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Acetylation of trehalose mycolates is required for efficient MmpL-mediated membrane transport in *Corynebacterineae*

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ABSTRACT

Pathogenic species of *Mycobacteria* and *Corynebacteria*, including *Mycobacterium tuberculosis* and *Corynebacterium diphtheriae*, synthesize complex cell walls that are rich in very long chain mycolic acids. These fatty acids are synthesized on the inner leaflet of the cell membrane and subsequently transported to the periplasmic space as trehalose monomycolates (TMM), where they are conjugated to other cell wall components and to TMM to form trehalose dimycolates (TDM). *Mycobacterial* TMM, and the equivalent *Corynebacterium glutamicum* trehalose corynomycolates (TMCM), are transported across the inner membrane by MmpL3, or NCgl0228 and NCgl2769, respectively, although little is known about how this process is regulated. Here we show that transient acetylation of the mycolyl moiety of TMCM is required for periplasmic export. A bioinformatic search identified a gene in a cell wall biosynthesis locus encoding a putative acetyltransferase (*M. tuberculosis* Rv0228 / *C. glutamicum* NCgl2759) that was highly conserved in all sequenced *Corynebacterineae*. Deletion of *C. glutamicum* NCgl2759 resulted in the accumulation of TMCM, with a concomitant reduction in surface transport of this glycolipid and syntheses of cell wall trehalose dicorynomycolates. Strikingly, loss of NCgl2759 was associated with a defect in the synthesis of a minor, and previously uncharacterized, glycolipid species. This lipid was identified as trehalose mono-acetylcorynomycolate (AcTMCM) by mass spectrometry and chemical synthesis of the authentic standard. The *in vitro* synthesis of AcTMCM was dependent on acetyl-CoA, while *in vivo* [³H]-acetate pulse-chase labelling showed that this lipid was rapidly synthesized and turned over in wild type and genetically complemented bacterial strains. Significantly, the biochemical and TMCM/TDCM transport phenotype observed in the Δ NCgl2759 mutant was phenocopied by inhibition of the activities of the two *C. glutamicum* MmpL3 homologues. Collectively, these data suggest that NCgl2759 is a novel TMCM mycolyl acetyltransferase (TmaT) that regulates transport of TMCM and is a potential drug target in pathogenic species.

INTRODUCTION

Pathogenic *Mycobacteria* and *Corynebacteria* are the cause of human diseases such as tuberculosis (TB), leprosy and diphtheria. *Mycobacterium tuberculosis*, the etiological agent of TB, is estimated to latently infect one-third of the world's population and cause more than 1.3 million deaths annually. While considerable progress has been made in decreasing both the incidence and mortality of TB, the threat from coinfection with HIV-AIDS, and the spread of multidrug and extensively drug-resistant strains is ongoing ⁽¹⁾. The intrinsic resistance of these bacteria to common antibiotics and disinfectants, as well as many host microbicidal responses is partly attributable to their unusual lipid-rich cell wall structure ⁽²⁾, which comprises an asymmetric outer membrane covalently linked to an arabinogalactan-peptidoglycan complex in the periplasmic space. This outer membrane layer is dominated by mycolic or corynomycolic acids, long chain α -alkyl β -hydroxyl fatty acids, that are either linked to terminal arabinose residues on the arabinogalactan ⁽³⁾ or are constituents of outer layer glycolipids, including trehalose monomycolate (TMM) and trehalose dimycolate (TDM) ⁽⁴⁾. Recent studies have shown that mycolic acids are also covalently linked to some cell wall proteins ⁽⁵⁾. Enzymes involved in mycolic acid synthesis are targets of current front line anti-TB drugs, including isoniazid and ethionamide, highlighting the essential role that these fatty acids play in *M. tuberculosis* survival ⁽⁶⁾.

Mycobacterial mycolic acids are very long chain (C₆₀-C₉₀) species, which can contain additional alkene, cyclopropane, hydroxy, methoxy, epoxy, and keto groups ^(3, 7). The enzymes involved in the biosynthesis of mycobacterial mycolic acids, or shorter (C₂₂ to C₃₆), less modified *Corynebacterium glutamicum* corynomycolic acids, have been extensively studied ⁽⁸⁻¹²⁾. Long chain (C₁₆-C₁₈, C₂₄-C₂₆) fatty acids are initially synthesized by a type-1 fatty acid synthase, and then elongated by a multi-enzyme fatty acid synthase type-2 complex. Following additional functionalization, the C₂₃-C₂₇ α -branch is condensed with meromycolic acid by the cytoplasmic polyketide synthase, Pks13, to form a 2-alkyl-3-keto fatty acid ⁽¹¹⁾. This precursor is reduced by CmrA to form the mature mycolic acid ^(10, 13) that is subsequently linked to the 6-hydroxyl of the disaccharide, trehalose, to form TMM in mycobacteria or TMCM in corynebacteria. While early studies suggested that the transport of mycolic acids across the inner cell membrane involved the linkage of these fatty acids to another phospholipid, such as polyprenol-phosphomannose ⁽¹⁴⁾, more recent studies suggest that most, if not all, mycolic acids are transported to the periplasmic space as TMM/TMCM ⁽¹⁵⁾. TMM/TMCM is subsequently utilized by mycolyltransferases that are located in the cell wall and are involved in the transfer the mycolic acids to the cell wall arabinogalactan, or from one TMM/TMCM molecule to another to form TDM/TDCM ⁽⁹⁾. In mycobacteria, these include members of the antigen 85/fibronectin-binding proteins (Fbps) ^(2, 16), which are differentially located in the outer leaflet of the cell membrane (FbpA, FbpB) or the cell wall fraction (FbpC) ⁽¹⁷⁾. In *C. glutamicum*, the outer membrane corynomycolic acid transferases, Cop1, Cmt1 and Cmt2 are thought to be responsible for the synthesis of TDCM, while the essential enzyme Cmt4 may mediate other mycolyltransfer reactions ^(18, 19). The free trehalose produced by the periplasmic or cell wall mycolyltransferases is subsequently salvaged by a specialized ABC transporter system that transports trehalose from the periplasm to the cytosol and is essential for *M. tuberculosis* virulence ⁽²⁰⁾.

Several recent studies strongly suggest that transport of TMM from the cytoplasmic to the periplasmic leaflet of the cell membrane is mediated by the polytopic membrane protein, MmpL3 ^(15, 21-23). MmpL3 is a member of the **m**ycobacterial **m**embrane **p**rotein **l**arge (MmpL) family that typically contain 12 transmembrane domains and have been implicated in the transport of other cell wall components. MmpL3 was identified as the likely target of a panel of inhibitors that were found to have potent bactericidal activity against *M. tuberculosis* in several independent high-content phenotypic screens ^(15, 21-24). Treatment with these inhibitors, which included AU1235, SQ109 and BM212, led to the accumulation of TMM and inhibited the formation of outer membrane TDM and the mycoylation of AG ^(15, 21, 22). MmpL3 is highly conserved in all species of

mycobacteria and is an essential protein in *M. tuberculosis* ⁽²⁵⁾. Conditional deletion of MmpL3 in *M. smegmatis* phenocopied the chemical inhibitors, resulting in the loss of TDM and AG-linked mycolic acids ⁽²⁶⁾. Interestingly, *C. glutamicum* appears to contain two MmpL family members that are the functional homologues of MmpL3 involved in TCM transport. Deletion of *NCgl0228* or *NCgl2769* alone had no effect on *C. glutamicum* growth or TCM synthesis and export, whereas disruption of both genes resulted in complete ablation of TCM, TDCM and AG-linked corynomycolic acids ⁽²⁶⁾. The latter phenotype indicates that the *C. glutamicum* MmpL3 homologues are involved in regulating the synthesis of TCM, as well as the transport of this glycolipid across the cell membrane. While these studies provide compelling evidence that MmpLs are involved in the transport of trehalose mycolates and the provision of mycolate donors for synthesis of essential cell wall components, it is not known if or how this transport step is regulated and whether TMM/TCM is the only substrate for these transporters.

We have developed a bioinformatic approach to identify mycobacterial genes that are conserved across the Corynebacterineae suborder and likely to be involved in cell wall biosynthesis. One of the genes identified in this search, *Rv0228* in *M. tuberculosis* and *NCgl2759* in *C. glutamicum*, encodes a putative polytopic membrane protein with an acyltransferase domain. Detailed biochemical analysis of a *C. glutamicum* mutant lacking *NCgl2759* indicates that this protein mediates the transient acetylation of newly synthesized TCM and that this modification is required for efficient transport of TCM to the periplasmic space and cell wall mycolyltransferases. This phenotype can also be recapitulated by genetic deletion of the MmpL transporter, *NCgl0228*, combined with chemical inhibition of the second MmpL transporter, *NCgl2769*, indicating that both transporters recognize acetylated TCM. Our findings suggest that acetylation plays a key role in regulating TCM export and assembly of the outer membrane and that enzymes involved in TCM acetylation/deacetylation could be targets of new anti-TB therapies.

RESULTS

NCgl2759 is a putative membrane acyltransferase that is conserved across the Corynebacterineae - In order to identify new genes involved in cell wall synthesis we undertook a bioinformatic search for genes that (i) are of unknown function and conserved across the Corynebacterineae suborder, (ii) genetically linked to loci involved in cell wall synthesis in one or more Corynebacterineae and (iii) are essential for growth of *M. tuberculosis* ⁽²⁷⁾. This search led to the identification of the *M. tuberculosis* gene *Rv0228* which is predicted to encode a polytopic membrane protein (with nine transmembrane domains) containing a putative acyltransferase domain. Although the *Rv0228* gene is not genetically linked to other known cell wall biosynthesis genes, its ortholog in *C. glutamicum*, *NCgl2759*, is adjacent to a cell wall biosynthesis locus involved in AG and mycolic acid biosynthesis ⁽²⁸⁾. *M. tuberculosis* *Rv0228* was shown to be essential in a genome-wide screen ⁽²⁹⁾ and to be highly conserved in Corynebacterineae with orthologs in *M. leprae*, *M. avium* subspecies *paratuberculosis*, *C. glutamicum* and *Corynebacterium diphtheriae*, displaying 86%, 87%, 55% and 53% amino acid similarity, respectively, to the *M. tuberculosis* ortholog (Fig S1). The high level of genomic synteny in the genes flanking *M. tuberculosis* *Rv0228* (*Rv0224-0227c*) and *C. glutamicum* *NCgl2759* (*NCgl2760-2762* and *NCgl2764*) provided further evidence that each of these loci share common functions in each organism.

