The Reductase That Catalyzes Mycolic Motif Synthesis Is Required for Efficient Attachment of Mycolic Acids to Arabinogalactan*

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Mycolic acids are essential components of the cell walls of bacteria belonging to the suborder Corynebacterineae, including the important human pathogens Mycobacterium tuberculosis and Mycobacterium leprae. Mycolic acid biosynthesis is complex and the target of several frontline antimycobacterial drugs. The condensation of two fatty acids to form a 2-alkyl-3-keto mycolate precursor and the subsequent reduction of this precursor represent two key and highly conserved steps in this pathway. Although the enzyme catalyzing the condensation step has recently been identified, little is known about the putative reductase. Using an extensive bioinformatic comparison of the genomes of M. tuberculosis and Corynebacterium glutamicum, we identified NCgl2385, the orthologue of Rv2509 in M. tuberculosis, as a potential reductase candidate. Deletion of the gene in C. glutamicum resulted in a slow growing strain that was deficient in arabinogalactan-linked mycolates and synthesized abnormal forms of the mycolate-containing glycolipids trehalose dicorynomycolate and trehalose monocorynomycolate. Analysis of the native and acetylated trehalose glycolipids by MALDI-TOF mass spectrometry indicated that these novel glycolipids contained an unreduced β-keto ester. This was confirmed by analysis of sodium borodeuteride-reduced mycolic acids by gas chromatography mass spectrometry. Reintroduction of the NCgl2385 gene into the mutant restored the transfer of mature mycolic acids to both the trehalose glycolipids and cell wall arabinogalactan. These data indicate that NCgl2385, which we have designated CmrA, is essential for the production of mature trehalose mycolates and subsequent covalent attachment of mycolic acids onto the cell wall, thus representing a focus for future structural and pathogenicity studies.

Several species of the suborder Corynebacterineae are important human pathogens, most notably Mycobacterium tuberculosis, the causative agent of tuberculosis (TB). Despite intensive efforts to control the disease, TB kills approximately 2 million people per year (1). The prevalence of drug-resistant strains, including the recently identified extremely drug-resistant strains, has been labeled a global health emergency, underlying the need for new anti-mycobacterial therapies. Several current TB drugs target enzymes that synthesize the unique cell wall, an important bacterial virulence factor. The core of the cell wall consists of type 4 peptidoglycan covalently linked to arabinogalactan (AG), a polysaccharide of arabinose and galactose sugars which is, in turn, covalently linked to long chain 2-alkyl and 3-hydroxy fatty acids, the mycolic acids (2). These molecules vary in length from C60 to C90 in mycobacteria, but the equivalent species in corynebacteria, the corynomycolates, are significantly smaller (C22–C36) (3). The covalently bound mycolates form the inner leaflet of an outer lipid bilayer with a number of noncovalently bound glycolipids, including trehalose monomycolate (TMM) and trehalose dimycolate (TDM) (2), forming the outer leaflet. The corynebacterial equivalents, trehalose monocorynomycolate (TMCM) and trehalose dicorynomycolate (TDCM), have identical structures except that they contain the shorter corynomycolates (4). TDM, also known as cord factor, is a well established immunostimulatory compound with toxic properties (5, 6). The outer lipid bilayer forms the main permeability barrier around Corynebacterineae and is the major factor in the natural resistance of these bacteria to many antibiotics, the intracellular environment of the macrophage, and environmental stresses (7).

The structure and biosynthesis of mycolic acids have been intensively studied because of their functional importance, uniqueness, and the finding that many drugs, including the front line TB drug isoniazid, target several enzymes involved in their biosynthesis (8). Although the chain length of mycolic acids varies between Corynebacterineae, many chemical features of the mycolate molecules are conserved, most notably the mycolic motif (Fig. 1A). Several of the enzymes involved in

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4 The abbreviations used are: TB, tuberculosis; GC-MS, gas chromatography-mass spectrometry; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; AG, arabinogalactan; TMM, trehalose monomycolate; TMCM, trehalose monocorynomycolate; TDM, trehalose dimycolate; TDCM, trehalose dicorynomycolate; HPTLC, high performance thin layer chromatography; AcPIM2, acylated phosphatidylinositol dimannoside; for, forward; rev, reverse.
early steps of mycolic acid biosynthesis have recently been identified in Corynebacterium glutamicum, a species that can tolerate the loss of mycolic acids allowing the characterization of viable mutants that cannot be derived in mycobacteria. In a recent important study, the polyketide synthase Pks13 was identified as the condensase required for condensation of the two fatty acids to form 2-alkyl, 3-keto mycolates (11). All of these genes are essential for life in M. tuberculosis (12), highlighting the importance of the pathway in the human pathogen. Mature mycolates generated by this pathway are thought to be lighting the importance of the pathway in the human pathogen.

