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In vitro and in vivo characterization of a *Mycobacterium tuberculosis* mutant deficient in glycosyltransferase Rv1500

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Abstract

In *Mycobacterium marinum*, the homologue of Rv1500 of *M. tuberculosis* encodes a glycosyltransferase. Initially, it was suggested that this gene is involved in the synthesis of phosphatidylinositol mannosides (PIMs), generating Ac_2PIM_7 from Ac_2PIM_5. Phosphatidylinositol mannoside and its related compounds lipomannan (LM) and lipoarabinomannan (LAM) have been shown to modulate the host response to an infection with *M. tuberculosis*. Here, we generated a deletion mutant of Rv1500 in *M. tuberculosis* H37Rv, and analyzed the mutant using a biochemical approach as well as in vitro and in vivo infection models. Inactivation of Rv1500 did not lead to an altered expression pattern of PIMs in *M. tuberculosis* H37Rv. We found phosphatidylinositol (PI), PIM_2, $AcPIM_2$, Ac_2PIM_2, and $AcPIM_6$ in both strains, but were unable to detect Ac_2PIM_7 or Ac_2PIM_5 either in the wild type or the mutant strain. Uptake and growth of H37Rv and Rv1500 mutant strains in murine bone marrow-derived macrophages was identical, and TNFα and IL-12p40 production in mouse macrophages and dendritic cells was induced to similar levels following infection with either strain. Aerosol challenge of mice showed that wild type and Rv1500 mutant strains had identical growth rates in infected organs over time. We verified mRNA expression of Rv1500 in H37Rv and conclude that Rv1500 must serve a redundant role in viability and virulence of *M. tuberculosis*.
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Keywords: *Mycobacterium tuberculosis*; Rv1500 deletion mutant; PIM synthesis; Lipid analysis; In vitro and in vivo characterization

Introduction

The infection with *Mycobacterium tuberculosis*, the agent of tuberculosis, causes approximately 1.6 million deaths each year (World Health Organisation, 2007). One third of the world's population is afflicted with latent infection. The individual risk to develop active disease amounts to about 10% over a lifespan (Rook et al., 2005).

Cell wall components are crucial virulence determinants of *M. tuberculosis*. They have been shown to play an important role in host–pathogen interaction (Karakousis et al., 2004). The genus *Mycobacterium* possesses a cell

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wall of unique structure. It includes the mycolic acid–arabinogalactan–peptidoglycan complex (MAPc), and an outermost capsule (Crick et al., 2001). In addition, the mycobacterial envelope contains abundant lipid-rich compounds such as phthiocerol dimycocerosate (PDIM), lipomannan (LM), lipoarabinomannan (LAM), sulfolipids, and the 19-kDa lipoprotein (Karakousis et al., 2004), trehalose-based lipids such as cord factor (trehalose-6,6-dimycolate), as well as acyltrehaloses and lipooligosaccharides (LOSs) (Brennan and Nikaido, 1995).

Chatterjee et al. (1992) postulated that LM and LAM are multiglycosylated phosphatidylinositol mannosides (PIMs), and from this structural relationship they inferred a biosynthetic relationship. Morita et al. (2004) hypothesized that 4-fold mannosylated PIM might be the branch point at which the biosynthesis of polar PIMs, LM, and LAM, diverges. LAM (Karakousis et al., 2004) and PIM (Apostolou et al., 1999; de la Salle et al., 2005; Fischer et al., 2004; Gilleron et al., 2003; Apostolou et al., 1999) are able to modulate the immune system of the host. Dependent on the capping motif and the degree of acylation (Nigou et al., 2001) of LAM the nature of the immune modulatory effect can be proinflammatory or antiinflammatory. Thus, it is important to understand the biosynthesis of these compounds in molecular detail to explain this multifaceted interaction with the immune system of the host.

