Identification of a Membrane Protein Required for Lipomannan Maturation and Lipoarabinomannan Synthesis in Corynebacterineae

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Mycobacterium tuberculosis and related Corynebacterineae synthesize a family of lipomannans (LM) and lipoarabinomannans (LAM) that are abundant components of the multilaminate cell wall and essential virulence factors in pathogenic species. Here we describe a new membrane protein, highly conserved in all Corynebacterineae, that is required for synthesis of full-length LM and LAM. Deletion of the Corynebacterium glutamicum NCgl2760 gene resulted in a complete loss of mature LM/LAM synthesis. Structural studies, including monosaccharide analysis, methylation linkage analysis, and mass spectrometry of native LM species, indicated that the ΔNCgl2760 t-LM comprised a series of short LM species (8–27 residues long) containing an α-1–6-linked mannose backbone with greatly reduced α-1–2-mannose side chains and no arabinose caps. The structure of the ΔNCgl2760 t-LM was similar to that of the t-LM produced by a C. glutamicum mutant lacking the mptA gene, encoding a membrane α-1–6-mannosyltransferase involved in extending the α-1–6-mannan backbone of LM intermediates. Interestingly, NCgl2760 lacks any motifs or homology to other proteins of known function. Attempts to delete the NCgl2760 orthologue in Mycobacterium smegmatis were unsuccessful, consistent with previous studies indicating that the M. tuberculosis orthologue, Rv0227c, is an essential gene. Together, these data suggest that NCgl2760/Rv0227c plays a critical role in the elongation of the mann backbone of mycobacterial and corynebacterial LM, further highlighting the complexity of lipoglycan pathways of Corynebacterineae.

The bacterial suborder Corynebacterineae includes important human and animal pathogens such as Mycobacterium tuberculosis (tuberculosis), Mycobacterium leprae (leprosy), and Corynebacterium diphtheriae (diphtheria). These bacteria synthesize a complex and highly distinctive cell wall, which confers intrinsic resistance to host antibacterial factors, antibiotics, and adverse environmental conditions (1). The inner layers of the mycobacterial/corynebacterial cell wall comprise peptidoglycan and arabinogalactan polysaccharide as well as covalently linked long chain mycolic acids that form the inner leaflet of an outer membrane (2, 3). A diverse range of non-covalently linked glycolipids and waxes form the outer leaflet and define the surface properties of these bacteria. Many of the cell wall components are essential for the growth of pathogenic mycobacteria species, hampering efforts to functionally characterize genes involved in cell wall assembly via standard gene deletion approaches (4–7). In contrast, the non-pathogenic Corynebacterium glutamicum can tolerate the loss of major cell wall components, making it a widely used model organism for studying pathways involved in core cell wall biosynthesis (8–18).

One class of non-covalently bound glycolipids synthesized by all mycobacteria/corynebacteria are the phosphatidylyl-myoiinositolmannosides (PIMs). The PIMs are abundant cell wall components in their own right and are membrane anchors for the lipomannans (LM) and lipoarabinomannans (LAM). These lipoglycans are essential for both the viability and in vivo survival of pathogenic mycobacterial species, due to their capacity to modulate the host immune response during infection (7, 19–24). Many steps of the PIM → LM → LAM biosynthetic pathway are known (Fig. 1A; reviewed in Refs. 2 and 7). Phosphatidylinositol (PI) is mannosylated by GDP-mannose (GDP-Man)-dependent transferases PimA (26) and PimB (16) and

The abbreviations used are: PIM, phosphatidylyl-myoiinositolmannosides; LM, lipomannan(s); LAM, lipoarabinomannan(s); t-LM, truncated LM; PI, phosphatidylinositol; Man, manno; PPM, polypropen phosphomannose; TMCM, trehalose mononocorynomycolate; Gl-A, glucopyranosyluronic acid diacylglycerol; Gl-X, mannosyl-glucuronic acid diacylglycerol; TDCM, trehalose dicorynomycolate; AcTMCM, trehalose acetyl-monocorynomycolate; HPTLC, high-performance thin layer chromatography; SCQ, single crossover; DCO, double crossover; ESI, electrospray ionization; BHI, brain heart infusion; DIG, digoxygenin; PMAA, partially methylated alditol acetate.