Inactivation of the C. glutamicum NCgl2759 gene - In contrast to *M. tuberculosis* and other mycobacteria, *C. glutamicum* can tolerate the loss of several major cell wall components, particularly outer layer non-covalently linked (glyco)lipids ^(10, 30). As *Rv0228* has been shown to be essential in *M. tuberculosis* under standard culture conditions ⁽²⁹⁾, we investigated the function of the *C. glutamicum* orthologue, *NCgl2759* (Fig 1A). Deletion of the *C. glutamicum* *NCgl2759* gene was accomplished using a two-step recombination strategy (see Material and Methods). Following integration of the suicide plasmid *pK18mobsacBΔNCgl2759* and two

rounds of selection, kanamycin-sensitive, sucrose-resistant colonies were observed after 8 days growth. PCR analysis showed that colonies with normal size (>2 mm diameter) retained the wild type *NCgl2759* gene, whereas all small colonies (< 0.2 mm diameter) displayed the expected profile for gene knockouts (data not shown). Deletion of *NCgl2759* in the latter strains was confirmed by Southern blot hybridization. A probe derived from *NCgl2759* hybridized to two fragments in wild-type *C. glutamicum* of 5.6 kb and 4.0 kb in size, and to one band in the mutant of 9.0 kb following digestion of genomic DNA with *Bgl*II/*Nco*I (Fig 1B). This was the expected profile for a knockout mutant since one *Bgl*II site was located in the deleted portion of *NCgl2759* (Fig 1A, B). The slow growth phenotype of the mutant was maintained in liquid media (Fig 1C). Transformation of the Δ *NCgl2759* mutant with a shuttle plasmid containing the gene, pSM22:*NCgl2759*, restored the growth rate to wild-type levels whereas transformation with the pSM22 vector alone did not (Fig 1C).

Loss of NCgl2759 results in changes in the profile and surface transport of trehalose mycolate glycolipids - HPTLC analysis of the lipid fraction of *C. glutamicum* wild type, the Δ *NCgl2759* mutant and complemented strains revealed marked changes in the relative abundance of different trehalose mycolates. In particular, the Δ *NCgl2759* mutant contained reduced levels of the polar TDCM and accumulated high levels of TMCM. TDCM was restored to wild type levels, and TMCM levels reduced, following complementation of the mutant with pSM22:*NCgl2759*, but not with an empty plasmid (Fig 2). The accumulation of TMCM in the Δ *NCgl2759* mutant was not reflected by an overall increase in corynomycolic acid synthesis, as shown by GC/MS analysis of the total lipid fraction. These analyses also showed that the mutant synthesized the same prominent mycolic acid species (C32:0 and C34:1) as the wild type bacteria (Table S1). Corynomycolic acids are also covalently linked to the AG component of the cell wall. GC/MS analysis of the AG-linked corynomycolic acids, released after saponification of the delipidated cell pellet, revealed comparable levels of corynomycolic acids in wild type and Δ *NCgl2759* fractions (Fig 3A, Table S2). Collectively, these analyses indicate that loss of *NCgl2759* is associated with a defect in the conversion of TMCM to TDCM, but has little effect on the covalent linkage of a minor subpool of corynomycolic acids to the cell wall.

The accumulation of TMCM in the *NCgl2759* mutant and the defect in TDCM biosynthesis would be consistent with a defect in the transport of newly synthesized TMCM to the periplasmic space/outer membrane where it is converted to TDCM. We therefore investigated the subcellular distribution of TMCM/TDCM in the wild type and mutant bacteria using differential solvent extraction. As previously shown⁽³¹⁾, extraction of live bacteria with 1-butanol results in the selective removal of 'outer membrane' lipids such as TDCM, but not cell (inner) membrane lipids such as phosphatidylinositol or cardiolipin (Fig 3B). In wild type bacteria, approximately 75% of the ¹⁴C-TMCM was extracted by 1-butanol, indicating that a major fraction of the steady-state pool of TMCM is located in the periplasmic space/outer membrane. In striking contrast, <5% of the TMCM was extracted in 1-butanol in the *NCgl2759* mutant (Fig 3B,C). These analyses indicate that loss of *NCgl2759* is associated with a defect in the transport of TMCM from the site of synthesis in the inner membrane to the periplasm/outer membrane.

Synthesis of a minor lipid species, Lipid-X, is ablated in the Δ NCgl2759 mutant – HPTLC analysis revealed the presence of a minor glycolipid species in total lipid extracts of wild type bacteria, which was absent in lipid extracts from the Δ *NCgl2759* mutant (Fig 2). This species, termed Lipid-X, migrated just above TMCM and keto-TMCM, a key intermediate in the TMCM pathway⁽¹⁰⁾. The synthesis of Lipid-X was restored after complementation of the Δ *NCgl2759* mutant with a plasmid containing *NCgl2759* (Fig 2). Lipid-X was purified by HPTLC and subjected to GC/MS compositional analysis after methanolysis and TMS derivatization, revealing the presence of glucose and C32:0 and C34:1 corynomycolic acids. Trehalose was also detected in these analyses, indicating that Lipid-X is a mycolated trehalose. To confirm that Lipid-X contained a trehalose headgroup, purified Lipid-X was subjected to mild base treatment and the released headgroup analyzed by

HPTLC. A single glycan headgroup was detected that co-migrated with authentic trehalose (Fig S2). These analyses suggested that Lipid-X comprises TMCM with a base- and acid-sensitive modification. To further define the nature of this modification, wild type and $\Delta NCgl2759$ mutant lipids were extracted from the same region of the HPTLC and analyzed by LC-MS (Fig S3A). As expected, the major band detected in this region of the HPTLC corresponded to unmodified TMCM species that were detected as NH_4^+ or Na^+ ion adducts. Pseudomolecular ions for the $[TMCM-NH_4]^+$ adducts were observed consistent with the presence of C32:0 (m/z 838), C34:1 (m/z 864) to C36:2 (m/z 890) corynomycolic acids (Fig S3B), which closely matched the composition detected by GC-MS analysis (Table S1, S2). Wild type lipid extracts contained two additional bands that were resolved from TMCM after two rounds of HPTLC purification. Several of the masses in this cluster (m/z 836.9, 864.8, 891.0) corresponded to the previously characterized keto-TMCM (Fig S3C, and were detected in a comparable fraction from the $\Delta NCgl2759$ mutant (Fig S3E). Additional masses at m/z 880.8, 906.8 and 932.9 were detected in the HPTLC fractions generated from wild type lipids, but were absent in the $\Delta NCgl2759$ mutant extracts (Fig 4, S3C, E). These masses correspond to the major molecular species of TMCM with an acetyl (CH_3CO- , 42 Dalton) substituent on either the trehalose glycan headgroup or the single corynomycolic acid (Fig 5). Such a modification is consistent with its sensitivity to both base and acid treatment and the annotation of Rv0228/NCgl2759 as a putative acyltransferase. To define the location of the acetyl group in Lipid-X, the major molecular species of unmodified TMCM (m/z 838.8) and Lipid X (m/z 880.9) were subjected to MS/MS fragmentation (Fig 4). Fragmentation of the NH_4^+ -pseudomolecular ion of unmodified TMCM yielded two pairs of diagnostic fragments ions at m/z 641.7/623.7 and m/z 479.9/461.6 (where each pair is separated by 18 mass units, due to loss of water) corresponding to loss of unmodified hexose (m/z 197.1) and hexose₂ (m/z 359). MS/MS of Lipid X also resulted in loss of hexose (m/z 683) and hexose₂ (m/z 521). However, these fragments were paired with ions at m/z 623 and m/z 461, respectively, which differ by 60 mass units corresponding to loss of acetate. These results indicate that the acetyl group is exclusively located on the corynomycolic acid lipid moiety (Fig 4B). To unequivocally resolve the structure of Lipid-X we undertook the total synthesis of non-modified TMCM (C32:0) and the proposed structure of Lipid-X, AcTMCM (C32:0) using a $CsHCO_3$ -mediated substitution of a trehalose tosylate with synthetic (+)-corynomycolic acid or O-acetyl-(+)-corynomycolic acid as the key step (Text S1). The MS/MS fragmentation patterns of these defined, chemically-synthesized materials were identical to those obtained from *C. glutamicum* (Fig S4), confirming the proposed structure of Lipid-X as AcTMCM. Additional Lipid-X molecular species containing acetylated C34:1 and C36:2 corynomycolic acids (m/z 906 and 932) were also detected in the wild type, but not the $\Delta NCgl2759$ lipid fractions. Identical fragmentation of these species was observed after MS/MS (data not shown). Importantly, the expression of all these species was restored in the $\Delta NCgl2759$ mutant following complementation with the plasmid pSM22:NCgl2759 (Fig S5). Finally, MS/MS analysis of the major glycolipid species in the $\Delta NCgl2759$ HPTLC fraction that migrated just above TMCM indicated that this fraction contained predominantly keto-TMCM (Fig S3D,E) ⁽¹⁰⁾. The accumulation of this species in the mutant provided additional evidence for NCgl2759 having a role in TMCM metabolism.

AcTMCM (Lipid-X) is a transient intermediate that precedes formation of TDCM - To determine whether AcTMCM is an end-product or metabolic intermediate, wild type and $\Delta NCgl2759$ mutant bacteria were pulse-chase labelled with [¹⁴C]-acetate and sequentially extracted with 1-butanol and chloroform:methanol to simultaneously analyze the rates of synthesis/turnover of individual species as well as their transport from the inner membrane to the periplasmic space/outer membrane. As shown in Fig 5, label was rapidly incorporated into both the inner and outer membrane pools of TMCM in wild type bacteria during the 5 min pulse. Label in these TMCM pools was depleted during the subsequent chase, with the inner membrane pool having a much faster turnover than the outer membrane pool. Depletion of label in the outer membrane TMCM fraction was associated with concomitant increase in labelling of the outer membrane TDCM (Fig 5). Significantly, a labelled glycolipid species with the same HPTLC mobility as AcTMCM was detected in the inner membrane fraction and found to chase out following depletion of TMCM (Fig 5A,B). Markedly different labelling kinetics

and subcellular trafficking were observed in the $\Delta NCgl2759$ mutant. In particular, a much higher level of ^{14}C -labelling was observed in the inner membrane pool of TMCM during the pulse and there was negligible depletion of label out of this pool during the chase (Fig 5A,B). The accumulation of labelled TMCM in the inner membrane pool was associated with a marked reduction in newly synthesized TMCM in the outer membrane and negligible synthesis of new TDCM. The transport of TMCM to the outer membrane and synthesis of TDCM was restored by complementation of the mutant with the pSM22:*NCgl2759* plasmid encoding native *NCgl2759* (Fig S6). Collectively, these analyses suggest that TMCM is rapidly transported from the inner membrane to the outer membrane fraction in wild type bacteria and that synthesis of AcTMCM is linked to, and may precede, TMCM transport. Inhibition of TMCM acetylation in the $\Delta NCgl2759$ mutant effectively prevents the chase of label out of the inner membrane pool of TMCM and leads to a block in the appearance of TMCM in the outer membrane pool and synthesis of TDCM.