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**Bioinformatics Analysis**—Identification of the conserved mycolate reductase and other conserved cell wall genes was undertaken by performing a FASTA BLAST comparison of every gene found in the C. glutamicum ATCC 13032 Kitasato gene list located at the TIGR comprehensive microbial resource against the M. tuberculosis H37Rv genome using the FASTA sequence analysis program. E values and best matches in the M. tuberculosis genome were assigned based on these results. Amino acid similarity percentage values were assigned according to the results found in the protein versus all alignment menu located for each gene in the aforementioned C. glutamicum ATCC 13032 Kitasato gene list. Sequences were compared utilizing ClustalW.

**Strains and Culture Conditions**—E. coli DH5α was cultured in Luria-Bertani media at 37 °C. All C. glutamicum strains, including the wild type strain ATCC 13032, were grown in BHI media (Oxoid) at 30 °C. 15 g/liter agar was used for preparation of solid media, and 10% sucrose (w/v) was added when necessary. C. glutamicum electrocompetent cells were prepared according to a protocol (23) except that electroporation was performed at the following settings: 2.5 kV, 200 ohms, and 25 microfarads. Kanamycin (30 μg/ml) and ampicillin (100 μg/ml) were added to media when necessary.

**Construction of the ΔNCgl2385 C. glutamicum Mutant and Complementation Plasmid**—The entire NCgl2385 gene and flanking regions were amplified by PCR as a 2.28-kb fragment using Proofstart DNA polymerase (Qiagen) and the primers NCgl2385for (5'-CCCGAATTCCGGGCGGAAGAATTAGG-3') and NCgl2385rev (5'-TGCGGATCCGTAGGACCAGGCTCTGCAAC-3'), according to the manufacturer's instructions, and cloned into the BamHI/EcoRI sites (underlined) of pUC18 (24). A 344-bp sequence internal to the gene was removed by Xhol/DralIII digestion, and the remaining sequence was religated and subcloned into the BamHI/EcoRI sites of the suicide plasmid pK18mobsacB (25). Following electroporation, kanamycin-resistant transformants were tested for the occurrence of single homologous recombination events by Southern blot hybridization using a probe prepared using the digoxigenin hybridization kit (Roche Applied Science). A verified single crossover clone was cultured on BHI plates containing 10% sucrose to select for double homologous recombination events. Deletion of the gene was screened by PCR using the primers NCgl2385for and NCgl2385rev and then confirmed by Southern blot hybridization analysis. One confirmed deletion strain, designated ΔNCgl2385, was selected for further analysis. Complementation of the mutant was performed by inserting the entire NCgl2385 gene in addition to 241 bp of
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upstream sequence and 109 bp of downstream sequence into the unique PvuII site of plasmid pSM22, which carries the Corynebacterium origin of replication repA and the kanamycin resistance gene aphA3 (26), creating pSM22::NCgl2385. The complementation plasmid and empty pSM22 plasmid control were then electroprotopated into C. glutamicum ΔNCgl2385 and transformants selected on kanamycin plates.

Extraction and Analysis of Cell Wall Lipids—All strains were grown to exponential phase (A\text{600 nm} = 7). Cell wall glycolipids were extracted as described previously (27). Briefly, PIMs and trehalose mycolates were extracted in chloroform:methanol (2:1 v/v) and chloroform:methanol:water (1:2:0.8 v/v). More polar components, such as LM/LAMs, were extracted from the delipidated pellet by reflux in 50% ethanol and AG-linked mycolates released from the resultant pellet with 0.1 M potassium hydroxide (2 ml, 48 h, 25 °C) (28). Extracted glycolipids were analyzed on Silica Gel 60 HPTLC plates (Merck) developed in chloroform, methanol, 13M NH3, 1 M ammonium acetate, water (180:140:9:9:23, v/v) and detected with orcinol-H\text{2SO}_{4} (28). The LM/LAM and mycolate fractions were analyzed after methanolysis, trimethylsilyl derivatization, and GC-MS, as described below. Arabinogalactan levels were determined by subjecting the delipidated, base-treated cell pellets to solvolysis in methanolic HCI and GC-MS analysis of the released methylglycosides and corynomycolate methyl esters.

