Regulated Alteration of Mycolic Acid Structure in the Cell Wall of Mycobacterium Tuberculosis

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Introduction

Worldwide, 9 to 10 million new cases of tuberculosis occur each year [1]. Tuberculosis is caused by Mycobacterium tuberculosis, which has the distinct ability to persist in a human host for many years. Part of its long term survival strategy may involve its highly protective cell envelope. A large portion of the coding capacity of the M. tuberculosis genome is devoted to lipid biosynthesis and degradation [2]. About 40-60% of the cell wall dry weight is estimated to be made up of lipids [3]. Mycolic acids, which are α-alkyl, β-hydroxy fatty acids, are a major constituent of these lipids [4,5], and as such, their role in tuberculosis pathogenesis as well as M. tuberculosis persistence has been the subject of many investigations [6-11]. Structural alterations and relative differences in the amount of different classes of mycolic acids (α-, keto- and methoxy-mycolates) in the cell wall can exert a profound effect on clinical outcomes of infected hosts [7,12-15].

M. tuberculosis contains 4 homologous copies of an operon designated mcel-f, which resemble ATP-Binding Cassette (ABC) transporters possibly involved in lipid importation [2,16,17]. Shimono et al. showed that M. tuberculosis disrupted in one of these operons mcel failed to elicit a strong Thi-type immune response and caused a formation of poorly organized mouse lung granulomas comprised mostly of lipid-rich foamy macrophages [18]. Casali et al. showed that mcel operon is negatively regulated intracellularly by mcelR, located immediately upstream of the operon [19]. Uchida et al. showed that a mutant disrupted in mcelR gene causes accelerated immunopathologic response in mice, in which there is rapid progression to death of the animal following massive granuloma formation in their lungs [20]. These studies indicated that differential in vivo expression of the mcel genes correlates with distinct pro-inflammatory responses in mice.

The mce operon proteins are located in the cell wall [21-24]. A phylogenomic analysis of the M. tuberculosis mce operons revealed that related operons are widely distributed among members of Actinomycetales and that they encode a subfamily of ABC lipid uptake transporters that may be involved in remodeling the cell envelope [16]. Intriguingly, the product of the first gene (fadDS) of the mcel operon, FadD5, has high sequence similarity (43%) to the E. coli fatty-acyl-CoA synthetase FadD [25]. In M. tuberculosis, FadD5 is predicted to catalyze the first step in fatty acid degradation [25]. In addition, the M. tuberculosis Mce1R protein is 33% identical to the FadR transcriptional repressor protein found in E. coli, which binds fatty-acyl CoA and induces the expression of genes involved in fatty acid degradation and transport [26]. More recently, Dunphy et al. showed that M. tuberculosis disrupted in fadDS is diminished in growth in vitro in minimal medium supplied with mycolic acid as the sole carbon source [27].

Taken together, these observations suggest that the mcel operon is involved in fatty acid transport and metabolism. This led us to examine in more detail the cell wall lipid composition of the mcel operon mutant. Since the operon is regulated intracellularly [19], it may

Abstract

The hallmark of infection caused by Mycobacterium tuberculosis, the agent of tuberculosis, is its ability to persist in a human host for many years. The lipid-rich cell wall of M. tuberculosis serves as an effective barrier against many chemotherapeutic agents and toxic host cell effector molecules, and it may contribute to the mechanism of persistence. M. tuberculosis strains mutated in a 13-gene operon called mce1, which encodes a putative ABC lipid transporter, induce aberrant granulomatous response in mouse lungs. Because of the postulated role of the mce1 operon in lipid importation, we compared the cell wall lipid composition of wild type and mce1 operon mutant M. tuberculosis strains. High resolution mass spectrometric analyses of the mce1 mutant lipid extracts showed unbound mycolic acids to accumulate in the cell wall. Quantitative analysis revealed more than 10-fold greater amount of free mycolates in the mutant compared to that of the wild type strain. The mycolates from the wild type and mce1 operon mutant strains showed no difference in the fractions covalently attached to trehalose or arabinogalactan, indicating that the accumulation of free mycolic acid was not due to a defect in substrate mycolation. No differences were observed in colony morphology between wild type and mce1 mutant strains. Since the mce1 operon's expression is regulated in vivo in wild type M. tuberculosis, the above observations suggest that bacterial cell wall can undergo dynamic structural alterations during a course of infection, which may serve as an adaptive strategy for this organism to maintain persistence in a host.