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acylated by PatA (27) in cytoplasmic reactions that form AcPIM2 and Ac2PIM2. These acylated PIM2 species can undergo additional mannosylation to form acylated PIM6 as an end product or to form hyperglycosylated LM and LAM in periplasmic reactions requiring polyrenol phosphomannose (PPM) donors. In mycobacteria, PimE uses PPM to elongate a periplasmic reactions requiring polyprenol phosphomannose (PPM) donors. In mycobacteria, PimE uses PPM to elongate a backbone of LM (17) following its activation by lipoprotein LpqW (29, 30). However, the role of MptB in mycobacteria is unclear, possibly due to gene redundancy (17). PPM-dependent mannosyltransferases MptC and MptD add α1–2-mannose side chains (31) essential for virulence (32), whereas further extension of the α1–6 backbone is performed by MptA (12), forming LM. LM is converted to LAM by a series of α1–5-arabinosyltransferases, including EmbC (33, 34), AftB, AftC (15), and AftD (5), and then capped with additional sugars or organic acids in pathogenic species (35, 36).

Studies in C. glutamicum have revealed a second lipoglycan pathway, termed LM-B, which is distinct from the preserved LM-A/LAM pathway (Fig. 1B). Although both pathways share many common enzymes, LM-A species are based on a glucopyranosyluronic acid diacylglycerol (GI-X) anchor rather than PI (16, 18). On the cytoplasmic side, Gl-A is mannosylated by MgtA (previously named PimB), producing mannosyl-glucuronic acid diacylglycerol (GI-A), which is extended by MptB (17, 29) and other enzymes common to both pathways to produce LM-B, the major LM pool of C. glutamicum (37, 38).

Here we show that deletion of NCgl2760, the orthologue of the essential M. tuberculosis Rv0227c gene (39, 40), results in a defect in the elongation of both LM-A and LM-B intermediates and loss of mature LAM (12). Interestingly, this gene sits within a group of genes involved in synthesis of trehalose corynomycolates, including the recently described tmaT/NCgl2759 gene (Fig. 2A). We conclude that NCgl2760 has a direct role in regulating LM-A/LAM and LM-B synthesis, possibly by regulating the activity of LM mannosyltransferases and/or access of PIM intermediates to these enzymes, that it may be a therapeutic target in pathogenic mycobacteria species.

Results

Identification of a Corynebacterial Orthologue of M. tuberculosis Gene, Rv0227c—We have recently shown that the C. glutamicum gene NCgl2759 (orthologue of the essential M. tuberculosis gene, Rv0228) encodes an acetyltransferase that converts trehalose monocorynomycolate (TMCMC) to an acetylated form (AcTMCMC) and is essential for efficient transport of TMCMC across the inner membrane for conversion to trehalose dicorynomycolate (TDCMC) (41). To determine whether other genes in this locus are involved in trehalose corynomycolate biosynthesis, we investigated the function of NCgl2760, the next gene in the genome of C. glutamicum ATCC 13032. NCgl2760 encodes a putative protein of 272 residues and is the orthologue of M. tuberculosis Rv0227c, sharing 16.4% identity and 24.9% similarity (supplemental Fig. S1) and synteny to Rv0227c and related genes in other mycobacterial genomes (Fig. 2A). NCgl2760 is predicted to contain a single transmembrane domain and a large periplasmic domain (Fig. 2B).

Inactivation of the NCgl2760 Gene—To investigate NCgl2760 function, a two-step recombination strategy was used to create an NCgl2760 mutant for phenotypic characterization (Fig. 3). Deletion of the gene was achieved using the suicide vector pK18moblacB (42), which carries a kanamycin resistance gene (aph) and Bacillus subtilis sacB gene, conferring sensitivity to sucrose. Potential deletion mutants were identified by PCR screening (data not shown) and then confirmed by Southern blotting analyses (Fig. 3). In contrast to the NCgl2760 null mutant (41), the ΔNCgl2760 strain grew at the same rate as the wild-type (parental) strain on solid media. Growth curves performed in liquid brain heart infusion (BHI) medium confirmed the absence of any detectable growth defect arising from deletion of the NCgl2760 gene (data not shown). This contrasts with the situation in M. tuberculosis, where the gene is regarded as essential (39, 40).

Complementation of C. glutamicum ΔNCgl2760—Before phenotypic characterization of the mutant, complementation
and control strains were created to determine whether any cell wall defect was due solely to disruption of NCgl2760. To complement the mutant, ΔNCgl2760 was transformed with pSM22: NCgl2760, a plasmid carrying a full-length NCgl2760 gene plus 180 bp of upstream sequence, which may include the native promoter. The empty pSM22 vector was also introduced into ΔNCgl2760 as a control.