MALDI-TOF Analysis of Trehalose Glycolipids—Trehalose glycolipids were purified by HPTLC, and silica scrapings were extracted in chloroform:methanol:water (1:2:0.8 v/v). The extracts were dried and glycolipids recovered by biphasic partitioning in 1-butanol:water (1:1 v/v). Purified glycolipids (∼1 nmol) were analyzed directly or after O-acetylation by MALDI-TOF mass spectrometry. MALDI-TOF spectra were acquired on a Voyager-DE STR mass spectrometer (PerSeptive Biosystems) in the reflector mode. Native and acetylated samples (1 μl) were dissolved in 1-butanol and loaded onto a metal plate with 1 μl of matrix saturated α-cyano-4-hydroxycinnamic acid (Sigma) in 60% 1-propanol and 0.1% trifluoroacetic acid (Pierce). Mass spectra were collected in positive ion mode using an extraction delay time set at 100 ns and an accelerating voltage of 25 °C. Isogalactotrihexosylceramide was used as an external standard (Sapphire Bioscience). Purified glycolipids were O-acetylated in 200 μl of pyridine:acetic anhydride (1:1 v/v) for 2 h at 80 °C. Solvents were removed under vacuum prior to analysis by MALDI-TOF.

Gas Chromatography-Mass Spectrometry of Cell Wall Components—Cell wall extracts and purified trehalose glycolipids were subjected to solvolysis in 0.5 M methanolic HCl (50 μl, 80 °C, 16 h). Samples were neutralized with pyridine (10 μl), dried under vacuum, and the released methyl glycosides and corynomycolates derivatized in N-methyl-N-(trimethylsilyl) trifluoroacetamide + 1% trichloromethylsilane (50 μl; Pierce) for 1 h at 25 °C. Derivatized samples were analyzed by GC-TOF-MS (Agilent GC 6890N interfaced with a Leco PegasusII TOF). GC was performed with a DB5-MS DG column (30 m × 0.25 mm inner diameter, 250 μm film thickness) and the following oven temperature program: 1 min at 70 °C followed by a 12.5 °C/min temperature ramp to 295 °C and then a 25 °C/min ramp to 320 °C. Electron impact ionization was performed at 70 eV, an ion source temperature of 250 °C, and mass spectra were collected from 50 to 550 m/z. In some cases, the trehalose glycolipids were reduced with NaB\text{3H}_{4} prior to methanolysis. Glycolipids were resuspended in 50% 1-propanol containing NaB\text{3H}_{4} (20 mg/ml) for 2 h at 4 °C. Samples were acidified with glacial acetic acid and dried, and reduced glycolipids were recovered by phase partitioning in 1-butanol:water (1:2 v/v).

For head group analysis, HPTLC-purified glycolipids were treated with base (0.1 M NaOH in methanol, 37 °C, 2 h) and the glycan moieties recovered in the aqueous phase after 1-butanol:water phase partitioning. The glycan fraction was desalted on a mixed bed ion exchange resin (AG-50-X50 (H\text{+}))/AG3 X12 (OH\text{−}), dried, and derivatized in N-methyl-N-(trimethylsilyl)trifluoroacetamide + 1% trichloromethylsilane (50 μl) for 1 h at 25 °C, prior to analysis by GC-MS.

