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The *Mycobacterium tuberculosis* protein Rv2387 is involved in cell wall remodeling and susceptibility to acidic conditions

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ABSTRACT

The distinctive cell walls of mycobacteria are characteristic features of these bacteria. Individual cell wall components influence diverse mycobacterial phenotypes, such as colony morphology, virulence and stress resistance. To investigate the role of the hypothetical protein Rv2387, we constructed a *Mycobacterium smegmatis* strain that heterologously expressed this ORF, and we observed that the *M. smegmatis* strain expressing Rv2387 exhibited altered colony morphology and cell wall lipid composition, leading to a marked decrease in the resistance against acidic conditions. This study demonstrates that due to its impact on cell wall remodeling, Rv2387 might play an important role in mycobacterial physiology.

1. Introduction

Tuberculosis, caused by *Mycobacterium tuberculosis*, remains a formidable threat worldwide. Despite several decades of intense chemotherapy, tuberculosis still claims approximately 1.7 million lives annually, and in 2016, there were 10.4 million new infections worldwide [1]. One reason for the success of *M. tuberculosis* lies in its extraordinary ability to resist the diverse stress factors within macrophages, enabling the bacterium to avoid being killed by the host immune system [2]. The cell wall is a defining feature of mycobacteria. This unique and highly complex cell wall, which consists of lipid-rich, hydrophobic structures, is responsible for the acid-fast staining properties, distinctive colony morphology and resistance to various antimycobacterial drugs [3–5]. The mycobacterial cell wall forms an asymmetric lipid bilayer that is composed of up to 60% (wt/wt) lipids [6]. In addition to mycolic acids, various types of complex lipids exist in the cell envelope, including lipoglycans (e.g., lipoarabinomannan [LAM]), trehalose-containing glycolipids, phthiocerol dimycocerosates, phenolic glycolipids, and glycopeptidolipids (GPLs) [7]. Different lipids appear to play different roles and are known to contribute to the virulence and pathogenesis of the bacilli [8,9]. The mycobacterial cell wall is a precursor to the interface between the bacilli and the environment. Alterations in the cell wall composition can have dramatic effects on many phenotypes, including colony morphology and drug resistance [5,10,11].

The conserved hypothetical protein Rv2387 is predicted to be involved in the host-pathogen interaction [12]. The transcription of *M. tuberculosis rv2387* was up-regulated upon exposure to various antibiotics [13]. Moreover, an *MMAR_3708* (homologous to *rv2387*) mutant was identified by the screening of a transposon mutation library of *Mycobacterium marinum* for altered colony morphology [14]. All these reports suggest an important role for Rv2387 in the cell wall properties and physiology of mycobacteria.

In the present study, as a first step toward evaluating the possible role of Rv2387 in the physiology of *M. tuberculosis*, we took advantage of the absence of the *rv2387* gene in the nonpathogenic *Mycobacterium smegmatis* and generated two recombinant

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Mycobacterium strains. We cloned the *rv2387* gene from the *M. tuberculosis* strain H37Rv and expressed this gene in the *M. smegmatis* strain mc^2155. We found that the morphology and surface architectures of *M. smegmatis* expressing Rv2387 were significantly altered. Moreover, our studies demonstrated that MS_Rv2387 had a modified cell wall fatty acid profile, and the ability of this strain to resist acidic conditions was reduced.

2. Materials and methods

2.1. M. smegmatis culture and transformation

M. smegmatis mc^2155 bacteria were grown in Middlebrook 7H9 medium (BD Difco, USA) supplemented with 0.2% (w/v) glucose, 0.2% (v/v) glycerol and 0.05% (v/v) Tween 80 (Sangon Biotech, China). For preparation of competent cells, the culture was allowed to grow until the mid-log phase. The culture was then centrifuged, washed four times with 10% glycerol, and resuspended in 1/100 t h of the culture volume in ice-cold 10% glycerol. Then, 400-μl aliquots of the cells were stored at −80 °C until further use. Prior to transformation, the cells were thawed on ice, and 1 μg of plasmid was added. The cells were incubated on ice for 10 min and transferred to a prechilled cuvette with a 1-mm gap width. One milliliter of the culture medium was added immediately, and the cells were incubated at 37 °C for 4 h. The transformants were selected on 7H10 agar plates containing 20 μg/ml kanamycin (Sangon Biotech, China).