Disruption of NCgl2760 Results in Accumulation of a Truncated LM Species—Cell wall components of ΔNCgl2760 and the complementation strains were analyzed and compared with the parental WT strain, C. glutamicum ATCC 13032. No changes were found in the levels or composition of free lipids, including the major PIMs and trehalose corynomycolates (supplemental Fig. S2). However, marked differences were observed in the LM/LAM profile of the ΔNCgl2760 mutant, following analysis of purified LM/LAM by PAGE (Fig. 4A). As expected, WT C. glutamicum produced two distinct populations of lipoglycans corresponding to LM (LM-A and LM-B co-migrate) and LAM (Fig. 4A, lane 1). In contrast, the ΔNCgl2760 strain (lane 3) and ΔNCgl2760 bearing empty pSM22 (lane 5) lacked mature LM or LAM but synthesized a faster-migrating “LM” species that we designated t-LM (truncated LM). Synthesis of mature LM and LAM was fully restored by complementation of ΔNCgl2760 with a plasmid-encoded copy of the gene (Fig. 4A, lane 4), confirming that the loss of LM/LAM and appearance of t-LM were due solely to deletion of the NCgl2760 gene.

ΔNCgl2760 and ΔmptA Mutants Synthesize a Similar Truncated LM—The LM/LAM phenotype of ΔNCgl2760 appeared to be very similar to that of a previously described C. glutamicum cell wall mutant lacking the mptA gene, which encodes an α(1→6)-mannosyltransferase responsible for extending the mannann backbone of LM-A and LM-B precursors (12). Disruption of mptA results in the synthesis of truncated LM-A- and LM-B-based lipoglycans that have a short mannann backbone and lack arabinose side chain elaborations (12). To allow direct comparison of these two lines, we deleted the mptA gene in C. glutamicum 13032 (see supplemental Fig. S3). As expected, LM/LAM analysis of the ΔmptA strain revealed a lack of full-length LM and LAM and the presence of a faster migrating lipoglycan species on SDS-PAGE (Fig. 4A, lane 2). Significantly, the faster migrating LM lipoglycan extracted from ΔmptA cells had the same migration on SDS-PAGE gels as the ΔNCgl2760 t-LM species (Fig. 4A, lanes 2 and 3), supporting the conclusion that the ΔNCgl2760 mutant has a defect in LM elongation.

To further define the nature of the LM defect in ΔNCgl2760, purified lipoglycans from WT and mutant lines were subjected to monosaccharide composition analysis. Whereas the WT LM/LAM fraction contained an average molar ratio of myo-inositol/mannose/arabinose of 1:100:7, the LM/LAM fractions of ΔNCgl2760 and ΔmptA had molar ratios of 1:30:0.13 and 1:23:0.17, respectively. Complementation of ΔNCgl2760 with the plasmid containing NCgl2760, but not empty pSM22, restored the molar ratio of myo-inositol/mannose/arabinose to 1:83:6. These analyses are consistent with loss of NCgl2760 being associated with a complete loss of LM arabinose side chains as well as an overall reduction in mannann backbone chain length.

Methylation linkage analysis of the lipoglycan fractions from WT and mutant bacteria confirmed that the t-LM of the ΔNCgl2760 mutant lacked the terminal arabinose side chains with a concomitant increase in terminal mannose residues (Fig. 5). Interestingly, these analyses showed that the ΔNCgl2760 t-LM had fewer 2,6-linked mannose residues (with associated increase in 6-linked mannose), indicating fewer mannose side chains branching off the α1→6-linked mannann backbone (Fig. 5C). Analysis of the ΔmptA LM fraction (Fig. 5B) gave an almost identical branching pattern, consistent with both mutant lines producing short LM species containing an α1→6-linked mannann backbone with few mannose side chains and no arabinose capping.

LC-ESI-TOF-MS analysis of the LM fractions of WT and mutant bacteria provided further information on the nature of the defect in LM maturation. Direct analysis of WT LM by LC-ESI-TOF-MS allowed the detection of a polydisperse mixture of 117 different LM species (differing by 162 mass units) in the 2712–9190 Da size range (after deconvolution; Fig. 6A). The masses of these species were consistent with the presence of a mannann chain containing 10–50 mannose residues and a lipid
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The lipid anchor. Similarly, 112 LM species were detected in the lipoglycan species had a size between 2200 and 4900 Da, very

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predicted mannan chain length (number of mannose residues).