Keywords: Mycolic acid; Mycobacterium tuberculosis; Mce operon; Mass spectrometry; Tuberculosis

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be involved in regulating *M. tuberculosis* cell wall lipid composition during infection. The ability to remodel the cell wall during infection may be a part of *M. tuberculosis*’s adaptive strategy developed against the constantly changing environment of the host granulomas.

**Materials and Methods**

**Generation of the mce1 operon mutant**

The construction of the mce1 operon mutant was previously described [18]. This mutant was constructed in a wild type H37Rv background by a strategy described by Parish and Stoker [28]. Briefly, following digestion with appropriate restriction enzymes the desired PCR products flanking the region targeted for deletion were subcloned into the p2NIL vector. Unmarked mutant was generated by a two-step counter selection process as previously described [28].

**Growth and maintenance of Mycobacterial strains**

All *M. tuberculosis* strains were grown in Middlebrook 7H9 broth (Difco, MD) containing 10% ADC (Beckton-Dickinson, MD), 0.2% glycerol (Fisher Scientific, NJ) and 0.05% Tween 80 (Fisher Scientific, NJ). The broth cultures were grown at 37°C until mid-logarithmic or stationary phase depending on experiment. The agar plates were incubated at 37°C until visible colonies appeared.

**Lipid extraction for mass spectrometry**

Total lipid fractions were extracted as follows. Mycobacterial cultures were grown to stationary phase, and 7-10ml of culture were pelleted and extracted with 4 mL of chloroform, methanol (2:1 in scintillation vials (Fisher, NJ) for 2 hours on a Genie 2 Vortex (Fisher, NJ) set at the lowest speed. This solvent extract was then pelleted at 3,500g. If after centrifuging a bubble of water was apparent on top of the solvent layer, additional chloroform: methanol (2:1) was added until it dissolved. The solvent was then saved at -80°C until use and the biomass was discarded.

We also extracted lipids from the outer layer of the cell wall. This procedure was adapted from Dobson et al. and Converse et al. [29,30]. Mycobacterial cultures were grown to stationary phase as described above, and 7-10ml of culture was pelleted and extracted with 4 mL of chloroform, methanol (2:1) in scintillation vials (Fisher, NJ) for 2 hours on a Genie 2 Vortex (Fisher, NJ) set at the lowest speed. This solvent extract was then centrifuged at 3,500g. If after centrifuging a bubble of water was apparent on top of the solvent layer, additional chloroform: methanol (2:1) was added until it dissolved. The solvent was then saved at -80°C until use and the biomass was discarded.

**Preparation of samples for quantification of free mycolic acids**

*M. tuberculosis* strains were grown in Middlebrook 7H9 medium until they reached an OD$_{600}$ of 1.0. Ten milliliters of each culture (wild type, mce1 mutant and complemented *M. tuberculosis* strains) were used to extract surface lipids (methodology described above). Extracted surface lipids were further filtered with a 0.2 µm filter and distributed into 500 µl aliquots. These samples were then spiked with purified *M. tuberculosis* mycolic acids (Sigma) to produce final concentrations of 0, 1, 2, 5 and 10 µM added mycolic acid. We then analyzed these samples using ESI-MS and plotted the mass spectral intensities of mycolic acid ions against the added standard mycolic acid concentrations to generate standard addition curves, from which the amounts of mycolic acid present in the bacterial lipid extracts were determined (Supplementary Figure 1).