RESULTS

Identification of New Cell Wall Biosynthesis Genes in Corynebacte- riaeae—Our identification of potential reductase candidates formed part of a more general strategy to identify conserved cell wall biosynthesis genes. Because of the striking similarities between the cell wall components of mycobacteria and corynebacteria, we hypothesized that the majority of cell wall biosynthesis processes would be conserved between both genera. To identify new genes involved in these processes, we comprehensively referenced every putative open reading frame in the C. glutamicum ATCC 13032 Kitasato genome against the annotated genome of M. tuberculosis H37Rv using the BLAST algorithm (14). Conserved genes of unknown function that are essential or result in slow growth phenotypes when mutated in M. tuberculosis, as determined by the transposon mutagenesis studies of Sassetti et al. (12), were considered to include candidates for cell wall biosynthesis genes of major structural components, because the vast majority of characterized cell wall genes are refractory to deletion or give rise to slow growing mutants when disrupted. Hence, we compacted the 2,993 predicted proteins of C. glutamicum to a list of only 83 that fulfilled these criteria.

We then narrowed our search to focus on potential candidates for the conserved reductase proposed to function late in mycolic acid biosynthesis. Of the 80 uncharacterized genes found in M. tuberculosis with putative reductase domains (15), only two essential genes, Rv0561 and Rv2073c, and one gene, Rv2509, which results in a slow growth phenotype upon deletion, were conserved in C. glutamicum. Rv2073c was considered an unlikely candidate because it is absent from the highly decayed genome of Mycobacterium leprae, a species that despite a much reduced genome still synthesizes mycolic acids, and despite repeated attempts we were unable to delete the C. glutamicum orthologue of Rv0561c, suggesting that it encodes an essential function. The remaining candidate, Rv2509, is highly conserved in all sequenced genomes of the Corynebacteriaeae, including M. leprae (16). The homologues of Rv2509 in M. leprae and Mycobacterium avium subspecies paratuber- culosis, and its orthologues in C. glutamicum and Corynebacterium diphtheriae, display 93, 94, 70, and 67% amino acid similarity, respectively, to the M. tuberculosis protein (Fig. 2). In the M. tuberculosis genome, Rv2509 is annotated as a 28-kDa puta-
tive short chain type dehydrogenase/reductase based on its amino acid sequence similarity to a range of reductases, the presence of an N-terminal TG\(\times\)G motif that represents a potential binding site for the cofactors NAD(H) or NADP(H), a putative active site YXXXL motif at residues 157–161, and the presence of a number of other residues highly conserved in the short chain type reductase family (Fig. 2).

**Deletion of NCgl2385 in C. glutamicum Results in a Slow Growth Phenotype**—To investigate whether Rv2509 is the reductase involved in mycolic motif formation, we deleted its orthologue in *C. glutamicum*, NCgl2385. A 344-bp segment internal to the gene was deleted using a two-step recombination strategy (see “Materials and Methods”). Potential mutants were screened

![FIGURE 2. ClustalW amino acid sequence alignment of *M. tuberculosis* Rv2509 and orthologues in *M. leprae* (ML0429), *M. avium* subspecies *paratuberculosis* (MAP_2318), *C. glutamicum* (NCgl2385), and *C. diphtheriae* (DIP1812). A putative binding site for the cofactor NAD(H) or NADP(H) is shown by the horizontal line, and the putative active site tyrosine is indicated by #. Residues highly conserved in other short chain dehydrogenases/reductases (29) are shown below the aligned sequences. Residues surrounded by black are completely conserved among the sequences, and residues surrounded by gray are similar, and residues surrounded in white are not conserved.