Fig. 6). In marked contrast, the

lated a range of LM species with masses of 2626–5500 Da (8–27 mannose residues; Fig. 6C), a single crossover strain (lane 2), and the ΔNCgl2760 mutant (lane 3). Positions of DIG-labeled λ DNA standards digested with HindIII are indicated in kb. Bands showing that the NCgl2760 gene has been deleted (a 6.2-kb NruI fragment in B and a 1.8-kb Sall fragment in C) are indicated by asterisks.

anchor comprising a PI moiety with 2–3 fatty acyl chains. The plots in Fig. 6 show the masses (m/z, after deconvolution) of all detected LM species (from low to high molecular weight) and predicted mannan chain length (number of mannose residues). Supplemental Fig. S5 explains, step by step, the development of Fig. 6. In marked contrast, the ΔNCgl2760 t-LM fraction completely lacked LM species above 5500 Da and instead accumulated a range of LM species with masses of 2626–5500 Da (8–27 mannose residues; Fig. 6C). As in the WT fraction, a number of different series (91 species in total) were detected in this fraction that differed by m/z 162 and acyl composition of the lipid anchor. Similarly, 112 LM species were detected in the ΔmptA mutant (Fig. 6B), with a size distribution of 2206–8179 Da (8–43 mannose residues). However, the majority of these lipoglycan species had a size between 2200 and 4900 Da, very

similar to ΔNCgl2760. Interestingly, up to 72% of LM species detected in the ΔNCgl2760 and ΔmptA mutants had identical molecular weights, indicating a very similar LM phenotype. An ΔmptA mutant, in which the LM-B pathway is inactive (38) (Fig. 4B), also produced a series of LM species (78 species; Fig. 6D), with a size distribution of 3222–8859 Da (13–48 mannose residues), 32 of which were common to ΔNCgl2760. Interestingly, the C. glutamicum NCgl2759 mutant, lacking the acetyltransferase involved in TMCM transport, synthesized LM that was indistinguishable from WT LM (data not shown), indicating that the LM phenotype observed in ΔNCgl2760 bacteria is not an indirect consequence of defects in TMCM transport. Collectively, these analyses indicate that loss of NCgl2760 is associated with a defect in LM elongation, leading to an accumulation of early LM precursors containing an α1–6-linked mannan backbone (~20 residues) with minimal mannose side chains.

Evidence That the Orthologue of NCgl2760 in M. smegmatis, MSMEG_0317, Is an Essential Gene—To investigate whether the putative orthologue in M. smegmatis (Fig. 2 and supplemental Fig. S1) also functioned in LM/LAM biosynthesis, we attempted to delete the MSMEG_0317 gene. A DNA fragment was synthesized containing 226 bp from the left and right flanking regions of MSMEG_0317 and cloned into an M. smegmatis suicide vector encoding streptomycin resistance and sucrose sensitivity via a sacB gene. This construct was electroporated into M. smegmatis mc^2155, selecting for single crossover (SCO) clones in which the plasmid had integrated into the chromosome. Southern blotting confirmed integration at the MSMEG_0317 locus, and this SCO strain was cultured on Middlebrook 7H10 plates containing 10% (w/v) sucrose to select for potential double crossover (DCO) strains. PCR analysis of 50 clones revealed that all were WT revertents resulting from ejection of the plasmid from the chromosome, which restored the MSMEG_0317 gene (data not shown). The lack of any ΔMSMEG_0317 mutants raised the possibility that the gene is essential in M. smegmatis.

If MSMEG_0317 is essential, introduction of a second copy at another site in the chromosome of the SCO strain should allow a DCO strain to be derived. The MSMEG_0317 gene was cloned into the integrative expression vector, pMV361 (43),
and then electroporated into MSMEG_0317 SCO. The resultant strain was cultured as described above to select potential DCO strains. This time, in 5 of 17 strains tested, the MSMEG_0317 gene had been replaced by the deleted copy (Fig. 7). These differences are statistically significant ($p$ value = 0.000067 ($p < 0.05$)), providing strong evidence of essentiality.

Our findings suggest that, in contrast to the situation in C. glutamicum, M. smegmatis may require the presence of full-length LM and/or LAM for viability.

Discussion

We have recently shown that the C. glutamicum acetyltransferase TmaT (TMCM mycolyl acetyltransferase; NCgl2759) catalyzes the acetylation of the major cell wall glycolipid TMC and that this modification is important for periplasmic transport of this glycolipid (41). A subsequent study showed that the mycobacterial orthologue has the same function (44). Based on these findings, we hypothesized that the adjacent gene, NCgl2760, which is syntenic in both corynebacteria and mycobacteria, would also have a role in corynomycolate biosynthesis. However, deletion of NCgl2760 had no effect on the steady state levels of TMCM or TDCM. Further analyses revealed that loss of NCgl2760 leads to a profound defect in the elongation of LM precursors and the synthesis of mature LM and LAM. This defect was completely reversed by genetic complementation with NCgl2760. Thus, NCgl2760 appears to have a role in the synthesis of lipoglycans that are structurally unrelated to TMCM, despite the location of its encoding gene.