Previously, Alexander et al. (2004) identified a gene coding for a glycosyltransferase, which they thought to catalyze the synthesis of 7-fold mannosylated PIM by adding two mannose residues to Ac_2PIM_5. They generated a deletion mutant of *M. marinum* and found the mutant to have an impaired LAM/LM synthesis and a reduced ability to invade macrophages. They also reported accumulation of a cell wall component, which they identified as Ac_2PIM_5. They were able to restore the wild-type phenotype by transforming ORF Rv1500 of *M. tuberculosis* H37Rv in the *M. marinum* mutant strain. From these results, they concluded that Rv1500 encoded a mannosyltransferase, which synthesizes Ac_2PIM_7 from Ac_2PIM_5 (Alexander et al., 2004).

In this study, we generated a deletion mutant of Rv1500 in *M. tuberculosis* H37Rv to examine the role of the putative mannosyltransferase in cell wall synthesis. Here we show that in *M. tuberculosis* the deletion of Rv1500 does not affect the expression pattern of PIMs and that the mutant strain revealed no alteration or attenuation in in vitro and in vivo infection models, even though we confirmed the expression of this open reading frame on the mRNA level.

Materials and methods

Bacteria

M. tuberculosis H37Rv and the mutant strain were cultivated in 7H9 broth or on 7H10 plates (Difco Laboratories, Inc., Detroit, Mich., USA), supplemented with 0.5% glycerol, 10% ADS (0.5% bovine albumin fraction V, 0.2% glucose, 140 mM NaCl), and 0.05% Tween 80 for 7H9. For infection experiments, *M. tuberculosis* cultures grown to mid-log phase were harvested, aliquoted, and frozen at −80 °C. After thawing, viable cell counts were determined by plating serial dilutions on Middlebrook 7H10 agar supplemented with 10% bovine serum (Biowest, Nuaillé, France). To ensure proper dispersion of *M. tuberculosis*, the bacterial suspension was drawn through a nonpyrogenic needle (Microlance 3, BD, Drogheda, Ireland) prior to every infection experiment. *Escherichia coli* HB101 was used as a host for molecular cloning and grown in Luria Bertani (LB) broth or on LB agar plates. Media were supplemented with 50 μg/ml hygromycin for mycobacteria, 100 μg/ml ampicillin, and 50 μg/ml kanamycin, respectively, for *E. coli* HB101.

Cloning and deletion of Rv1500

Rv1500 of *M. tuberculosis* H37Rv was obtained from a genomic cosmid library by colony blot hybridization. Construction of the library was described earlier (Bange et al., 1999). Briefly, partially digested genomic DNA of *M. tuberculosis* H37Rv was inserted into the *Bcl*I site of the cosmid vector pYUB412, generating a genomic library with an average insert size of between 35 and 40 kb. The DNA probe for hybridization was generated by PCR using the following primers: Rv1500-1: 5′-CGCCTCTCGATCGTAACG-3′ and Rv1500-2: 5′-TTCCTCAACCAACCGCCG-3′. DNA amplification was performed in 35 cycles using 57.5 °C for annealing, 72 °C for elongation, and 94 °C for denaturation. Following the protocol given by the manufacturer, the 389-bp DNA probe was gel-purified and labeled with a DIG DNA-labeling and detection kit (Roche, Mannheim, Germany) and was used for colony blot hybridization and for Southern blot hybridization.

A 3925-bp *BamH*I/*Bgl*II DNA fragment from cosmid pSMA13 carrying Rv1500 was subcloned, and an internal 321-bp DNA fragment was deleted in frame with *Bgl*I and *PshA*I resulting in the construct pSM74 (Fig. 1A). An *Xba*I fragment of pYUB657 (Pavelka and Jacobs, 1999) containing the hygromycin resistance cassette and the *sacB* gene as a counter selectable marker was inserted into the *XbaI* site of pSM74. This construct, pSMA28, was transformed in *M. tuberculosis* H37Rv by electroporation and plated on hygromycin (50 μg/ml) to select for integration of pSMA28 via single cross over. Integration of pSMA28 was confirmed by PCR, using Coint-1 5′-TCTGATCGACTCAGACTT GG-3′ and Coint-2 5′-GAAGAACACCGAGCTCA CC-3′, and Southern blot hybridization, using the DNA probe generated with Rv1500-1 and Rv1500-2,

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