![FIGURE 3. Disruption of NCgl2385 and growth characteristics of the mutant and complementation strains. A, schematic demonstrating the genomic arrangement of wild type *C. glutamicum* (top) and NCgl2385 deletion (\(\Delta\)NCgl2385, bottom) strains, the location of ClaI sites, and the resultant fragments to be expected on Southern blot hybridization. NCgl2385f and NCgl2385rev denote the primers used to amplify the hybridization probe. NCgl2385 is shown as a filled arrow, and flanking genes are open arrows. The XhoI/DraIII fragment internal to NCgl2385 that was deleted to create \(\Delta\)NCgl2385 is shaded. B, Southern blot hybridization demonstrating disruption of NCgl2385. Lane 1, digoxigenin-labeled \(\Delta\)H9004 HindIII molecular weight DNA markers; lane 2, wild type *C. glutamicum* genomic DNA digested with ClaI; lane 3, \(\Delta\)H9004 NCgl2385 genomic DNA digested with ClaI. C, growth curves of wild type *C. glutamicum* (B), \(\Delta\)NCgl2385 (A), \(\Delta\)NCgl2385 + pSM22 (○), and \(\Delta\)NCgl2385 + pSM22-NCgl2385 (□). Measurements were taken from duplicate cultures. The growth curves of wild type and \(\Delta\)NCgl2385 + pSM22-NCgl2385 (upper curves) and \(\Delta\)NCgl2385 and \(\Delta\)NCgl2385 + pSM22 (lower curves) overlap almost exactly because of restoration of the growth rate by reintroduction of the gene to the mutant strain.
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first by PCR (data not shown), and disruption of the gene was confirmed by Southern blot hybridizations following digestions of genomic DNA with ClaI (Fig. 3, A and B). A probe derived from NCgl2385 hybridized to two fragments in the wild type parent strain of 1.2 and 1.7 kb in size and to one band in the mutant of 2.5 kb. This was the expected profile for a knock-out mutant because one ClaI site was located in the deleted portion of NCgl2385. The mutant grew as small colonies on solid media, and this slow growth phenotype was maintained in liquid media (Fig. 3C). Transformation of the ΔNCgl2385 mutant with the plasmid pSM22:NCgl2385, containing the entire NCgl2385 gene plus 241 bp of upstream sequence, restored a normal growth rate, whereas transformation with the empty pSM22 vector did not (Fig. 3C). The observed growth defect was consistent with the slow growth phenotype described for a M. tuberculosis Rv2509 knock-out strain (12).

The ΔNCgl2385 Mutant Produces Novel Forms of TDCM and TMCM and Is Deficient in Arabinogalactan-bound Corynomycolates—HPTLC analysis of noncovalently linked lipids and glycolipids extracted from wild type C. glutamicum and the ΔNCgl2385 mutant indicated that the two glycolipid species in the mutant, provisionally identified as modified TMCM and TDCM species (designated TMCM* and TDCM*), exhibited faster HPTLC mobilities than the wild type glycolipids (Fig. 4). The level of these faster migrating glycolipid species was also elevated in the mutant compared with wild type cells (Fig. 4). In contrast, the HPTLC mobility and levels of other glycolipids, including AcPIM2 and a novel species that may correspond to the recently characterized GlcA-diacylglycerol (32), were unchanged (Fig. 4). To confirm that the faster migrating glycolipid species in the mutant were indeed distinct forms of TMCM and TDCM, these species were purified by HPTLC and subjected to compositional and MALDI-TOF analysis. Monosaccharide analysis showed that TMCM/TDCM from wild type cells and TMCM*/TDCM* from the ΔNCgl2385 mutant contained glucose as the only monosaccharide constituent. After mild base treatment and analysis of the released glycan head group by GC-MS, all species were found to contain comparable levels of trehalose (data not shown). When TMCM and TDCM from wild type cells were analyzed by MALDI-TOF in positive ion mode, a series of sodiated molecular ions were identical consistent with the presence of molecular species of TMCM and TDCM containing C32:0, C34:1, and C36:1 corynomycolates (Fig. 5). The corynomycolate composition was confirmed by GC-MS analysis (Fig. 6 and Fig. 8). MALDI-TOF-MS analysis of the TMCM* and TDCM* species from the ΔNCgl2385 mutant revealed a similar cluster of sodiated molecular ions, although the mass of these species were 2 or 4 atomic mass units smaller than authentic TMCM and TDCM, respectively (Fig. 5). These mass differences could reflect an additional degree of unsaturation in the trehalose glycolipids of ΔNCgl2385 or the presence of an unreduced β-keto ester motif (see below). Following complementation of the ΔNCgl2385 mutant with a plasmid containing the NCgl2385 gene, the HPTLC mobility of trehalose glycolipids and levels of expres-
Characterization of Specific Defect in Corynomycolate Biosynthesis in the ΔNCgl2385 Mutant—To define whether the defect in mycolic acid biosynthesis in ΔNCgl2385 reflects increased fatty acid desaturation or the absence of a reductase activity, the trehalose glycolipids isolated from the wild type C. glutamicum and the ΔNCgl2385 mutant were O-acetylated and reanalyzed by MALDI-TOF-MS. As expected, this treatment resulted in an increase in the mass of authentic TDCM by 336 atomic mass units (equivalent to 8 OAc), because of the acetylation of the six free hydroxyls in the trehalose head group and the two hydroxyl groups on the corynomycolic acids (Fig. 7). In contrast, the mass of acetylated TDCM* only increased by 252 atomic mass units compared with the underivatized species, indicating the presence of only six free hydroxyl groups (Fig. 7). As TDCM and TDCM* contain the same glycan head group, these results suggest that TDCM* is exclusively modified with corynomycolates containing the –keto ester rather than mature hydroxylated corynomycolates. Similarly, MALDI-TOF analysis of O-acetylated TMCM and TMCM* indicated the addition of 8 and 7 acetyl groups, respectively (data not shown). Collectively these data suggest that the ΔNCgl2385 mutant lacks the capacity to synthesize mature corynomycolates.