The LM/LAM phenotype observed following disruption of NCgl2760 was very similar to that generated by deletion of the C. glutamicum gene encoding MptA, a polyprenol-phosphate-mannose-dependent $\alpha1$–6-mannosyltransferase that extends the mannan backbone of LM-A and LM-B intermediates (12) following initial $\alpha1$–6-Man extension by MptB (17) and $\alpha1$–2-Man side branching by MptC/D (31). Direct comparison of the ΔmptA and ΔNCgl2760 mutants revealed truncated species.
with similar SDS-PAGE migration and similar LM/LAM profiles (Figs. 4 and 6). Lipoglycans from both mutants lacked arabinose, as expected, and had significantly less 2,6-linked Man, suggesting that Man side branches are normally added after full extension of the α-1–6-linked Man backbone and are concentrated at the distal end of LM, consistent with previous findings (12).

Interestingly, neither MptA nor NCgl2760 are required for normal growth of C. glutamicum, in marked contrast to the essentiality of these genes in pathogenic and non-pathogenic species of mycobacteria (12). Notwithstanding the similar mutant phenotypes, the encoded MptA and NCgl2760 proteins share no other common characteristics. MptA is an integral membrane protein with 13 predicted transmembrane domains and a DXD motif of a GT-C glycosyltransferase, consistent with transfer of mannose from a PPM donor. NCgl2760 has a single membrane protein with 13 predicted transmembrane domains and a DXD motif, is also located (12). However, it has no motifs suggesting an enzymatic function, making NCgl2760 the second essential gene.

Based on these very similar phenotypes, we propose that NCgl2760 plays a key role in regulating steps in the LM/LAM biosynthetic pathway. The fact that this mutant produces LM species with a more restricted molecular size distribution than the ΔmptA mutant suggests that NCgl2760 may act just before the elongation steps catalyzed by MptA (Fig. 8). NCgl2760 could either directly or indirectly influence MptA function and/or regulate access of the LM substrate or the PPM donor to MptA. Structural studies may provide important insights into the role of NCgl2760 and its mycobacterial orthologues.

In contrast to the situation in C. glutamicum, the mycobacterial orthologues are essential for normal growth (39, 40). It
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has previously been proposed that the *M. tuberculosis* orthologue, Rv0227c, may have a role in invasion and infection of host cells (45). Specifically, evidence was proposed for surface expression of the Rv0227c protein based on electron microscopy and Western blotting studies, consistent with the presence of a putative signal sequence and membrane anchor in the corynebacterial orthologue (Fig. 2B). High activity binding peptides were identified within Rv0227c, some of which inhibited entry to the cell lines tested and could be considered candidates for inclusion in an anti-tuberculosis vaccine. Although our findings do not rule out a direct role for Rv0227c in host cell invasion, our data suggest that Rv0227c has a role in the synthesis of LM and ultimately LAM, which are ligands for host cell receptors (e.g. Toll-like receptors and C-type lectins, including dendritic cell-specific intercellular adhesion molecule-3 grabbing nonintegrin (DC-SIGN) and the macrophage mannose receptor (MMR) (for a review, see Ref. 7)) with binding influencing pathogen uptake and subsequent interactions between pathogen and host.

*C. glutamicum* has proved a useful model system for identifying pathways of cell wall biosynthesis in the actinomycetes, because it is more permissive to loss of cell wall components than mycobacteria. Indeed, *Rv0227c* is reported to be essential for *in vitro* growth of *M. tuberculosis* H37Rv based on high-density mutagenesis and sequencing studies (39, 40). Furthermore, we show here that the *M. smegmatis* orthologue is essential under standard growth conditions (Fig. 7). *C. glutamicum* may be more tolerant to loss of enzymes involved in LM/LAM biosynthesis because it has two pathways of glycolipid anchor biosynthesis: the PI-based LM-A pathway and the GlcA-Gro-Ac2-based LM-B pathway (Fig. 1). However, our findings indicate that NCgI2760 influences both the LM-A and LM-B pathways. This is also the case for a number of previously characterized GT-C type mannosyltransferases involved in synthesis of the mannan backbone and mannose side branches (12, 17, 31), showing that the same reactions occur on different lipid anchors. It remains unclear why LM-B species fail to progress further down the pathway to form LAMs.

Based on our findings in *C. glutamicum*, it is likely that *M. tuberculosis* Rv0227c fulfills a similar function in LM/LAM biosynthesis and is a possible drug target (39, 40). Indeed, interfering with Rv0227c activity should affect *M. tuberculosis* viability while also blunting the immunosuppressive properties of bacteria *in vivo*. The predicted location of Rv0227c within the periplasmic space may also increase access of the functional domain of this protein to inhibitors.