2.2. Construction of recombinant M. smegmatis expressing Rv2387

The full length of the *rv2387* gene was amplified from *M. tuberculosis* H37Rv genomic DNA by PCR using specific primers (forward primer, 5′-GCGGAATTCATGCTGCATGAGTTCTG-3'; reverse primer, 5′-TTATGCATGCACCGATCGAAGCCC-3′). The PCR product was digested with *EcoR* I and *Sph* I and ligated to the mycobacterial expression vector pNIT-myc [15,16] to generate the recombinant plasmid pNIT-*rv2387*. The recombinant plasmid was verified by restriction digestion and DNA sequencing (BGI, China). *M. smegmatis* was subjected to electroporation with pNIT-*rv2387* to generate a recombinant *M. smegmatis* (MS_Rv2387) strain, and transformants were selected on a 7H10 agar plate containing 20 μg/ml kanamycin. *M. smegmatis* transformed with the pNIT-mycvector (MS_Vec) was used as a control group.

2.3. Expression of Rv2387 in M. smegmatis

The MS_Rv2387 and MS_Vec cultures were grown in 7H9 medium supplemented with 0.2% glucose, 0.2% glycerol and 0.05% Tween 80 until the absorbance at 600 nm (OD_{600}) reached 0.6. In addition, the cultures were treated with 28 mM ε-caprolactam (inducer of pNIT-myc) for 16–20 h. The bacterial cell pellets were harvested and washed thrice with phosphate-buffered saline containing Tween 80 (PBS-T). The resulting pellets were then resuspended in lysis buffer (50 mM Tris-Cl (pH 8.0), 300 mM NaCl, 1 mM PMSF and 1 mM DTT) and vortexed. The bacterial cells were lysed by ultrasonication, and the lysed cells were centrifuged to collect the supernatant. The samples were subjected to SDS-PAGE, and the Myc-tagged Rv2387 protein was detected by a mouse anti-Myc antibody (Tiangen, China).

2.4. Cellular aggregation and colony morphology

To study cellular aggregation, the bacilli were grown to an OD_{600} of 0.6–0.8 in 7H9 medium with 0.05% Tween 80 and 28 mM ε-caprolactam in a 37 °C shaking incubator. The cultures were kept at room temperature for 30 min to allow the cell aggregates to settle, and the aggregates were then photographed with a Nikon Digital camera (Japan).

For colony morphology analysis, the MS_Rv2387 and MS_Vec strains were plated onto Middlebrook 7H10 plates with or without 0.05% (v/v) Tween 80 and incubated for 5–6 days at 37 °C. A Nikon Digital camera (Japan) was used to obtain photomicrographs of the colonies.

2.5. In vitro growth and stress assays

For *in vitro* growth curves, cultures were inoculated in triplicate with a starting OD_{600} of ~0.02. Twenty-eight micromolar ε-caprolactam was added to the cultures, and the OD_{600} was measured at various time points over a 70-h growth period. To assess the growth curve of recombinant *M. smegmatis* under acidic conditions, the pH of the culture was adjusted to 5.5. Growth was monitored by determining the OD_{600} at various time points over 96 h.

2.6. Spot tests

Caprolactam-induced MS_Vec and MS_Rv2387 were subjected to acidic stress for 6 h, serially diluted (1:10) and spotted (3 μl) onto 7H10 medium. Photographs were taken after three days of incubation at 37 °C. The experiments were repeated at least 3 times.

2.7. GC-MS analysis of fatty acid methyl esters

Fatty acids were extracted from midexponential-phase cultures of *M. smegmatis* as previously described [17]. The fatty acid concentrations were determined by GC-MS (Thermo Fisher Trace GC 1310-ISQ LT single quadrupole EI MS, A1-1310 autosampler) with a Thermo TG-5MS capillary column. The detailed analytical procedure has been described previously [18].

2.8. SEM analysis

MS_Vec and MS_Rv2387 were grown until the OD_{600} reached 1.0 in 7H9 medium containing inducer. Cultures were harvested by centrifugation, and the harvested pellets were then resuspended in 2.5% glutaraldehyde solution. The samples were dehydrated in an ascending series of ethanol. After critical point drying, the samples were sputtered with platinum (IB-3, Eiko) and observed by scanning electron microscopy (SEM) (JSM-6390 LV, JEOL Ltd.).

2.9. Statistical analysis

Data were analyzed using Student's two-tailed t-tests. Statistical significance was defined as a P-value$\leq$0.05. Error bars represent standard deviations (SDs).

3. Results

3.1. Expression of the rv2387 gene in M. smegmatis

The Mtb ORF *rv2387* is approximately 1.2 kb (Fig. 1A) in size and encodes a 45-kDa protein. To understand the mechanism via which Rv2387 affects mycobacterial physiology, we generated two recombinant *M. smegmatis* strains. The MS_Rv2387 strain was engineered to express a Myc-tagged Rv2387 protein from a recombinant pNIT-Myc vector, while the MS_Vec strain harbored the backbone vector. The immunoblotting analysis using the anti-Myc antibody indicated the presence of an ~50-kDa Rv2387-Myc fusion protein in the cell lysates of MS_Rv2387 cells but not in MS_Vec cells (Fig. 1B). These results indicate that the Rv2387

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