



A GMC Oxidoreductase Homologue Is Required for Acetylation of Glycopeptidolipid in *Mycobacterium smegmatis*

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Supporting Information

ABSTRACT: The *Mycobacterium tuberculosis* Rv3409c gene is required for modulation of the Toll-like receptor 2 (TLR-2) signaling response in infected macrophages. Although each is annotated as encoding a cholesterol oxidase, neither Rv3409c nor its ortholog MSMEG1604 is required for the metabolism of cholesterol in mycobacteria. Here we report that a unique lipid, L1334, accumulates in a MSMEG1604 transposon mutant in the *Mycobacterium smegmatis* cell envelope. L1334 is a polar glycopeptidolipid that is hyperphosphorylated and in which the 6-deoxytalose moiety is not acetylated. The alteration of L1334 acetylation is consistent with a reduced level of interference with TLR-2 signaling in mutant infected macrophages.

Recently, the ability of mycobacteria to catabolize cholesterol has been the subject of intense study because of a requirement for cholesterol metabolism in models of *Mycobacterium tuberculosis* persistence in infection.^{1,2} *M. tuberculosis* Rv3409c and its *Mycobacterium smegmatis* ortholog MSMEG1604 were proposed to function in sterol metabolism on the basis of their similarities to well-characterized cholesterol oxidases, ChoEs,³ which catalyze the conversion of cholesterol to cholest-4-en-3-one. Interestingly, disruption of the Rv3409c gene in *M. tuberculosis* leads to attenuated survival in macrophages and in the mouse model of infection.⁴ Rv3409c contributes to inhibition of the bactericidal activity of macrophages by interfering with Toll-like receptor 2 (TLR-2)-mediated signaling, thereby enhancing interleukin-10 production and suppressing NO production.⁵

In *Rhodococcus equi* and *Streptomyces*, the cholesterol oxidase *choE* genes are required for catabolism of cholesterol as a sole carbon source. However, gene disruption studies of MSMEG1604 in *M. smegmatis* and Rv3409c in *M. tuberculosis* demonstrated that this gene is not required by either mycobacterium species to mineralize cholesterol.^{6–9} Furthermore, assays to directly detect cholestenone formation with purified protein or lysates containing expressed MSMEG1604 found no evidence of product formation,^{6,7} despite reports that cell lysates containing expressed Rv3409c and cholesterol produce hydrogen peroxide.^{4,10} Both Rv3409c and MSMEG1604 share a low level of amino acid identity (~24%) with experimentally characterized cholesterol oxidases (*choE*) from *R. equi* and *Streptomyces* sp.⁷ as well as other glucose–methanol–choline (GMC) oxidoreductase superfamily members;¹¹ the conserved amino acids are predominantly in

the FAD binding region. The low level of identity and similarity between substrate binding amino acids and the absence of a cholesterol catabolism phenotype and cholesterol oxidation catalytic activity lead to the conclusion that the gene function has been misassigned.

Fortuitously, in a phenotypic screen to identify genes associated with glycopeptidolipid (GPL) production, Reyrat and co-workers isolated an MSMEG1604 mutant Myc11.^{12,a} The colony morphology of Myc11 changed from smooth to rough, although mutant colonies were still stained by the hydrophobic dye phenol red. On the basis of the cell wall morphology phenotype, we undertook differential mass spectrometric profiling of the mutant lipidome as a first step in understanding the biochemical function of MSMEG1604 and its orthologs.

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectral analysis revealed that the majority of the lipids produced by wild type (WT) and Myc11 were the same. However, the relative amounts of the GPL species were reduced in Myc11 (Figure S1 of the Supporting Information). Moreover, a relatively abundant new lipid appeared in the Myc11 mass spectral profile (Figure 1). The primary molecular ion for the new lipid is at 1334 Da, and the lipid is henceforth termed L1334.

Two complementation strains were constructed [Myc11:pMs100 and Myc11:pMs101 (Tables S1 and S2 of the Supporting Information)]. Complementation of Myc11 with MSMEG1604 abrogated production of L1334, and the WT GPL profile was restored (Figure 1 and Figure S1 of the Supporting Information). Moreover, complementation restored the red, smooth phenotype. Single colonies of WT *M. smegmatis* mc²155 and Myc11 grew to a 1 cm diameter after 2 weeks at 37 °C with glycerol as the carbon source. Single colonies of complemented Myc11 were 80–90% the size of WT colonies, suggesting that the constructs introduced some additional metabolic burden.