During the course of these experiments, we noticed that the synthesis of a ^{14}C -labelled lipid species with a faster HPTLC mobility than TDCM was increased in the $\Delta NCgl2759$ mutant. This species was predominantly recovered in the outer membrane fraction and had an HPTLC mobility similar to keto-TDCM. Consistent with this assignment, a glycolipid species with the same HPTLC mobility and outer wall location, as shown by 1-butanol extraction, was synthesized in the *C. glutamicum* $\Delta NCgl2385$ mutant that lacks the keto-mycolic acid reductase, CmrA (Fig 3B,C) ⁽¹⁰⁾. These analyses strongly suggest that keto-TMCM, the first committed intermediate in TMCM biosynthesis, can be transported to the outer membrane and used to synthesize keto-TDCM. Interestingly, nearly all of the keto-TMCM in the outer membrane fraction was converted to keto-TDCM indicating that this reaction is quite efficient. While some labelling of a putative keto-TDCM was observed in wild type bacteria, this appears to be a minor pathway, indicating that the conversion of keto-TMCM to TMCM and AcTMCM via the activities of CmrA and *NCgl2759* normally predominate.

Our biochemical analysis of the $\Delta NCgl2759$ mutant had suggested that the attachment of mycolic acids to cell wall AG was not disrupted in this mutant. In support of this conclusion, analysis of the rate of incorporation of [^{14}C]-acetate-labelled mycolic acid into the base-labile AG/cell wall fraction, revealed similar linear rates of AG-mycolic acid conjugation in wild type and mutant bacteria (Fig 5B).

NCgl2759 utilizes acetyl-CoA - To investigate the possible acetyl donor utilized by *NCgl2759*, cell free extracts were prepared from wild type and $\Delta NCgl2759$ mutant bacteria and incubated with [^{14}C]-acetyl-CoA. Addition of [^{14}C]-acetyl-CoA to cell lysates of wild type bacteria generated a distinct labelled species that co-migrated with AcTMCM. An equivalent band was not labelled in $\Delta NCgl2759$ mutant lysates (Fig 6A). However, [^{14}C]-acetyl-CoA-charged cell lysates derived from $\Delta NCgl2759$ or the CmrA $\Delta NCgl2385$ mutant did contain a faint band with a similar HPTLC mobility to AcTMCM. The presence of this band in both mutants suggests that it is an unrelated lipid species or that other enzyme activities with overlapping specificities to both the reductase and acetyltransferase poorly compensate for loss of each of these enzymes. In a complementary set of experiments, wild type and mutant bacterial lysates were incubated with [^{14}C]-TMCM and the synthesis of AcTMCM measured after 60 min incubation in the presence of unlabelled acetyl-CoA. Again, AcTMCM was synthesized in wild type lysates, but not in $\Delta NCgl2759$ mutant lysates (Fig 6B). Significantly, labelled TMCM was converted to TDCM in both bacterial cell lines, suggesting that the *in vivo* requirement for *NCgl2759* for the synthesis TDCM is abolished *in vitro*, possibly reflecting a result of mixing of intracellular and periplasmic enzymes to the same location as the periplasmic corynomycolic acid transferases, and that the transport step is redundant following cell lysis.

Genetic and chemical inhibition of the two MmpL transporters NCgl0228 and NCgl2769 phenocopies loss of NCgl2759 - Recent studies have suggested that MmpL3 is required for transport of TMM across the inner membrane of mycobacteria ^(15, 21, 22, 26), while the putative MmpL3 orthologues, *NCgl0228* and *NCgl2769* have

a similar function in *C. glutamicum* ⁽²⁶⁾. While individual *C. glutamicum* mutants lacking either *NCgl0228* or *NCgl2769* have no apparent phenotype, disruption of both genes results in the complete loss of corynomycolic acid and TCMC biosynthesis, suggesting that the expression of at least one of these proteins is required for the function of the polyketide synthase, Pks13 ⁽²⁶⁾. In order to investigate the consequences of inhibiting the function of both transporters without disrupting mycolic acid and TCMC biosynthesis, *C. glutamicum* mutants lacking individual MmpL3 proteins were generated (see Fig S7, S8) and treated with the MmpL3 inhibitor, AU1235 ⁽¹⁵⁾. Treatment of the $\Delta NCgl2769$ MmpL3 mutant with AU1235 had no effect on the transport of TCMC to the outer membrane and conversion to TDCM (Fig 7, with Fig S9 serving as a control). In marked contrast, treatment of the $\Delta NCgl0228$ mutant with AU1235 resulted in a dramatic decrease in TCMC levels in the outer membrane fraction and effective inhibition of TDCM (Fig 8). Control experiments in which the inhibitor was omitted showed a slight decrease in TCMC levels in the surface lipid extract in the $\Delta NCgl0228$ mutant compared to wild-type and the $\Delta NCgl2769$ mutant (Fig S9), suggesting that NCgl0228 is the more dominant transporter of the two in wild-type bacteria. Overall, these data indicate that AU1235 selectively targets NCgl2769 and that AU1235 treatment of the $\Delta NCgl0228$ mutant can therefore be used to inducibly block the transport of TCMC to the outer membrane/periplasmic space without affecting corynomycolic acid synthesis.

The phenotype generated by genetic deletion of *NCgl0228* and chemical inhibition of NCgl2769 was very similar to that observed in the $\Delta NCgl2759$ mutant (Fig 3B). To confirm that loss of both transporters does indeed result in a block in transport of TCMC, the $\Delta NCgl0228$ mutant was pulse chase labelled with [¹⁴C]-acetate in the presence or absence of AU1235. The effect of AU1235 was observed 5 min after its addition. In common with the $\Delta NCgl2759$ mutant, addition of AU1235 to the $\Delta NCgl0228$ strain greatly reduced the rate at which label was chased from TCMC in the inner membrane, and both the appearance of labelled TCMC in the outer membrane fraction and synthesis of TDCM (Fig 8). These analyses indicate that loss of NCgl2759 phenocopies the combined inhibition of both MmpL transporters, suggesting that AcTCMC is the substrate utilized by both transporters.

DISCUSSION

TMM and TCMC are major components of the mycobacterial and corynebacterial cell walls, respectively, and critical mycolic acid donors for a large family of cell wall mycolyltransferases. Recent studies suggest that these glycolipids are synthesized on the cytoplasmic leaflet of the cell membrane and then transported to the periplasmic side of the cell membrane by MmpL3 in mycobacteria, or two orthologues of this polytopic membrane protein in *C. glutamicum*, *NCgl0228* and *NCgl2769*. How the MmpL3 proteins recognize TMM/TCMC and the regulation of this key step in cell wall assembly have not been previously investigated. In this study we provide evidence that the transport of newly synthesized *C. glutamicum* TCMC to the periplasm is dependent on the expression of NCgl2759, which acetylates the mycolic acid moiety of TCMC to form acetyl-TCMC (Fig 9A). We propose that transient acetylation of TCMC plays a key role in regulating the export of this major glycolipid.

NCgl2759 was originally identified in a screen for genes of unknown function that are (1) conserved across mycobacteria and corynebacteria, (2) located close to, or within, genetic loci involved in cell wall synthesis and (3) essential for growth of *M. tuberculosis* under standard culture conditions. NCgl2759 is predicted to encode a polytopic membrane protein with ten transmembrane domains and a putative acyltransferase domain. NCgl2759 orthologues were identified in all Corynebacterineae, within similar syntenic genomic contexts. The presence of an orthologue in *M. leprae* which has a highly decayed genome ⁽³²⁾, as well as the apparent essentiality of the *M. tuberculosis* orthologue, Rv0228 ⁽²⁹⁾, indicated that the encoded proteins have an important and potentially conserved metabolic function across this group of bacteria. While all

Corynebacterineae share a common cell wall structure, we and others have shown that *C. glutamicum* is more permissive to loss of key cell wall components making it an attractive model system for dissecting conserved processes in mycobacterial/corynebacterial cell wall biosynthesis and assembly. While deletion of *C. glutamicum* NCgl2759 had only a modest effect on bacterial growth, biochemical and pulse-chase labelling studies revealed a marked defect in the transport of TMCM to the outer membrane/periplasm fraction and concomitant loss of TDCM synthesis. NCgl2759 does not show homology to any of the six exported corynomycyltransferases that are responsible for the synthesis of TDCM ⁽¹⁸⁾ and TDCM synthesis was not perturbed in the cell-free system, indicating that NCgl2759 is not directly involved in TDCM synthesis. On the other hand, we provide evidence that NCgl2759 encodes an acetyltransferase *in vivo* and *in vitro*. Specifically, deletion of NCgl2759 resulted in the loss of synthesis of a previously uncharacterized minor glycolipid (Lipid-X) that was shown by MS/MS and total synthesis of an authentic standard, to be trehalose acetyl-corynomycolic acid (AcTMCM). This structure differs from a previously characterized acetylated-TMCM isolated from *M. tuberculosis* in which the acetyl-group is located on the trehalose moiety ⁽³³⁾. Loss of NCgl2759 was also associated with loss of synthesis of Lipid-X/AcTMCM in *in vivo* pulse-chase labelling studies and in an *in vitro* assay using ¹⁴C-acetyl-CoA. Collectively, our data show that NCgl2759 catalyzes the acetylation of newly synthesized TMCM and we refer to this protein as TMCM mycolyl acetyltransferase (TmaT). The conclusion that TmaT is required for the MmpL-mediated transport of TMCM across the cytoplasmic (inner) membrane, was strongly supported by the pulse chase labelling studies and the finding that the combination of genetic and chemical inhibition of the two MmpL3-like transporters in *C. glutamicum* phenocopied the loss of TmaT.