**Experimental Procedures**

**Bacterial Strains, Culture Conditions, Transformation, and Genetic Manipulation—Escherichia coli** DH5α was grown in Luria-Bertani (LB) medium at 37 °C with aeration. *C. glutamicum* ATCC 13032 was grown in BH1 medium (Oxoid) or LBHIS (LB, BHI, sorbitol) (46) at 30 °C with aeration. When necessary, ampicillin was added to a final concentration of 100 μg ml⁻¹ and kanamycin at 50 μg ml⁻¹. *E. coli* plasmid DNA was isolated from 10 ml of an overnight culture using the High Pure plasmid isolation kit (Roche Applied Science), and *C. glutamicum* genomic DNA was extracted from ~0.5 g of cells using the Illustra DNA extraction kit (GE Healthcare), according to the manufacturer’s instructions. When necessary, DNA was purified using an UltraClean 15 DNA purification kit (MoBio). PCRs were performed in a PTC-200 thermal cycler (MJ Research) using *Taq* polymerase (Roche Applied Science) or ProofStart DNA polymerase (Qiagen). Initial denaturation of template DNA was done at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, a 1-min primer-specific annealing step, and a 1-min/kb extension step at 72 °C. The program included a 10-min final extension step at 72 °C. PCR products were purified by extraction from 1% agarose gels using an UltraClean 15 DNA purification kit (MoBio). Endonucleases, T4 ligase, polynucleotide kinase system, and alkaline phosphatase were obtained from New England Biolabs and used according to the manufacturer’s instructions.

**Bioinformatic Identification and Analysis of NCgI2760—** The corynebacterial orthologue of mycobacterial Rv0227c was found using the BLASTp (47) algorithm. Protein topology predictions were performed using Protter version 1.0 (48). Amino acid sequence alignments were produced using Clustal Omega.
Construction of C. glutamicum ΔNCgl2760 and Complementation Strains—The NCgl2760 gene was deleted using a two-step allelic replacement strategy previously used in our laboratory to create other cell wall mutants (11, 16, 29, 41). A 1.0-kb fragment containing sequence from the left side of the NCgl2760 gene was amplified using Phusion Start DNA polymerase (Qiagen) and the primers NCgl2760-left_F (5’-GTCACC-CGGTGAAATATAGGAGCACC) and NCgl2760-left_R (5’-GGTTAGCCTCCCATATAGGAGCCCGG) and cloned into the XmnI/BamHI sites (underlined) of pUC19 (49), creating plasmid pUC-NCgl2760left. A 1.0-kb fragment containing sequence from the right side of the NCgl2760 gene was amplified using primers NCgl2760-right_F (5’-TAGCGGATCCCAATTTCAGGCGGCCGCCCAGCTGATGAGC) and NCgl2760-right_R (5’-GGTTAGCCTCCCATATAGGAGCCCGG) and cloned into the BamHI/XbaI sites (underlined) of pUC19, creating plasmid pUC-NCgl2760right. The right flanking sequence was then liberated from pUC-NCgl2760right using BamHI/XbaI and subcloned into Xba/BamHI-digested pUC-NCgl2760left, fusing the left and right flanking sequences to create plasmid pUC-ΔNCgl2760. This entire insert was then liberated using XmnI/XbaI and subcloned into XmnI/XbaI-digested pK18mobsacB, a suicide plasmid for C. glutamicum (50) that contains kanamycin and sucrose selection markers. The resultant plasmid, pK18mobsacB:ΔNCgl2760, was sequenced complementation plasmid (pSM22 (42), which contains the corynebacterial origin of replication repA and kanamycin resistance gene aphA3. A sequenced complementation plasmid (pSM22:NCgl2760) and pSM22 control plasmid were electroporated into the C. glutamicum ΔNCgl2760 deletion strain, followed by selection on kanamycin-supplemented BHI plates.

To complement the ΔNCgl2760 strain, the entire NCgl2760 gene plus 180 bp of upstream sequence was PCR-amplified using primers NCgl2760-comp_F (5’-TCGGATATCGGCGACTGAGTCGCTCCTCC) and NCgl2760-comp_R (5’-TCGGATATCGGCGACTGAGTCGCTCCTCC), digested with EcoRV (underlined), and cloned into the unique PvuII site of pSM22 (42), which contains the corynabacterial origin of replication repA and kanamycin resistance gene aphA3. A sequenced complementation plasmid (pSM22:NCgl2760) and pSM22 control plasmid were electroporated into the C. glutamicum ΔNCgl2760 deletion strain, followed by selection on kanamycin-supplemented BHI plates.