On the basis of the apparent differences in GPL species between WT and Myc11, we performed thin-layer chromatography (TLC) analysis of native GPLs isolated without alkali treatment, which deacetylates sugar hydroxyls. The TLC analysis again indicated that a unique lipid accumulated in Myc11 (Figure S2A of the Supporting Information). The polarity of this lipid was slightly higher than that of native GPLs isolated from WT *M. smegmatis* without alkali treatment.

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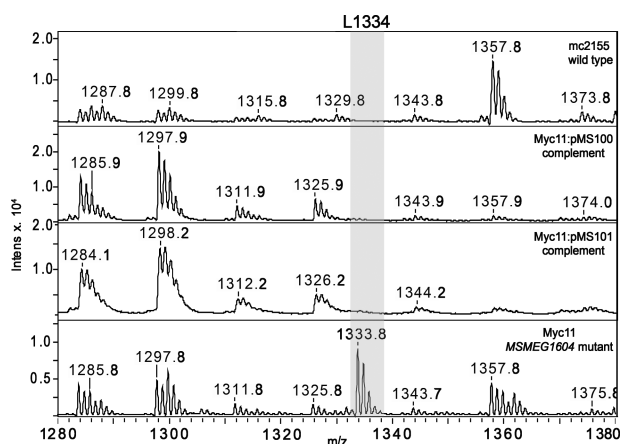


Figure 1. Enlarged region of the MALDI-TOF mass spectra of total lipids of *M. smegmatis* mc2155, the Myc11:pMS100 complement, the Myc11:pMS101 complement, and Myc11. The full spectra are shown in Figure S1 of the Supporting Information.

Neither Myc11:pMS100 nor Myc11:pMS101 produced this unique lipid, and the native GPL distribution was almost completely restored in the complemented strains, although the relative amounts were not identical to that of the wild type.

L1334 was purified to near homogeneity using silica column chromatography. The exact mass of L1334 is 1333.8617 Da (3 ppm tolerance). Consistent with a lipid structure, additional species were observed that differ from the molecular mass of L1334 by multiples of 28 Da, corresponding to a CH_2CH_2 moiety, i.e., at m/z 1305.8, 1362.1, and 1390.8 Da.

Myc11 was grown in minimal medium constituted with ^{15}N -labeled NH_4Cl . Comparison of the ^{15}N -labeled WT and ^{15}N -labeled Myc11 mass spectral profiles revealed a lipid in ^{15}N -labeled Myc11 with a molecular ion $[\text{M} + \text{Na}]^+$ at 1338 Da and loss of the 1334 Da molecular ion. Isolation of ^{15}N -labeled L1334 by preparative TLC confirmed the m/z of the molecular ion to be 1338.7 Da (Figure S2B of the Supporting Information). Therefore, on the basis of the exact mass and four nitrogen atoms, the molecular formula for L1334 was assigned as $\text{C}_{69}\text{H}_{122}\text{N}_4\text{O}_{19}\text{Na}$ (calcd $[\text{M} + \text{Na}]^+$, 1333.8601 Da; observed $[\text{M} + \text{Na}]^+$, 1333.8617 Da).

The molecular formula in combination with the TLC polarity data suggested that L1334 is a GPL derivative. We analyzed the composition of extracted total lipids from the WT and Myc11 strains with and without alkali treatment by TLC. L1334 is stable to alkali treatment, consistent with the absence of acetyl groups in its isolated form. Moreover, L1334 has the same polarity as deacetylated GPL-5 (Figure S2C,D of the Supporting Information), which also has a mass of 1334 Da (MNa^+). On the basis of the molecular formula, we proposed the deacetylated GPL-5 structure shown in Figure 2A.

The mass spectral fragmentation of L1334 revealed a loss of 164 Da from the parent ion ($[\text{M} + \text{Na}]^+ = 1333.86$ Da) that yielded a daughter ion at m/z 1169.79 Da (Figure S3 of the Supporting Information). A loss of 160 Da from this daughter yielded a MS^3 ion at m/z 1009.71 Da. This ion was further fragmented with a loss of 174 Da to yield a MS^4 ion at m/z 835.62 Da. The three neutral losses of 164, 160, and 174 Da were attributed to the loss of three sugar residues from the parent molecule. Notably, there was no loss of 174 Da from the 1169.79 Da daughter ion, confirming the assignment of the 160/174 disaccharide moiety. In addition, each of the MS^2 , MS^3 , and MS^4 ions loses neutral fragments corresponding to