TMCM functions as the mycolic acid donor for both TDCM synthesis and mycolyltransferases that covalently link mycolic acids to the cell wall arabinogalactan. Unexpectedly, loss of TmaT had no discernable effect on the assembly of arabinogalactan-linked mycolic acids. As all of the mycolyltransferases are thought to be located in the periplasm/outer membrane, this finding suggests that transport of TMCM to the periplasm continues at a low rate in the absence of acetylation and that mycoyltransferases involved in arabinogalactan conjugation utilize TMCM more efficiently than those involved in TDCM biosynthesis. The latter pathway may be critically dependent on the accumulation of high levels of TMCM as this glycolipid is both the mycolic acid donor and acceptor for these enzymes. Alternatively, TDCM may be converted back to TMCM by cell wall mycoyltransferases when TMCM is limiting. This reaction may utilize pools of free trehalose in the periplasmic space generated by AG-dependent mycolyltransferases and/or maintained by export of intracellular pools by an inner membrane trehalose transporter (Fig 9). Thus decreased TDCM levels in the Δ NCgl2759 (Δ tmaT) mutant could reflect reduced synthesis and/or increased turnover of TDCM as a result of reduced availability of TMCM in the periplasm. Regardless of the mechanism, these findings suggest that the TmaT-regulated export of TMCM plays an important role in regulating the rate of synthesis of different classes of cell wall components.

The precise role of TMCM acetylation in cytoplasmic membrane transport remains unclear. The acetyl group may function as a signal that is recognized by the MmpL proteins. Alternatively, acetylation may alter the chemical properties or conformation of the mycolyl chain and facilitate transport of these very long chain fatty acids across a putative hydrophobic pore formed by the MmpL proteins ⁽³⁴⁾. Intriguingly, reversible acetylation of yeast sterols has recently been shown to be required for the export of these apolar lipids ⁽³⁵⁾. Mycolic acids are thought to assume a compact conformation in which the aliphatic chain folds back on itself and is contained within a single leaflet of a membrane bilayer ⁽³⁶⁾. This conformation may have the same physical properties as eukaryotic sterols ⁽³⁷⁾ suggesting that acetylation may have similar functions in both trehalose mycolate and sterol transport. Whether acetylation of the trehalose headgroup ⁽³³⁾ also plays a role in TMCM transport remains unclear. As with sterol transport, our pulse-chase labelling studies as well as the characterization of outer membrane forms of TMCM strongly suggest that AcTMCM is deacetylated following transport to the

periplasmic space. While the coryne/mycobacterial deacetylase is yet to be defined, this enzyme could play important roles in determining the directionality of membrane transport and/or the conformation of the mycolyl chains in the outer membrane.

In mycobacteria, a single MmpL transporter, MmpL3, is responsible for transmembrane transport of TMM. In contrast, *C. glutamicum* expresses two MmpL orthologues, NCgl0228 and NCgl2769, that have redundant functions in TMM transport. Deletion of each of these transporters individually had no effect on TMM transport. However, deletion of both transporters completely abrogated corynomycolic acid biosynthesis, indicating that expression of at least one of these proteins is required for the activity of earlier enzymes in this pathway, as well as transmembrane transport⁽²⁶⁾. Other MmpL proteins, such as MmpS4 are thought to anchor enzymes associated with the synthesis of their substrate (i.e. GPLs)⁽³⁸⁾ and these findings suggest that expression of at least one of the MmpL3 homologues is required for the activity of upstream enzymes in mycolic acid synthesis. As it is not possible to study TMM transport in the Δ NCgl0228/ Δ NCgl2769 double knockout line, we generated individual Δ NCgl0228 and Δ NCgl2769 mutant lines and attempted to pharmacologically inhibit the activity of the remaining transporter. Remarkably, we found that the MmpL3 inhibitor, AU1235, selectively targets the MmpL homologue NCgl2769, but had no detectable inhibitory effect on NCgl0228⁽¹⁵⁾. Treatment of the Δ NCgl0228 mutant with AU1235 resulted in a TMM export phenotype that phenocopied the defect observed in the acetyltransferase null mutant, suggesting that AcTMM is transported by both MmpL proteins in *C. glutamicum*.

While our data suggest that AcTMM is the primary substrate transported by NCgl0228/NCgl2769, the keto-TMM intermediate can also be transported to the outer membrane and used to synthesize TDCM when downstream enzymes, such as the CmrA reductase, are deleted. It is not known if keto-TMM is recognized by NCgl0228/NCgl2769 or transported by other proteins. It is possible that enzymes involved in TMM synthesis form a complex that delivers AcTMM to the MmpL proteins, and that loss of individual components of this complex interferes with the selective targeting of specific TMM molecular species to these transporters. Regardless, TMM species containing the keto-corynomycolic acids are clearly utilized with equal efficiency as bulk TMM species for the synthesis of TDCM.

The proposed role of NCgl2759 in TMM synthesis and transport is summarized in Fig 9. Previous studies have shown that corynomycolic acids are synthesized on the cytoplasmic leaflet of the cell membrane by Pks13 and transferred to trehalose by an unknown mycolyltransferase to form keto-TMM^(10, 11). Keto-TMM is subsequently converted to TMM by CmrA⁽¹⁰⁾, a cytoplasmic enzyme. As shown here, TMM is converted to AcTMM by NCgl2759/TmaT. AcTMM is then flipped to the periplasmic space by the redundant transporters NCgl0228/NCgl2769. As loss of both transporters results in loss of corynomycolic acid synthesis, it is possible that Pks13 and/or other proteins required for corynomycolic acid biosynthesis are anchored to these transporters, as has been proposed for MmpL7-PpsE⁽³⁹⁾ and MmpL8-Pks2⁽⁴⁰⁾. Following transfer to the periplasmic space, AcTMM is apparently deacetylated to form mature TMM which is utilized by periplasmic CMTs⁽¹⁸⁾. Trehalose produced by mycolyltransferase reactions is recycled back into the cytoplasm by the LpqY-SucABC transporter complex⁽²⁰⁾. Our study indicates that TmaT has a key regulatory role in TMM transport and cell wall biogenesis in Corynebacteria. Given the extent to which TmaT is conserved in pathogenic mycobacteria and its essentiality in *M. tuberculosis*, this enzyme and/or the putative deacetylase may represent new drug targets.

METHODS

Bioinformatics analysis - *M. tuberculosis* strain H37Rv sequences were obtained from the Tuberculist World-Wide Web Server at the Institut Pasteur. *C. glutamicum* ATCC 13032 Kitasato sequences, in addition to protein

alignments, were obtained from The J. Craig Venter Institute Comprehensive Microbial Resource web site. Amino acid similarity percentages were assigned according to the results found in the protein versus all alignment menu located for each gene in the above *C. glutamicum* ATCC 13032 Kitasato web site. Sequences were compared using ClustalW.

Growth and manipulation of *Escherichia coli* and *C. glutamicum* – *E. coli* DH5 α was cultured in Luria-Bertani medium at 37°C. All *C. glutamicum* strains were grown in brain heart infusion medium (BHI, Oxoid) at 30°C. 15g/l of agar was used for preparation of solid media and 10% sucrose (w/v) added when necessary. *C. glutamicum* electrocompetent cells were prepared according to ⁽⁴¹⁾, except that electroporation was performed at the following settings: 2.5kV, 200 Ω and 25 μ F. Kanamycin (30 μ g/ml) and ampicillin (100 μ g/ml) were added to media as appropriate.

DNA Manipulations - All restriction endonucleases and DNA modification enzymes were obtained from Roche and New England Biolabs. Genomic DNA was extracted from *C. glutamicum* as described ⁽⁴²⁾. PCR was performed by standard procedures using Proofstart DNA polymerase (Qiagen) and amplifications of [95°C- 10mins, 30 cycles (94°C- 1 min, 55°C - 1min, 72°C- 3 mins), 72°C- 10 mins]. Probes for Southern blot hybridisations were digoxigenin (DIG) labelled using a DIG labelling kit (Roche). Samples sequence was determined using an Applied Biosystems 3730S capillary sequencer.

Targeted disruption of the *NCgl2759* gene – Gene deletion was performed by amplifying two fragments flanking the *NCgl2759* gene. The left fragment of 2439 bp was amplified using the primers Leftfor (5'-CATTGAGCTCGGTGTTGGGTTGTGTTGTTG-3') and Leftrev (5'-CTTCTCTAGATGACGATTGTGTTCCCACTG-3'), containing *SalI* and *XbaI* sites respectively (underlined) and cloned into the same sites in pUC18. Design of the amplification and cloning procedure took into account the presence of a transposon in the *NCgl2758* gene in the wild type strain. The right fragment of 929 bp was amplified using the primers Rightfor (5'-GCATCTAGACAATGACCGTTGCCAGTAT-3') and Rightrev (5'-TCTGAAGCTTGAAACGTCATCGCCTCATCT-3'), containing *XbaI* and *HindIII* sites respectively (underlined) and cloned into the same sites in pUC18. The right fragment was subsequently digested out with *XbaI*/*HindIII* and subcloned into same sites in the plasmid carrying the left fragment. Both fragments were then excised with *SalI*/*HindIII* and subcloned into the *SalI*/*HindIII* sites of pK18*mobsacB* to produce pK18*mobsacB*: Δ *NCgl2759*. Following electroporation of *C. glutamicum* ATCC 13032, kanamycin resistant colonies were tested for the presence of integration of the plasmid via a single crossover event by Southern blot hybridisation. Confirmed single crossover clones were then cultured on plates containing 10% sucrose to generate double cross-overs which were confirmed by PCR and Southern blot hybridisation. For complementation, a 1.52kb fragment consisting of *NCgl2759* and 347bp of upstream sequence was amplified using the primers A (5'-GTGGGATCCAGCAAATTAGTTCCCTTACCG-3') and B (5'-GCTTAAGCTTCGGATAACGAGAGTGTCCAA-3'), containing *BamHI* and *HindIII* sites respectively, cloned into the same sites in pUC18, subsequently excised, blunt ended and then subcloned into the *PvuII* site of pSM22 ⁽¹⁰⁾ to create pSM22:*NCgl2759*.

Targeted disruption of the *NCgl0228* and *NCgl2769* genes - 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. The *NCgl0228* synthetic DNA fragment was designed with a *BamHI* site at one end and an *XbaI* site at the other end, while the *NCgl2769* knockout fragment carried *BamHI* and *HindIII* sites instead. The synthetic sequences were cloned into pK18*mobsacB* at the appropriate restriction enzyme sites. 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 blot hybridisation (See Fig S4, S5).