**Quantitative mass spectrometry**

The extracted lipids were quantitatively analyzed with an LTQ Orbitrap XL hybrid mass spectrometer equipped with an Ion Max electrospray ionization source (ESI; Thermo Fisher Scientific, Waltham, MA) that was connected in-line with an Agilent 1200 series auto-sampler and quaternary pump (Santa Clara, CA). Sample solutions contained in 2-mL screw-top vials sealed with septa caps (Agilent) were loaded into the auto-sampler compartment prior to analysis. An injection volume of 50 µL was used for each sample. The injected sample aliquot was pumped to the ESI probe of the mass spectrometer for a period of 8 min with a flow of methanol delivered at a flow rate of 50 µL/min. Solvent (1:1 chloroform/methanol) blanks were run between samples, and the auto-sampler injection needle was rinsed with 1:1 chloroform/methanol after each sample injection to avoid cross-contamination between samples. The connection between the auto-sampler and the ESI probe of the mass spectrometer was made using PEEK tubing (0.005 i.d. × 1/16 o.d., Western Analytical, Lake Elsinore, CA). External mass calibration was performed prior to analysis with the standard LTQ calibration mixture containing caffeine, the peptide MRFA, and Ultramark 1621 dissolved in 51% acetonitrile/25% methanol/23% water/1% acetic acid solution (v/v/v/v). The voltages applied to the ESI source optics were adjusted for optimum desolvation and transmission of mycolate anions with Tune Plus software (version 2.4, Thermo), while directly infusing a 2:1 chloroform/methanol solution containing commercially obtained mycolic acid standard at a concentration of ~100 µM into the ESI probe at a flow rate of 5 µL/min, using a syringe pump. The optimized ESI source parameters were as follows: ion transfer capillary temperature 220°C, normalized sheath gas (nitrogen) flow rate 30%, ESI voltage 2.3 kV, ion transfer capillary voltage 1 V, and tube lens voltage 222 V. Mass spectra were recorded in the negative ion mode over the range m/z = 900 to 1600 with the Orbitrap mass analyzer, in profile format, with a full MS automatic gain control target setting of 1 × 10$^5$ charges and a resolution setting of 6 × 10$^3$ (at m/z = 400, FWHM). Mass spectra were processed with Xcalibur software (version 4.1, Thermo). Quantitative analysis of mycolic acids was performed by the method of standard additions [31].

**Fourier transform ion cyclotron resonance mass spectrometry**

Mass spectra were acquired on a Bruker Apex II Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (FT-ICR MS), equipped with a 7T superconducting magnet (Bruker Daltonics, Billerica,
MA). Lipid extracts of *M. tuberculosis* were ionized by electrospray ionization (Analytica, Branford, CT) in negative ion mode and were infused at a rate of 2µL/min in 2:1 chloroform:methanol. The ions were accumulated in an external hexapole for 0.5-2s (the time was adjusted to maximize ion signal without depleting spectral resolution) before being transferred to the ICR cell for high resolution analysis. Tandem mass spectrometry (MS²) experiments were performed by isolation of the desired precursor ion by a CHEF isolation sweep, followed by ion activation and dissociation by SORI-CID [32,33]. For SORI-CID, Ar gas was pulsed into the FT-ICR MS analyzer cell to raise the pressure in the cell to the low 10⁻⁶ mbar range, and the ions were then activated 500 Hz off-resonance for 250 msec. The amplitude of the off-resonance irradiation was adjusted to give nearly complete attenuation of the precursor ion signal. After a delay of several seconds to allow pump-down of the collision gas pulse, the product ions were detected under high-resolution conditions. Spectra were acquired by XMass 5.0.10 (Bruker Daltonics, Billerica, MA), and were composed of between 256k and 1M data points and were an average of between 32 and 128 scans. All spectra were internally calibrated by known compounds.