To obtain further evidence that the ΔNCgl2385 corynomycolates retain the –keto group, TDCM and TDCM* were reduced with sodium borodeuteride and the attached fatty acids subsequently released and analyzed by GC-MS (Fig. 8A). Sodium borodeuteride reduction should have no effect on mature TDCM-linked corynomycolates but will reduce the –keto group in TMCM*-linked corynomycolates, with incorporation of two deuterium atoms (one of which will subsequently be displaced by a trimethylsilyl group during derivatization). As shown in Fig. 8B, the major ions at m/z 313 and 371 in the mass spectrum of the mature C32:0 corynomycolate methyl ester are because of fragmentation on either side of the trimethylsilyl-modified hydroxyl group (30, 31). A diagnostic ion at m/z 342 results from secondary fragmentation of m/z 371 because of loss of the CHO (29 atomic mass units) (31). As expected, the mass spectrum of this corynomycolate methyl ester was not altered by reduction (data not shown). However, sodium borodeuteride reduction of TDCM* generated corynomycolic acids with the same GC retention times as wild type corynomycolic acids (data not shown), and the mass spectrum of the major methyl ester indicated the concomitant incorpo-
ration of a deuterium atom (Fig. 8C). Specifically, both of the major fragment ions were increased by 1 atomic mass unit locating the deuterium on the hydroxylated carbon. This was further confirmed by the fact that the secondary fragment ion, m/z 342, was unchanged, indicating that the silylated hydroxyl was linked to a deuterated carbon (Fig. 8C). These data confirm the structure of TDCM* to be that shown in Fig. 8D. Similar results were obtained when the corynomycolic acids of reduced TMCM* were analyzed (data not shown). Collectively, these data demonstrate that all the corynomycolic acids of the ΔNCgl2385 mutant contain a free keto group and that the mutant is therefore deficient in corynomycolic acid reductase activity.

DISCUSSION

It has previously been established that mycolic acids are formed by the condensation of two fatty acids followed by reduction of the β-keto ester motif to form the final β-hydroxy acid (17, 18). The recent discovery that Pks13 catalyzes the condensation reaction (11) and that a series of enzymes are involved in activation and carboxylation of fatty acids prior to this step (9, 10) has significantly increased our understanding of the synthesis of these important cell wall constituents. However, identification of the enzyme catalyzing the final step of mycolic acid formation, the reduction of the β-keto ester motif, has remained elusive, in part because of the large number of putative reductases in mycobacterial genomes.

In this study we have provided strong evidence that NCgl2385 is involved in generating the mycolic motif in C. glutamicum. Specifically, MALDI-TOF-MS and GC-MS analysis of the novel trehalose glycolipids and arabinogalactan-bound mycolic acids of the ΔNCgl2385 mutant demonstrated that all the mycolates of this mutant retain the β-keto ester motif. Re-expression of NCgl2385 in this mutant completely restored the synthesis of mature corynomycolates. The NCgl2385 gene is highly conserved in all sequenced genomes of members of Corynebacterineae and is likely to encode the same activity in all species, including the human pathogens M. tuberculosis, M. leprae, and Mycobacterium ulcerans. We propose that NCgl2385 is orthologous to Rv2509 from M. tuberculosis based on the strong amino acid similarity between the two predicted proteins (Fig. 2) and synteny between the regions in which the

![FIGURE 7. MALDI-TOF-MS analysis of O-acetylated TDCM.](image)

![FIGURE 8. GC-MS analysis of corynomycolates synthesized by C. glutamicum wild type and C. glutamicum ΔNCgl2385.](image)
two genes are found. Despite NCgI2385 being located in a locus generally dominated by corynebacterium-specific genes that are absent from mycobacterial genomes, its neighbor, NCgI2386, encodes a predicted protein sharing 74% amino acid similarity and 60% identity with the putative ribonuclease encoded by Rv2511. Rv2510c, a conserved hypothetical gene absent from all available corynebacterial genomes, splits Rv2509 and Rv2511 in M. tuberculosis. Thus, we have designated the orthologous genes NCgI2385 and Rv2509 as cmrA, for Corynebacterineae mycolate reductase A.