Extraction of cell wall glycolipids and lipopolysaccharides - *C. glutamicum* strains were grown to exponential phase (OD₆₀₀ nm=5-7) in BHI medium (Oxoid). Total lipid was extracted in chloroform:methanol (2:1, v/v) and chloroform:methanol:water (1:2:0.8, v/v) ⁽⁴³⁾. After removal of insoluble material by centrifugation (15,000 g, 10 min), extracts were dried under nitrogen and subjected to biphasic partitioning in 1-butanol and water (2:1, v/v). The organic phase was dried, and lipids were resuspended in 1-butanol. Cell surface exposed lipid was extracted by water saturated 1-butanol ⁽³¹⁾ at room temperature for 30 min followed by the residual lipid extraction by chloroform/methanol as for total lipid extraction. Lipid fraction was analyzed by high-performance thin layer chromatography (HPTLC) using aluminium-backed silica gel sheets (Merck). One-dimensional HPTLCs were developed in chloroform/methanol/13 M ammonium solution/ 1 M ammonium acetate/ water (180:140:9:9:23, v/v, solvent system A) or (180:140:9:9:2.3, v/v, solvent system B). Glycolipids were stained and visualized with orcinol:H₂SO₄. AG-linked mycolic acid was extracted from delipidated cell pellets after reflux in 50% ethanol to remove LM and LAM ⁽⁴⁴⁾. The pellet was saponified in 0.5M potassium hydroxide in methanol at 37°C, 2hr) and the extracts neutralized with 0.5 M acetic acid. The extracts were dried under N₂, and released mycolic acids recovered by 1-butanol:water biphasic partitioning (2:1, v/v). Mycolic acid species were analyzed by GC-MS after methanolysis and TMS-derivatisation ⁽¹⁰⁾. The LMs and LAM fraction was further purified by octyl-Sepharose chromatography after proteinase K treatment. The LM/LAM fraction was adjusted to 0.1 M ammonium acetate containing 5% 1-propanol and loaded onto an octyl-Sepharose column (1 ml) using the same buffer. Following elution of unbound material, LM and LAM were eluted with 30 and 40% 1-propanol (1 ml). LMs and LAMs were analyzed by SDS-PAGE and periodic acid-Schiff silver staining using reagents from the Silver SNAP stain kit 2 (Pierce) ⁽³⁰⁾.

Metabolic labelling – *C. glutamicum* was grown at 30°C in BHI medium to mid-log phase. For metabolic pulse-chase labelling, 15 ml bacterial culture was centrifuged (2,500 g, 10 min) and the cell pellet resuspended at 0.25 g/ml in PBS pre-warmed at 30°C. After incubation at 30°C for 15 min, labelling was initiated by addition of [¹⁴C]acetic acid (Amersham, 16.0 Ci/mmol, final concentration of 5 µCi/ml) and continued for 5 min. The culture was then chased by diluting in 10 volumes of BHI medium pre-warmed at 30°C. Aliquots were collected at indicated time points and snap-frozen in liquid nitrogen prior to analysis. After thawing, bacteria were rapidly centrifuged and [¹⁴C]acetate-labelled lipids extracted twice in chloroform:methanol (2:1, v/v) and once in chloroform:methanol:water (1:2:0.8, v/v). Dried lipids were suspended in 1-butanol:water (2:1, v/v) and lipids in the upper 1-butanol-rich phase analyzed by HPTLC and developed in solvent system A.

Mass spectrometric analysis - Total lipids were purified by preparative HPTLC and lipids extracted from silica scarapings using chloroform:methanol (1:1 v/v). The supernatant was dried under nitrogen and lipids recovered by 1-butanol:water biphasic partitioning (2:1 v/v). The 1-butanol phase was dried, and purified lipids were resuspended in 1-butanol and 10 times diluted in 5 mM ammonium formate in methanol. Then 8 µl was flow-injected (20 µl/min) to a quadrupole-linear iontrap hybrid MS, 4000 QTRAP (Applied Biosystems/MDS Sciex, Concord, ON, Canada). The mobile phase composition was water:methanol:tetrahydrofuran, 5:20:75 (v/v) with 5 mM ammonium formate. The ion spray voltage was set to 5500 V in the positive mode. The declustering potential was set at 80 V and the entrance potential was set at 10 V. The nebulizer and ion source gas were set to 20 psi. The ions source temperature was set to 350°C. For the product ion scan, collision energy was set to 35 V.

ABBREVIATIONS

TMCM, trehalose monocorynomycolate

TDCM, trehalose dicorynomycolate

TMM, trehalose monomycolate

TDM, trehalose dimycolate

TmaT, TMCM mycolyl acetyltransferase

AG, arabinogalactan
AcTMCM, trehalose acetyl-monocorynomycolate
keto-TMCM, trehalose monocorynketomycolate
m/z, mass-to-charge ratio
LC-MS, liquid chromatography mass spectrometry
GC-MS, gas chromatography mass spectrometry
HPTLC, high performance thin layer chromatography
CMT, corynomycolic acid transferase

ASSOCIATED CONTENT

Supporting Information

Nine supplementary figures (Figure S1-S9), two tables (Table S1, S2) and a text file (Text S1) accompany this paper. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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FIGURE LEGENDS

Figure 1. Disruption of *C. glutamicum* NCgl2759 results in a modest growth phenotype. A, Chromosomal loci of *C. glutamicum* wild type (top) and NCgl2759 deletion mutant (Δ NCgl2759, bottom) showing BglII/NcoI digestion sites. Thick lines indicate the hybridization probe. The NCgl2759 gene is indicated by a filled arrow while stripes indicate the sequence that was deleted in the Δ NCgl2759 mutant. Flanking genes are shown as open arrows. Thin arrows denote the four primers (Leftfor, Leftrev, Rightfor, Rightrev) that were used to amplify the left and right sequences and create the NCgl2759 deletion. B, Southern blots demonstrating disruption of NCgl2759. Lane 1, digoxigenin-labeled λ HindIII molecular weight DNA markers; lane 2, wild-type *C. glutamicum* genomic DNA digested with BglII/NcoI; lane 3, *C. glutamicum* Δ NCgl2759 genomic DNA digested with BglII/NcoI. C, growth curves of WT (■), Δ NCgl2759 (▲), Δ NCgl2759 + pSM22 (▼), Δ NCgl2759 + pSM22:NCgl2759 (◆). Measurements were taken from duplicate cultures. The growth curves of WT and Δ NCgl2759 overlap almost exactly because of restoration of the growth rate by reintroduction of the gene to the mutant strain.

Figure 2. Disruption of NCgl2759 results in altered glycolipid profile. Total lipids were extracted from *C. glutamicum* wild type, Δ NCgl2759, Δ NCgl2759 containing empty pSM22 and Δ NCgl2759 containing pSM22:NCgl2759 and analyzed by HPTLC. Glycolipids were detected by orcinol-sulfuric acid staining. The

identity of glycolipids indicated was based on the published reports and confirmation by LC-MS (not shown).

Figure 3. Disruption of *NCgl2759* has no effect on corynomycolic acid synthesis, but results in a selective defect in surface transport of TCMC and TDCM synthesis. A, Non-covalently linked corynomycolic acids, trehalose corynomycolates and AG-linked corynomycolic acids were sequentially extracted from wild type and $\Delta NCgl2759$ bacteria and levels quantitated by GC/MS (n=3, error bars are SD). B, Wild type, $\Delta NCgl2759$ (acetyltransferase mutant) and $\Delta NCgl2385$ (CmrA reductase) mutants were metabolically labelled with ^{14}C -acetate and sequentially extracted in 1-butanol (BuOH) and chloroform:methanol (2:1 v/v, C/M). Parallel cultures were directly extracted in chloroform:methanol:water (1:3:1 v/v, total lipid). The three fractions from each bacterial line were analyzed by HPTLC and labelled species detected by fluorography. C, Relative recovery of [^{14}C]-TCMC in the BuOH (outer membrane) and C/M (inner membrane) fractions. Label was quantitated on a linear HPTLC scanner.

Figure 4. Lipid X corresponds to acetylated forms of TCMC. MS/MS fragmentation of pseudo molecular ions for (A) TCMC_{C32:0} (m/z 838), (B) AcTCMC_{C32:0} (m/z 880) and (C) keto-TCMC_{C32:0} (m/z 836). TCMC and AcTCMC were derived from wild type bacteria, while keto-TCMC was derived from $\Delta NCgl2759$. All bands were purified by HPTLC prior to LC/MS/MS. The mass spectrum for AcTCMC is identical to that derived from synthetic AcTCMC (Supp Fig S4).

Figure 5. AcTCMC is a transient intermediate in TCMC biosynthesis and surface transport. A, *C. glutamicum* wild type and $\Delta NCgl2759$ bacteria were pulse-chase labelled with [^{14}C]-acetate. Bacteria were pulse labelled for 10 min, then suspended in fresh BHI medium without label and cell aliquots removed at indicated times. Pelleted bacteria were sequentially extracted in water saturated 1-butanol, and then chloroform:methanol (2:1 v/v) to generate fractions that were enriched in outer membrane and inner membrane/periplasmic space lipids. ^{14}C -labeled lipids in the 1-butanol (lower panel) and chloroform:methanol (1:2 v/v) fraction (upper panel) were analyzed by HPTLC and detected by fluorography. B, Incorporation of label into individual glycolipid species was determined by densitometry analysis of the HPTLC fluorographs. Incorporation of label into AG-linked mycolic acids was determined after saponification of the delipidated cells.

Figure 6. Synthesis of AcTCMC *in vitro*. Cell lysates of *C. glutamicum* wild-type and $\Delta NCgl2759$ mutant bacteria were prepared by sonication and *de novo* synthesis of AcTCMC measured in the presence of (A) [^{14}C]-acetyl-CoA or (B) [^{14}C]TCMC. Labeled lipids were analyzed by HPTLC. Free-MA, free mycolic acid. Incubation times are shown in minutes.

Figure 7. Inhibition of the two MmpL3 orthologues phenocopies the $\Delta NCgl2759$ trehalose mycolate defect. A, *C. glutamicum* mutants lacking the MmpL3 orthologues NCgl0228 or NCg2769 were generated and treated with the MmpL3 inhibitor, AU1235. After 30 min, wild type and mutant bacteria were metabolically labeled with [^{14}C]-acetate in the presence of inhibitor for 2hr. Labeled bacteria were sequentially extracted in water-saturated 1-butanol (BuOH) and chloroform/methanol (2:1 v/v) (C/M). In parallel experiments, labeled bacteria were extracted in chloroform:methanol (2:1 v/v) followed by chloroform:methanol:water (1:2:0.8 v/v/v) to generate a total lipid extract (Total lipid). Labeled lipids in the three fractions were analyzed by HPTLC. B, Quantitation of recovery of [^{14}C]-TCMC in the outer membrane/periplasmic fraction.