**Colony morphology characterization**

*M. tuberculosis* H37Rv and *mce1* mutant strains were grown for 11 days on 7H11 agar in a 5% CO₂ humidified incubator at 37°C. Colonies were examined with a Nikon Labophot-2 microscope (Merrell, NY) and photographed with a Digital Nikon 4300.

**Results**

*Mce1* operon mutant accumulates free mycolic acids

We extracted total lipid fractions from wild type, *mce1* mutant, and complemented strains of *M. tuberculosis* and compared them by MS. The lipid profiles were nearly identical for all three strains (Figure 1A). After zooming in on *m/z* 1100-1300, a clear region of difference between the *mce1* mutant and control strains was observed (Figure 1A inset). In this region, several lipid species were detected in the *mce1* mutant that were either absent in the controls or present in trace amounts. Analysis of the hexane extract by MS confirmed that these lipids were present in the cell wall (Figure 1B).

Using the high mass accuracy and tandem mass spectrometric capabilities of the Fourier Transform Ion Cyclotron Resonance (FTICR) mass spectrometer, we were able to assign molecular formulas and structures to the unknown lipids. The accurate masses were identified as follows: *m/z* 1136.166, 1236.225, and 1252.281 (Figure 2A). Since mycobacterial lipids are generally made of six elements (carbon, hydrogen, oxygen, nitrogen, phosphorus, and sulfur), the exact mass provided sufficient information to propose the molecular formula for these products. Tandem MS experiments were subsequently performed in order to gain structural information from these compounds. In each case, two product ions, *m/z* 367.35 and 395.39, were observed (Figure 2B), which are consistent with saturated fatty acids with 24 and 26 carbons, respectively. Interestingly, the alpha branch of mycolic acids

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**Figure 1:** MS of lipid profiles of wild type, *mce1* operon mutant and its complemented strain. (A) Total lipid extracts from wild type *M. tuberculosis* H37Rv (top), *Δmce* (middle), and complemented strain *Δmce::mce* (bottom). The lipids were found to accumulate in the *m/z* 1100-1300 range in the *Δmce*, but not the wild-type or complemented strain (insets). (B) A hexane extraction localized the accumulating lipids to the outer surface of the cell. *M. tuberculosis* H37Rv (top), *Δmce* (middle), and *Δmce::mce* (bottom). Zoom of the *m/z* 1100-1300 region.
from *M. tuberculosis* is generally composed of a saturated fatty acid containing 24 or 26 carbons.

To confirm their identity, mycolic acids were released from the arabinogalactan and trehalose by FT-ICR MS. No differences in mycolic acids were due to a defect in substrate mycolation, we analyzed the mycolic acids released from arabinogalactan by FT-ICR MS. No differences in mycolic acids were observed when the three strains were complemented strains (Figure 3A). Other than slight variations in ion abundance were observed in the wild-type or wild-type strain *M. tuberculosis* operon mutant does not have a defect in mycolation of arabinogalactan and trehalose

To rule out the possibility that the accumulating mycolic acids were due to a defect in substrate mycolation, we analyzed the mycolic acids from arabinogalactan by FT-ICR MS. No differences in mycolic acid abundance were observed in the mce1 operon mutant, the wild-type or complemented strains (Figure 3A). Other than slight variations in ion abundance, no differences were observed when the three strains were examined for defects in trehalose mycolation (Figure 3B).