Construction of NCgl2093 (mpta) and NCgl0452 (mgtA) Mutants—Both genes were deleted using synthetic gBlock gene fragments (Integrated DNA Technologies), whereby a 500-bp DNA sequence was custom-designed from ~220 bp of sequence upstream and downstream of the genes plus 48 bp containing the 5’- and 3’-end of each gene. Both synthetic DNA fragments were designed with a BamHI site at one end and an XbaI site at the other end for cloning into pk18mobsacB. These plasmid constructs were then electroporated into C. glutamicum ATCC 13032. Kanamycin-resistant colonies were tested for the presence of integration of the plasmid via a single crossover event by PCR. Confirmed single crossover clones were then cultured on plates containing 10% sucrose to generate double crossover strains that were confirmed by PCR and Southern blotting hybridization (supplemental Figs. S3 and S4).

Southern Hybridization—For Southern blotting analysis, 2 μg of genomic DNA was digested with appropriate restriction enzymes under optimal conditions for 16 h. Purified samples and digoxigenin (DIG)-labeled HindIII-digested λ DNA markers were separated on a 1% agarose gel followed by depurination, denaturation, neutralization, and capillary transfer onto a nylon membrane. The membrane was then hybridized at 65 °C with a gene-specific probe prepared by DIG labeling a 1.0-kb PCR product obtained using primers NCgl2760-comp_F and NCgl2760-comp_R.

Compositional Cell Wall Analysis—WT and mutant cells were harvested by centrifugation at logarithmic growth phase (A600 of 1–3) from 100 ml of culture in BHI. Free lipids, including PIMs and trehalose mycolates, were extracted and purified as described previously (41). The delipidated cell pellet was then subjected to ethanol reflux by suspending in 5 ml of 50% (v/v) ethanol and incubating at 100 °C for 2 h, with occasional vortex mixing. Samples were centrifuged at 800 × g for 5 min, the supernatant was collected, and the procedure was repeated two more times. Pellets were used for corynomycolic acid extraction. The supernatant was pooled, and ethanol was removed under a stream of N2 followed by freeze-drying. The dried pellets were reconstituted in 400 μl of water containing 0.02% (w/v) CaCl2 and 10 units of protease K, followed by incubation at 37 °C for 2–3 h. The digest was diluted with 50% propan-1-ol (50 μl) and 1 M ammonium acetate (50 μl) and loaded on a column of octyl-Sepharose (1 ml) equilibrated in 5% 1-propanol and 50 mM ammonium acetate. After washing the column, lipoglycans were eluted with 30, 40, 50, and 60% 1-propanol (1-ml volumes), and carbohydrate-containing fractions were identified by spotting 5-μl aliquots on high-performance thin layer chromatography (HPTLC) sheets and stained with orcinol-H2SO4 (51). Lipoglycan-containing fractions (in 30–40% propan-1-ol fractions) were pooled, dried under vacuum, and reconstituted in 30% (v/v) propan-1-ol (100 μl/100 mg pellet weight) for further analysis.

HPTLC Analyses—Extracted lipids were analyzed on aluminum-backed HPTLC silica sheets (Merck) in chloroform, methanol, 13 M ammonia, 1 M ammonium acetate, water (180: 140:9:9:2:3, v/v/v/v/v/v). Glycolipids were detected with orcinol in HCl (100 °C, 5 min) followed by charring at 150 °C to detect all lipids.

Polyacylamide Gel Electrophoresis of LM/LAM—Purified lipoglycan fractions were mixed with PAGE sample buffer and incubated at 98 °C for 5 min. Samples were then separated on a Mini-Protean®TGX™ precast gel (Bio-Rad), and LM/LAMs were visualized using the Pierce® silver stain kit according to the manufacturer’s instructions (Thermo Scientific). In detail, the gel was washed for 5 min in ultrapure water (two times); fixed for 15 min in 30% (v/v) ethanol, 10% (v/v) acetic acid solution (two times); washed for another 5 min in 10% (v/v) ethanol (two times); and washed for 5 min in ultrapure water (two times). Next, the gel was sensitized for 1 min (50 μl of...
sensitizer with 25 ml of water) and subsequently washed for 1 min in water (two times). Next, the gel was stained for 30 min (0.5 ml Enhancer with 25 ml of stain), washed two times for 20 s in ultrapure water, and finally developed (0.5 ml of Enhancer with 25 ml of Developer) for 2–3 min until bands appeared. The reaction was stopped by adding a 5% (v/v) acetic acid solution. The sugar content was also quantified by GC-MS after methanolysis and TMS derivatization (51).