Figure 8. Synthetic defect in transport of TCMC and synthesis of TDCM synthesis following treatment of the $\Delta NCgl0228$ mutant with AU1235. A, $\Delta NCgl0228$ bacteria were pulse-labelled with [^{14}C]-acetate (10 min in PBS), then suspended in fresh BHI medium containing either AU1235 or DMSO and aliquots removed

at indicated times. Cell surface exposed lipids were sequentially extracted in water-saturated 1-butanol (BuOH, lower panel) and chloroform/methanol (2:1 v/v) (C/M, upper panel) and labelled lipids analyzed by HPTLC (B) Relative [^{14}C]-acetate incorporation into TMCM (circles) and TDCM (squares) in cell surface lipid fraction (black) and residual total lipid fraction (white) and [^{14}C]-acetate incorporation into KOH/methanol extracted fraction of delipidated cells (triangles).

Figure 9. Mycolic acid transport pathways in *Corynebacteria*. A, Chemical structures and transitions of the key corynomycolic acid intermediates involved in inner membrane transport. B, Fatty acids synthesized by FAS-I are converted to (coryno)mycolic acids by the polyketide synthase, Pks13, that is located on the cytoplasmic leaflet of the cell membrane. Following transfer to trehalose, the mycolic acid keto group is reduced by CmrA to form TMCM and then acetylated by the integral membrane protein, TmaT (NCgl2759) to form AcTMCM. In this study we show here that acetylation is required for optimal transport of TMCM to the outer membrane as well as the formation of TDCM by periplasmic corynomycolic acid transferases (CMTs). A similar phenotype is observed following deletion of the two MmpL transporters, NCgl2769 and NCgl0228, indicating that AcTMCM is the major substrate for these transporters. As most of the TMCM in the outer membrane of wild type bacteria is non-acetylated, it is proposed that TMCM acetylation is reversed following transport, consistent with the pulse chase labeling studies. Interestingly, TMCM transport does not appear to be disrupted in the CmrA mutant, suggesting that MmpL transport proteins may also recognize keto-TMCM (single dashed arrow).