**Mce1 operon mutant contains 11 fold higher concentrations of free mycolic acids relative to wild type strain**

Using the methods of standard additions and MS, we quantified the amount of free mycolic acids present in the mce1 operon mutant, the wild type, and the complemented strains. We found that the amount of free mycolic acids in the mce1 operon mutant was roughly 11-fold higher than that in the wild type strain (Table 1). We obtained 24 ± 5, 2.3 ± 0.5 and 4.8 ± 2.3 μg of mycolic acids from 10 ml-growth of free mycolic acids from 10 ml-growth of free mycolic acids relative to wild type strain.
Since the mce1 operon is negatively regulated by mce1R when M. tuberculosis is intracellular [19], wild type M. tuberculosis should functionally behave like the mce1 operon mutant at some stage of infection. That is, free mycolic acids may indeed accumulate in the wild type cell wall in vivo. Changes in the relative proportion as well as structures of mycolic acids are associated with alterations in the fluidity and permeability of the mycolic acid layer, as well as the induction of immune response in mice [12]. Yuan et al. showed that M. tuberculosis overproducing O-methyltransferase encoded by mma3 led to increased methoxy-mycolate formation, and resulted in a reduced ability to survive in macrophage-like THP-1 cells [13].

Discussion

Here, we provide evidence for the accumulation of unattached mycolic acids in the cell wall of an M. tuberculosis strain disrupted in the mce1 operon. Complementation of the operon restored the wild type mycolate composition. These cell wall changes, however, were not reflected by obvious changes in colony morphology. The M. tuberculosis cell envelope contains 3 classes of mycolates-alpha, keto- and methoxy-mycolic acid [4]. Mycolates are attached to the arabinogalactan layer via a phosphodiester bond to the peptidoglycan layer [34]. Mycolates are attached to trehalose on the cell wall surface [35].

The mce1 operon mutant contained 11-fold higher amount of free mycolic acids than did wild type or complemented M. tuberculosis strains. Only trace amounts of free mycolic acids were detected in the wild type strain by MS, which also have been observed previously with high performance liquid chromatography [36].

How these free mycolates accumulate in the M. tuberculosis mce1 operon mutant is unclear. Several possibilities exist. A defect in mycolation of arabinogalactan or trehalose could cause accumulation of free mycolates. This was not found to be the case, as mass spectrometric analyses of the mycolate fractions from the wild type H37Rv and the mce1 operon mutant showed that there was no difference in the covalently attached mycolates between these two strains (Figure 3).

It is also possible that the free mycolates in the hexane extracted material represent contaminating cytoplasmic mycolates. However, the fact that all strains were treated similarly with hexane provides evidence to the contrary. Furthermore, in the cytoplasm, the newly synthesized mycolic acid is in the thioester linkage to polyketide synthase (Pks13) [34].

In arabinogalactan, mycolic acids are found in clusters of four on the terminal penta-arabinosyl units. McNeil et al. reported that in the cultured organism, only about two-thirds of these units were mycolated [37]. The method we used to examine strain differences in arabinogalactan mycolation may not have been sensitive enough to detect and quantify small changes; arabinogalactan may serve as a sink for free mycolates.

Recent observations suggest that the mce operons of M. tuberculosis are involved in lipid transport across the cell wall [16,17,27]. Pandey et al. showed that mce4 encodes a possible cholesterol import system [17]. Dunphly et al. showed that M. tuberculosis functionally disrupted in fadDS of the mce1 operon is unable to grow in minimal medium supplied with mycolic acid as the sole carbon source [27]. It grew similarly to the wild type in other long-chain fatty acids provided as a carbon source [27]. It was suggested that mycolic acids released from drying bacilli may be salvaged by live M. tuberculosis as an energy source during the bacterial persistent state.

Table 1: Quantitative analysis of mycolic acids extracted from approximately 10^6 wild type, mce1 operon mutant, and complemented M. tuberculosis H37Rv bacilli. Data presented here reflect the average of two independent experiments. Correlation coefficients from two different experiments are shown.

| M. tuberculosis strain | Correlation coefficient | Mycolic acid concentration (µM) | Mycolic acid quantity (µg) |
|------------------------|-------------------------|---------------------------------|--------------------------|
| Wild type H37Rv        | 0.985, 0.997            | 0.35±0.07                       | 2.3±0.5                  |
| mce1 operon mutant     | 0.996, 0.997            | 3.75±0.77                       | 24±5                     |
| mce1 operon complemented strain | 0.998, 0.993 | 0.75±0.35                       | 4.9±2.3                  |

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