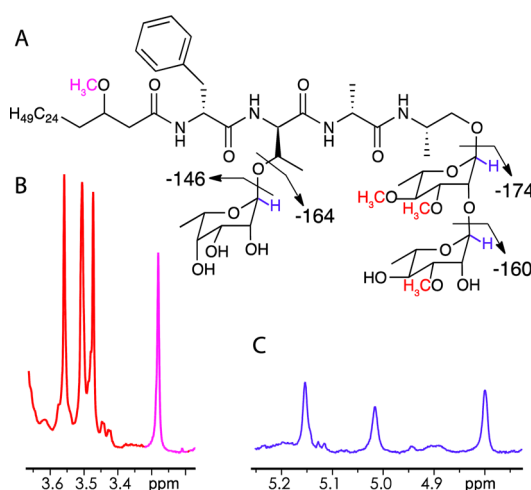


Figure 2. (A) Proposed structure of L1334. Arrows denote fragments lost in MS^n experiments shown in Figure S3 of the Supporting Information. Regions of ^1H nuclear magnetic resonance spectra corresponding to (B) four methyl ether and (C) three sugar anomeric hydrogen resonances.

loss of water and CH_3OH (Table S3 of the Supporting Information). These fragments indicate that the L1334 structure contains methoxy ethers. Moreover, no loss of acetoxy was observed, indicating the absence of acetylated residues in the D-allo-6-deoxytalose. The regiochemistry of methylation and dirhamnosylation is based on comparison to that of previously characterized *M. smegmatis* deacetylated GPL-5 that migrates like L1334 via TLC (Figure S2C of the Supporting Information).^{13–15}

The ^1H nuclear magnetic resonance (NMR) spectrum of L1334 is consistent with that of a GPL structure: (a) a tetrapeptide sequence, which includes phenylalanine, threonine, alanine, and alaninol; (b) a long fatty acyl chain; (c) three sugar residues; and (d) methyl ethers (Figure 2B,C). No acetyl resonances were observed. The NMR spectra confirmed the deacetylated, trisaccharide glycopeptidolipid structure of L1334.

Myc11 produces lipid L1334 in the late stationary phase. A similar hyperglycosylated GPL is formed in *M. smegmatis* under carbon starvation conditions, although the sugars are acetylated normally.^{14,15} Thus, the production of hyperglycosylated GPL is linked to nutrient availability.

GPLs are the major surface-exposed lipid components in *M. smegmatis*^{16–18} and affect colony morphology, sliding motility, and biofilm formation.^{19,20} Therefore, loss of acetylation is consistent with the Myc11 rough phenotype. The acetyltransferase responsible for acetylation of GPL is Atf1.²¹ However, the *MSMEG1604* gene is not located in the GPL biosynthetic gene cluster.²²

We assayed the Rv3409c protein with a variety of alcohol substrates, including cholesterol,⁶ methanol, ethanol, pentan-1-ol, choline, glucose, myoinositol, 2-phenylethanol, benzyl alcohol, rhamnose, 6-deoxytalose, and L1334. No enzymatic activity was detected with any of these substrates. Our data suggest that *MSMEG1604* and its orthologs encode a new member of the GMC oxidoreductase superfamily with an as yet unidentified substrate.

GPLs isolated from pathogenic non-tuberculosis mycobacteria, e.g., *Mycobacterium avium* and *Mycobacterium intracellulare*, stimulate the macrophage inflammatory response through TLR-2.²³ Acetylated GPL is required for this

stimulation,²⁴ and the number and pattern of *O*-acetyl groups on the 6-deoxytalose of GPL are important for TLR-2 signaling.^{24,25} Moreover, deacetylated GPLs are biologically inactive.²⁵ Similarly, lippomannan acetylation affects TLR-2 agonist activity in *M. tuberculosis*.²⁶

It has recently been shown that *M. tuberculosis* Rv3409c is indispensable for the modulation of TLR-2-mediated signaling.⁵ Disruption of MSMEG1604 leads to an alteration of GPL production in *M. smegmatis* and to the formation of an unusual GPL species that is nonacetylated and hyperphosphorylated. Our data suggest that Rv3409c controls acetylation of cell surface glycolipoproteins to modulate the host innate immune response.

■ ASSOCIATED CONTENT

■ Supporting Information

Materials and methods, three tables, and three figures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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■ ADDITIONAL NOTE

^aThe ORFs in the *M. smegmatis* genome were renumbered after 2005 by TIGR. Hence, the MSMEG1604 gene mentioned in this work was originally numbered MSMEG1603 as described by Sonden et al.¹²

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