Graphic

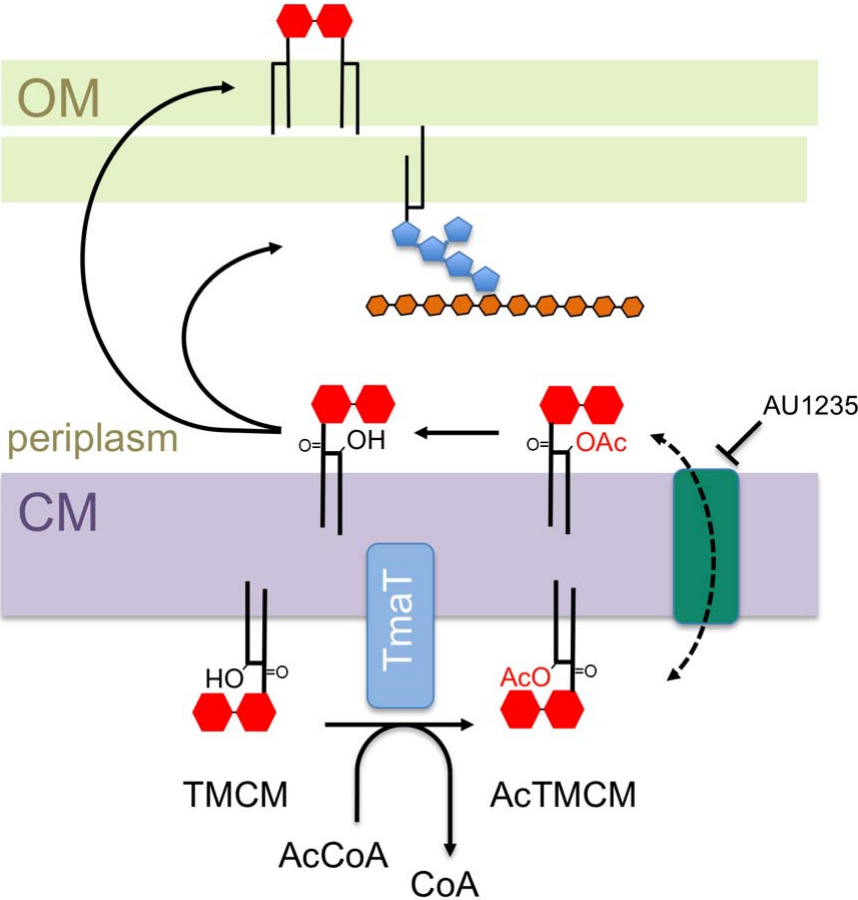


Figure 1

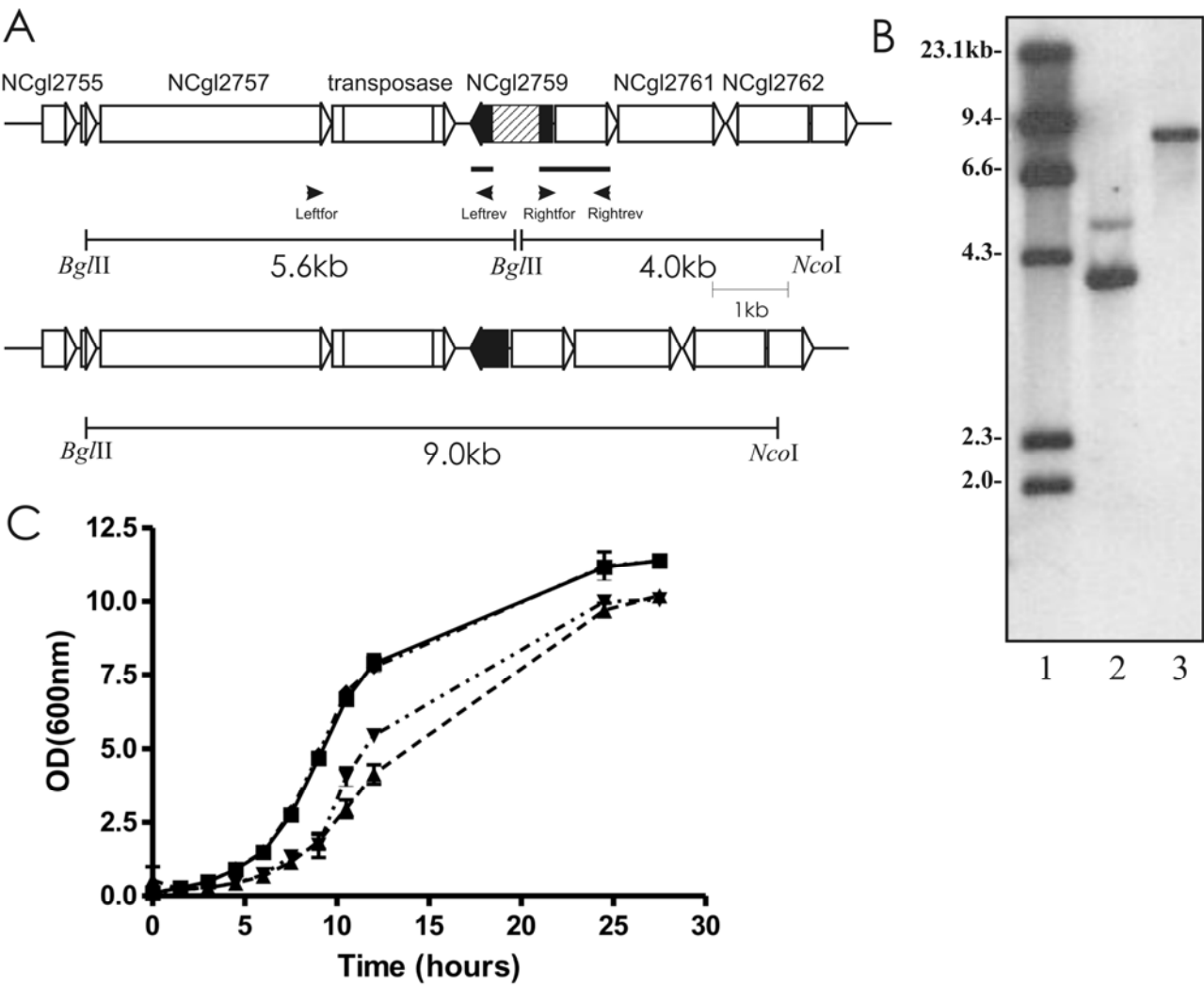


Figure 2

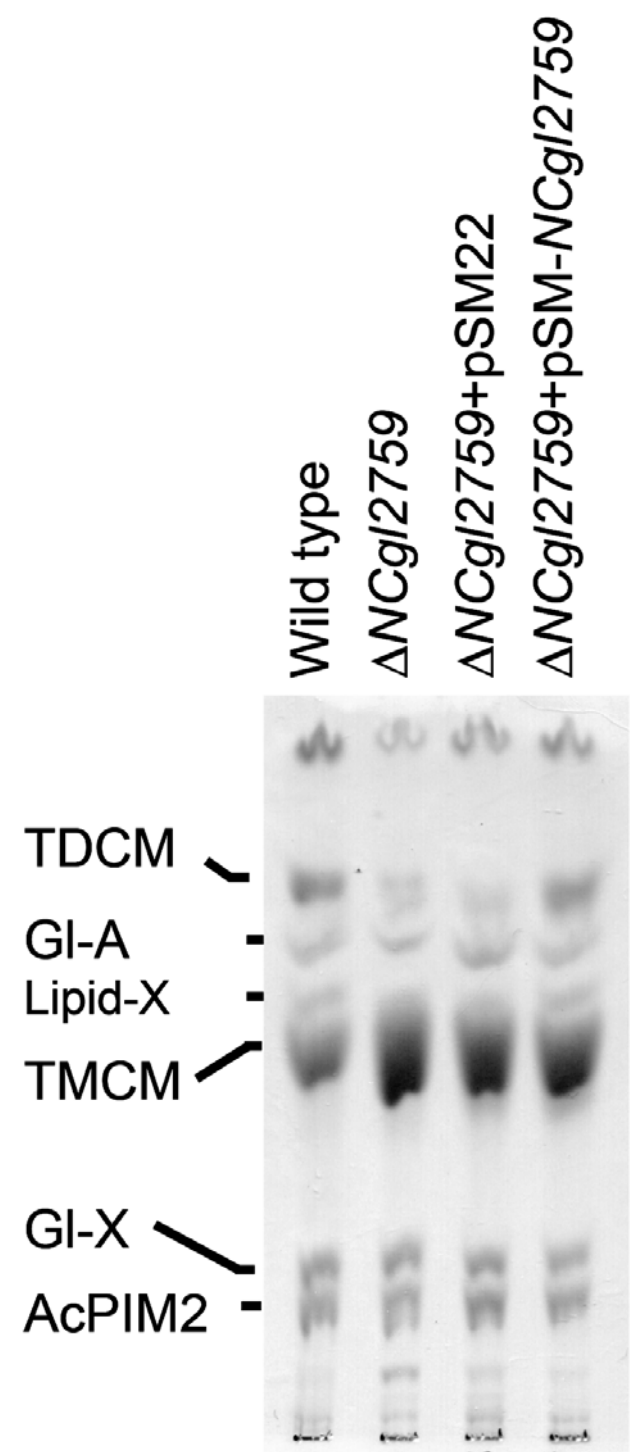


Figure 3

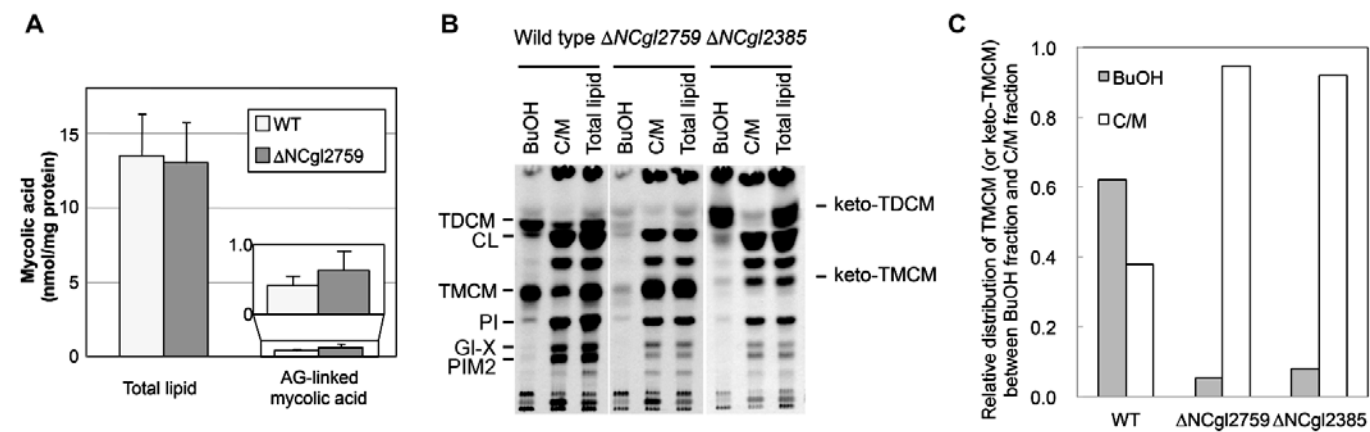


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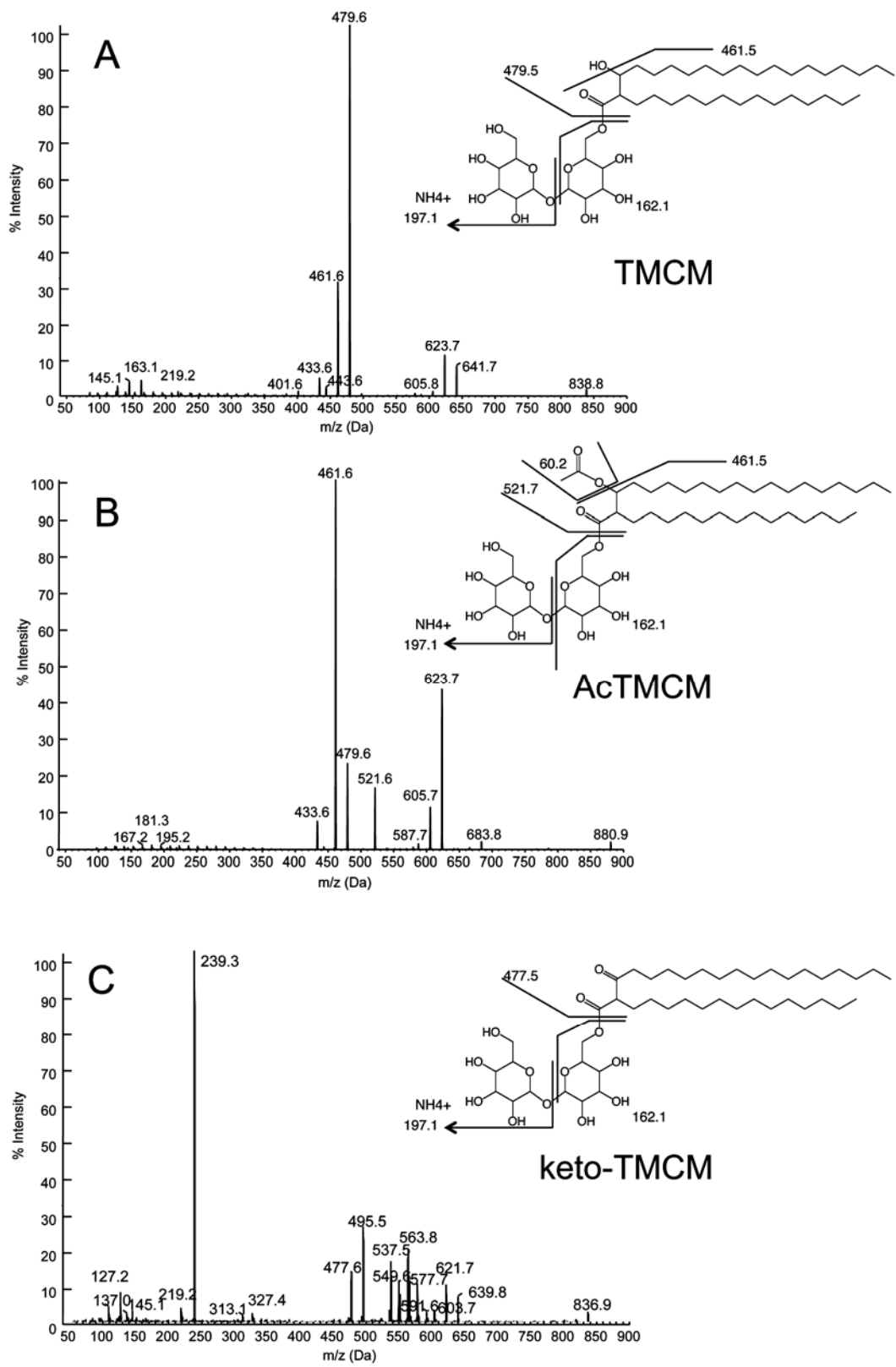


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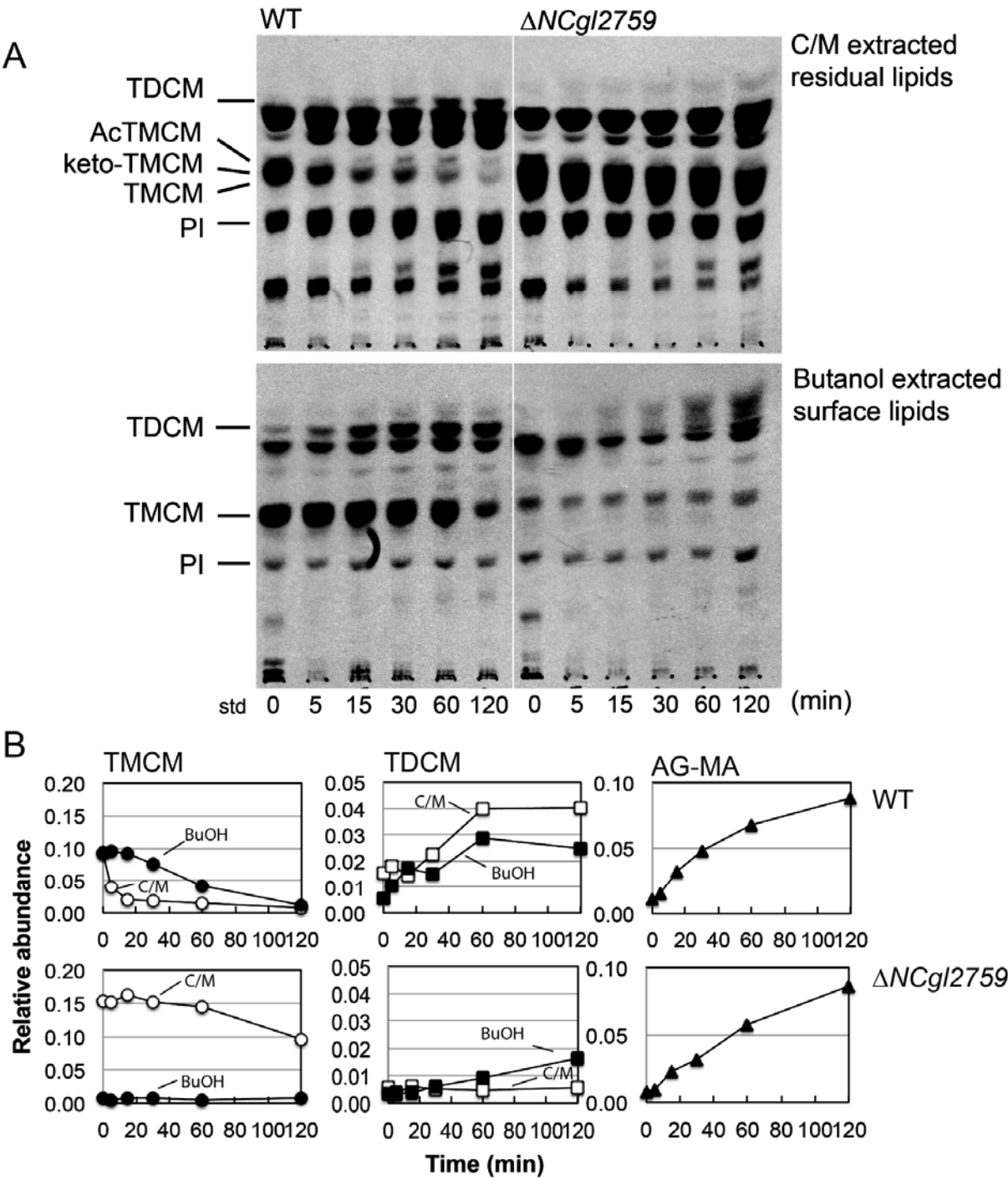