**Methodology of Extracted LM/LAM Samples for GC-MS Analysis**—LM/LAM samples (24 μl of stock, 1 mM) were transferred to a heat sealed capillary tube (250-μl Drummond microdispenser, Drummond Scientific Co.), together with 10 μl of 0.1 M formic acid, acetonitrile (1:1, v/v) was used to wash the lines for 10 min in water (two times). Next, the gel was stained for 30 min (room temperature), samples were dried down again, and the methylesters were derivatized by adding 35 μl of BSTFA + 1% TMCS (Thermo Scientific). GC-MS was performed on an Agilent HP 6890 GC system and an Agilent HP 5973 MSD (Agilent Technologies) in electron ionization mode as described previously (25).

**LC/MS-ESI-TOF Analysis of LM/LAM**—Purified LM/LAM was suspended in 5 mM ammonium carbonate/bicarbonate buffer (pH 8.16), and 4 μl was injected by direct infusion into a Agilent 6550 iFunnel Q-TOF LC/MS system (Agilent Technologies). The run was performed in negative ionization mode, with a mass range of 100–3200 Da. The reference nebulizer was set to 5 p.s.i.g. with a detection window of 100 ppm, a minimum height of 1000 counts, an acquisition rate of 0.4 spectra/s, and an acquisition time of 1589.4 ms/spectrum. The gas temperature was set to 220°C with a drying gas of 7 liters/min, dual AJS ESI 3500 V, Fragmentor 365 V, and Skimmer 65 V. The flow rate was set to 0.2 ml/min, and a solvent consisting of 0.1 M formic acid, acetonitrile (1:1, v/v) was used to wash the lines for 25 min. Data were analyzed using MassHunter, and the length of the mann chain was calculated, assuming the presence of a PI lipid anchor containing C16:1/18:1/16:0 fatty acids. Analyses were performed in duplicate.

**Linkage Analysis of LM/LAM**—Purified LM/LAM fractions were subjected to methylation linkage analysis as described previously (16). Permethylated, peracetylated sugars were analyzed on an Agilent HP 6890 GC and 5973 MSD (Agilent Technologies) in electron ionization mode, using a DB5 capillary column (J&W Scientific CP9013, 30 m, 250-μm inner diameter, 0.25-μm film thickness), with a 10-m inert duraguard. The injector insert and GC-MS transfer line temperatures were 270 and 250°C, respectively. The oven temperature gradient was set to 140°C (2 min), 140–230°C at 5.0°C/min and in the end held for 10 min. The run was also performed in SIM (selected ion monitoring) mode, selecting the ions 161, 162, 189, 190, and 205, which are specific for all partially methylated alditol acetates (PMAAs).

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**Construction of a Conditional Gene Knock-out of MSMEG_0317**—A 500-bp synthetic DNA fragment (Integrated DNA Technologies) was designed containing 226 bp of sequence upstream and downstream of the gene plus 48 bp containing the 5’- and 3’-end of the gene. For cloning purposes, the synthetic DNA was designed with a SacI site at the 5’-end and a PstI site at the 3’-end. The fragment was cloned into pMSS (a suicide vector for M. smegmatis carrying sacB and streptomycin resistance genes) digested with SacI/PstI to create plasmid pMSS-ΔMSMEG_0317. This plasmid was then electroporated into M. smegmatis mc^2^155 selecting for streptomycin resistance. Colonies were screened for sucrose sensitivity, indicating integration of the entire plasmid via an SCO event at the MSMEG_0317 locus, and a confirmed SCO clone was isolated.

To construct a conditional gene knock-out from the MSMEG_0317 SCO strain, MSMEG_0317 was PCR-amplified from M. smegmatis genomic DNA using primers MSMEG_0317F (5′-GCGAAATTCATAGCCTGCTGCGG) and MSMEG_0317R (5′-GCAAAGTTCAGATCGGTCCGTCGG) containing EcoRI and HindIII sites, respectively (underlined). The amplified fragment was then cloned into the integrative vector pMV361 vector (43), and a sequence-verified clone was electroporated into electrocompetent MSMEG_0317 SCO cells, selecting on kanamycin plates. Streptomycin-sensitive, sucrose-resistant colonies were derived from this strain as described above and analyzed by PCR to determine whether any were DCO strains using the primers MSMEG_0319 (5′-G-GTTCGCCAGCCACACCG) and MSMEG_0317R.

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**Author Contributions**—M. J. M., P. K. C., and R. L. C. designed the study and wrote the paper. T. C. J., R. B., Y. Y.-B., and A. K. R. contributed the experiments shown in Figs. 3–5 and supplemental Fig. S2. S. K. performed the experiments shown in Figs. 4–6 and supplemental Fig. S5 and prepared the figures. R. B. performed the experiments shown in supplemental Figs. S3 and S4. P. K. C. prepared Figs. 1–3, 7, and 8 and supplemental Fig. S1. All authors reviewed the results and approved the final version of the manuscript.

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