mg) added to a final volume of 100 μl. The reaction mixture incubated at 37°C for 2 h as described (Yokoyama and Ballou, 1989) and then terminated by the addition of 5 ml of 50% C<sub>2</sub>H<sub>5</sub>OH. The supernatant was recovered, dried and resuspended in 700 μl of water and loaded onto a 1 ml strong anion exchange SepPak cartridge (Supelco). The cartridge washed with H<sub>2</sub>O (5 ml), which was collected, dried and resuspended in 200 μl of H<sub>2</sub>O and 10% of the reaction product was quantified by scintillation counting using 5 ml of Ecolume (ICN Biomedicals, Costa Mesa, CA, USA) and the remaining material analysed by 15% SDS-PAGE-/autoradiography as described previously (Mishra et al., 2008a).

#### Acc-Man<sub>9</sub>-[<sup>14</sup>C]-Man<sub>x</sub> and selective cleavage by an $\alpha(1\rightarrow2)$ -D-mannosidase

The extracted Acc-Man<sub>9</sub>-[<sup>14</sup>C]-Man<sub>x</sub> products from the assays performed with *C. glutamicum* membranes were dried and subsequently incubated at 30°C for 24 h in 0.1 M acetate buffer, pH 5.0, with 5 mU of  $\alpha(1\rightarrow2)$ -D-mannosidase (Maras et al., 2000) as described previously (Ichishima et al., 1981; Yokoyama and Ballou, 1989). The reaction mixture was fractionated on a 50 ml Bio-Gel P-2 gel filtration column (30 cm × 1.5 cm; Bio-Rad) in H<sub>2</sub>O. The control Acc-Man<sub>9</sub>-[<sup>14</sup>C]-Man<sub>x</sub> before  $\alpha(1\rightarrow2)$ -D-mannosidase treatment eluted from the Bio-Gel P-2 column at fractions 11–13, and digested products (release of [<sup>14</sup>C]Manp) was retained and eluted in later fractions 33–39 based on a [<sup>14</sup>C]-mannose standard (Cooper et al., 2002).

#### Cell stimulation assays

Trypsin-released HEK293 cells were washed with and resuspended in culture medium at 1.11 × 10<sup>6</sup> cells ml<sup>-1</sup>. Cells (1 × 10<sup>5</sup>, 90 μl) were transferred to a 96-well U-bottom plate

(Greiner) and left for 2 h to let the cells re-adhere. After this, cells were stimulated (in triplicate) with 50 μg ml<sup>-1</sup> wild-type or mutant Cg-LM and Cg-LAM (pretreated with 1% hydrogen peroxide for 96 h as described (Birch et al., 2010) in a final stimulation volume of 100 μl. Unstimulated cells served as a control. Culture supernatants were harvested after 24 h by centrifugation and stored at -80°C for cytokine measurements using enzyme-linked immunosorbent assay (ELISA) against human IL-8 (Invitrogen).

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