Figure 6

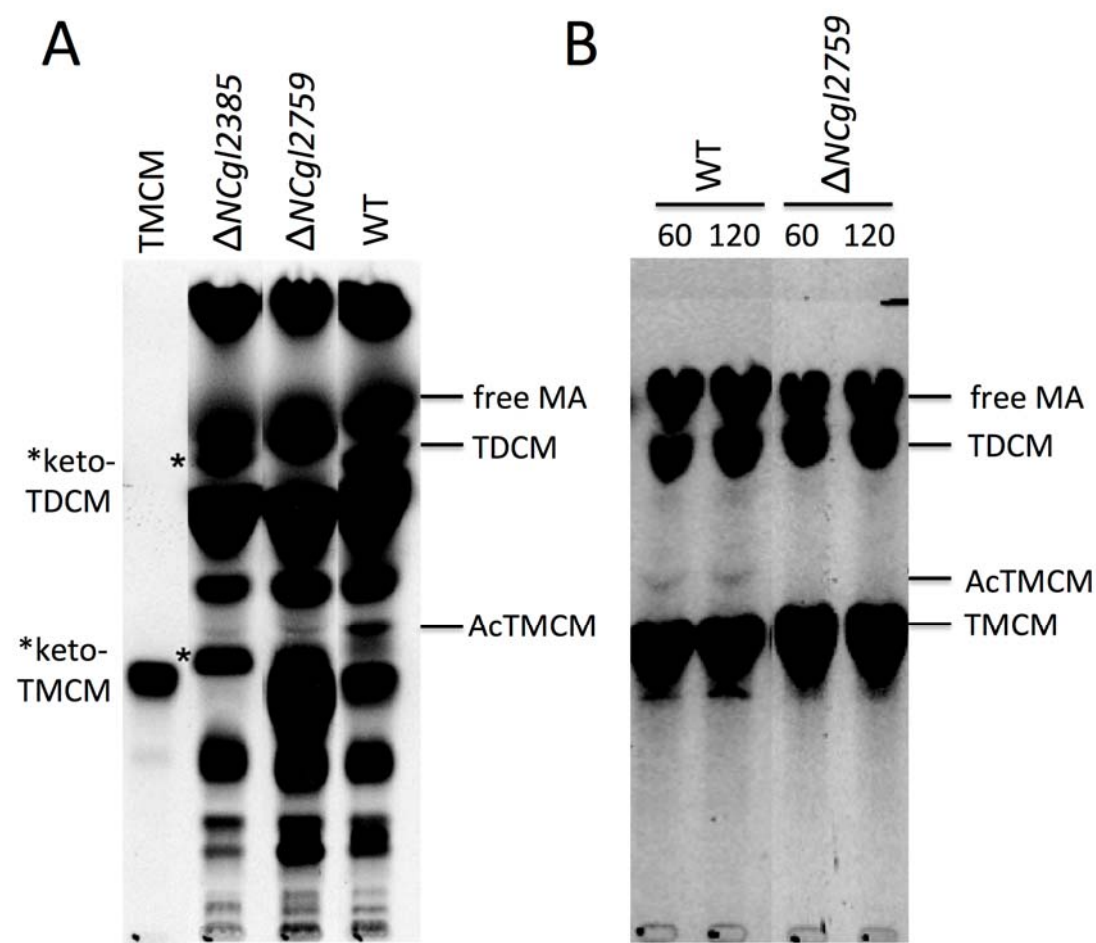


Figure 7

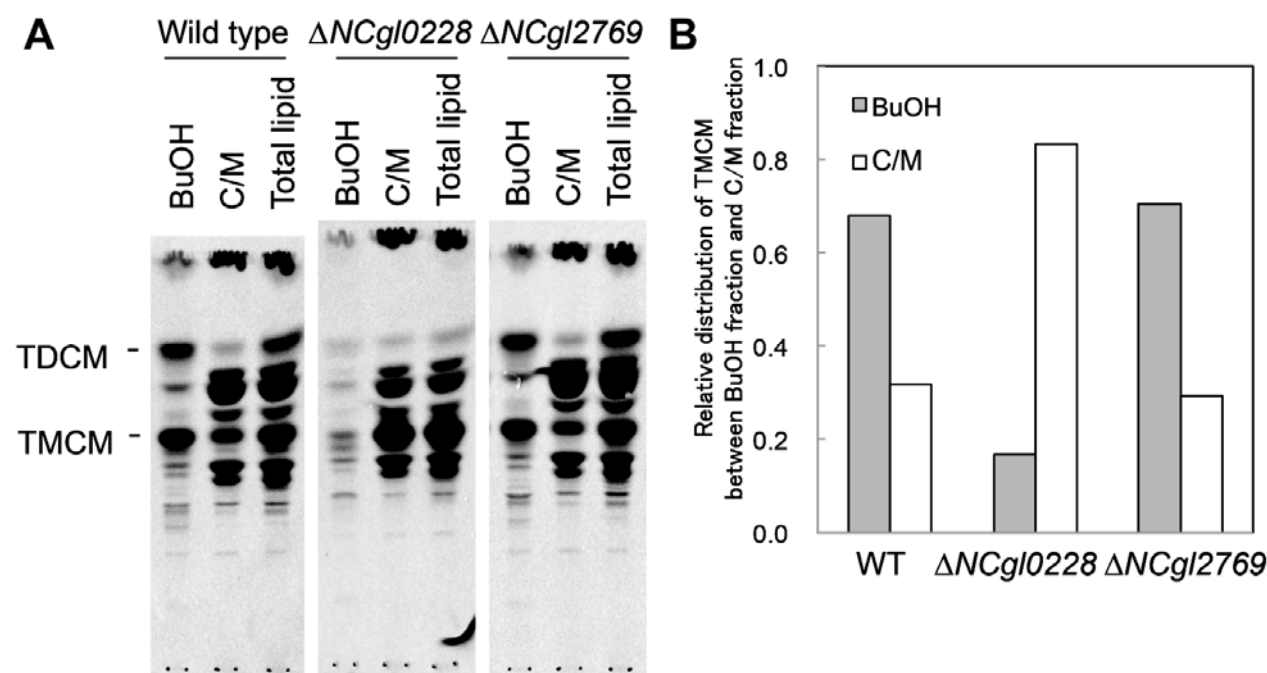


Figure 8

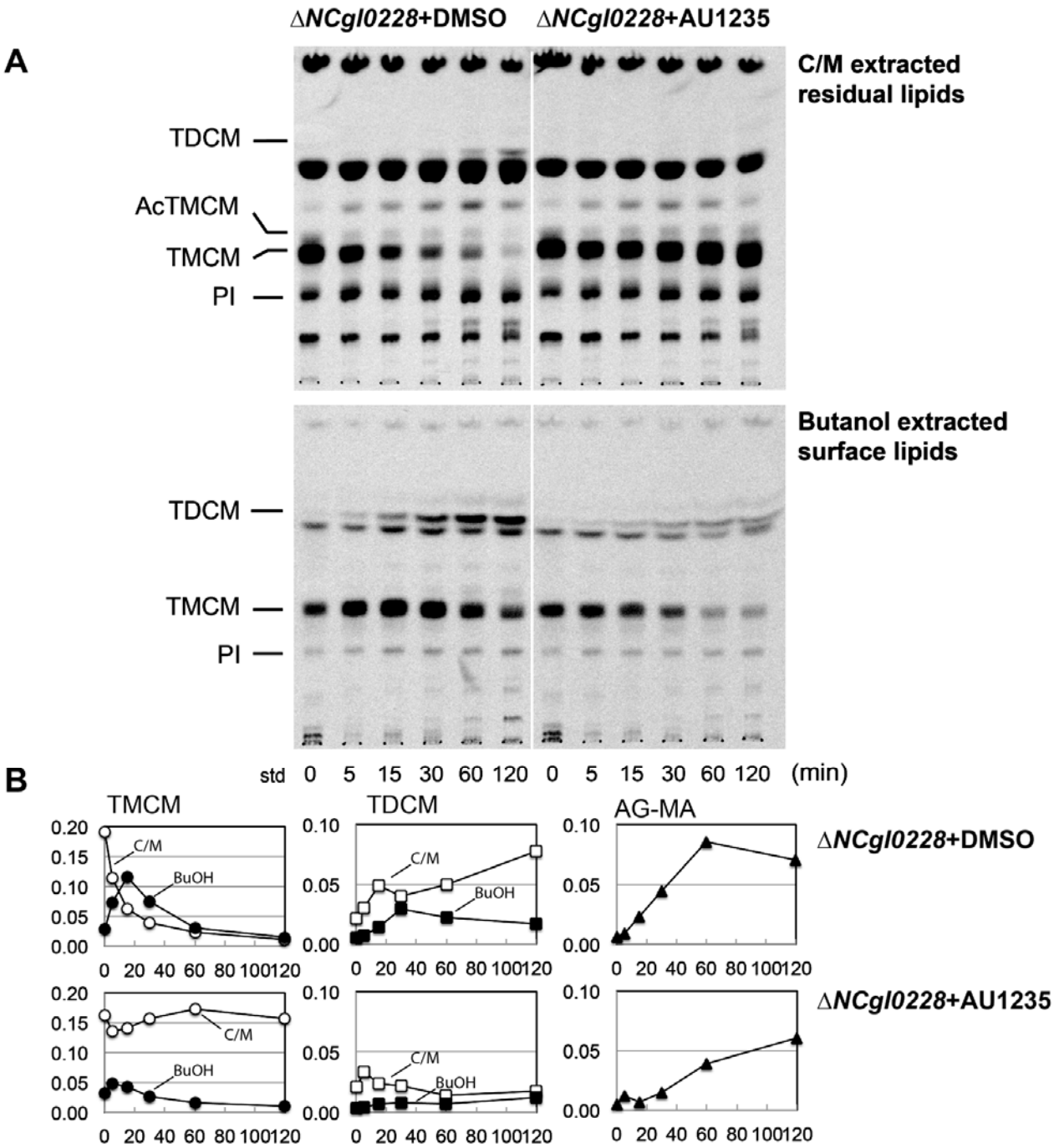


Figure 9