Deletion of cmrA in C. glutamicum resulted in elevated levels of TDCM and TMCM, but a marked decrease in the transfer of mycolates to cell wall arabinogalactan. This is the first reported case of a mutant lacking most AG-linked mycolates but retaining TDCM and TMCM. Our findings suggest that the reduction of the β-keto ester motif is important for subsequent mycoloyl-transfer reactions to AG but not for mycoloyl-transfer reactions that lead to the formation of TMC much or TDCM. Therefore, we propose that CmrA is highly conserved throughout the Corynebacterineae primarily because its activity is necessary for efficient formation of mycoloyl-AG. Currently, at least five mycoloyltransferases are known to exist in C. glutamicum (19) and three in M. tuberculosis (20), with a degree of functional redundancy occurring between the enzymes. Our data strongly suggest that the two types of transfer reaction differ in their dependence on the mycolate motif. One possible explanation is that the presence of the carboxyl group either (a) blocks recognition/binding of the transferases responsible for attaching mycolates to AG or (b) interferes with the catalytic mechanisms of these transferases, without affecting the transferases that form TDCM from TMCM. The work of De Sousa-D’Auria et al. (19) showed that the mycoloyltransferases from C. glutamicum differ in their substrate specificities with cMytA, cMytB, cMytD, and cMytF able to transfer a mycoloyl residue from one TMCM to another to form TDCM but only cMytA and cMytB capable of transferring mycoloyl residues to AG. Combining this information with our data, we suggest that the activities of cMytA and cMytB are blocked by the presence of the unreduced β-keto ester, whereas cMytD and/or cMytF remain unaffected.

It would be interesting to determine whether the same phenotype appears following disruption of the orthologous gene in mycobacteria. We have very recently isolated a transposon mutant of the orthologue of Rv2509 in M. smegmatis, and preliminary studies indicate that strain has a similar phenotype to the M. tuberculosis ΔNCgI2385 mutant. TDM and TMM extracted from the mutant have altered mobility on HPTLC plates; levels of AG-linked mycolic acids are reduced, and a growth defect has been observed (data not shown). Further studies will involve confirmation of these mycolipid defects, complementation of the transposon mutant with Rv2509 and its orthologues, and intracellular survival assays to assess the importance of AG-linked mycolates in vivo.

The lack of an obvious signal sequence or transmembrane domains suggests a cytosolic location for CmrA, although signal peptides are poorly defined in Corynebacterineae. Characterization of the mycoloyl-mannosylphosphopolyprenol carrier in M. smegmatis (MycPL) involved in transport of mycolates out of the cell demonstrated that the mycolic acids contained the mycolate motif, not the unreduced β-keto ester motif (21). A similar but highly unstable phospholipid was identified in C. diphtheriae, also containing a reduced mycolic motif (22). Together, this evidence is in agreement with an earlier model suggested by Portevin et al. (9, 11) and Takayama et al. (13), who proposed that reduction takes place in the cytosol while the mycolate is esterified to Pks13. A later model by Tropis et al. (4) proposed an extracellular location for the reduction reaction, following attachment of a mycolate to trehalose, which is not supported by the data presented here.

Future studies will focus on the structural characterization of CmrA and its interaction with mycolic acid intermediates, with the ultimate aim of rational inhibitor design. The strong sequence conservation between all orthologues suggests that the enzyme fulfills similar roles in mycobacteria; hence it is highly likely that CmrA is necessary for efficient esterification of AG with mycolic acids in devastating human pathogens like M. tuberculosis and M. leprae. Despite being nonessential in M. tuberculosis, targeting of CmrA with specific drugs would be expected to affect the intracellular growth and survival of the pathogen, making this enzyme an attractive target for future drug development.

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