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MOLECULAR ASPECTS

MadR1, a *Mycobacterium tuberculosis* cell cycle stress response protein that is a member of a widely conserved protein class of prokaryotic, eukaryotic and archeal origin

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SUMMARY

Stress-induced molecular programs designed to stall division progression are nearly ubiquitous in bacteria, with one well-known example being the participation of the SulA septum inhibiting protein in the SOS DNA damage repair response. Mycobacteria similarly demonstrate stress-altered growth kinetics, however no such regulators have been found in these organisms. We therefore set out to identify SulA-like regulatory proteins in *Mycobacterium tuberculosis*. A bioinformatics modeling-based approach led to the identification of *rv2216* as encoding for a protein with weak similarity to SulA, further analysis distinguished this protein as belonging to a group of uncharacterized growth promoting proteins. We have named the mycobacterial protein encoded by *rv2216* morphology altering division regulator protein 1, MadR1. Overexpression of *madR1* modulated cell length while maintaining growth kinetics similar to wild-type, and increased the proportion of bent or V-form cells in the population. The presence of MadR1-GFP at regions of cellular elongation (poles) and morphological differentiation (V-form) suggests MadR1 involvement in phenotypic heterogeneity and longitudinal cellular growth. Global transcriptional analysis indicated that MadR1 functionality is linked to lipid editing programs required for growth and persistence. This is the first report to differentiate the larger class of these conserved proteins from SulA proteins and characterizes MadR1 effects on the mycobacterial cell.

1. Introduction

A hallmark of *Mycobacterium tuberculosis* (*Mtb*) is its ability to evade host responses, survive stress conditions and tolerate drug treatment, resulting in the establishment and maintenance of a latent state of infection for long durations [1–3]. Studies of *Mtb* grown in artificial stress conditions and in animal models of infection as well as evidence from patient tissues substantiate that the bacilli can survive stresses by entering into a quasi-dormant state often referred to as non-replicating persistence (NRP). Despite the prominent role these bacteria have played in global disease, the regulatory elements controlling replication remain poorly understood, particularly in relation to stress-induced NRP.

Mounting experimental evidence indicates that the bacterial response to stress includes regulatory elements that govern cell division and alter morphology [4]. In other bacterial species, SOS response proteins, such as SulA (SfiA) in *Escherichia coli* and YneA in *Bacillus subtilis*, are known to regulate cell division and induce filamentation in response to drug exposure, DNA damage, radiation, or reactive oxidative intermediates [5,6]. These stress responsive regulatory proteins are used in a final survival strategy to halt cells until favorable growth conditions exist.

Mtb bacilli are known to filament following phagocytosis by host macrophages and under *in vitro* stress models of NRP, yet the underlying mechanisms facilitating these growth transitions remain unknown [3,7]. It was recently found that the Ssd and Soj_{Mtb} proteins induce cellular survival responses coupling filamentation with the induction of adaptive metabolic programs, demonstrating the association between these processes that are important

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characteristics of persistent mycobacterial infections [8,9]. However, evidence does not support a role for Ssd as the direct modulator of division progression through control of FtsZ protein polymerization dynamics, as has been shown for SulA and several other stress-associated division inhibitors [6,8,10]. Currently no genes encoding a protein known to directly participate in division cessation through septum control have been identified in any mycobacterial genome, raising the question as to how *Mtb* regulates cell cycle progression characterized by filamentation and transition into NRP during the establishment of a persistent infection.

To better understand the regulatory mechanisms controlling growth and division in mycobacteria, we set out to identify unannotated cell division regulatory proteins involved in division progression using consensus-modeling bioinformatics, morphological analysis, protein mapping and transcriptional analysis. This approach led to the identification of *rv2216* that encodes a SulA-like protein, and further analysis revealed that Rv2216 belongs to a group of widely conserved but uncharacterized growth promoting proteins of prokaryotic, eukaryotic and archeal origin, which we have named morphology altering division regulator protein 1 (MadR1). We show that MadR1 of *Mtb* contributes to morphological heterogeneity and affects elongation but not division. Furthermore, *madR1* overexpression induced a global transcriptional response promoting persistence-linked lipid metabolism at the plasma membrane. This is the first report defining the novel class of MadR1 proteins and characterizing their phenotypic effects in mycobacteria.

2. Methods

2.1. Strains, growth and SEM

Mtb H37Rv and *Mycobacterium smegmatis* MC^2 155 were grown at 37 °C in Middlebrook 7H9 liquid medium or on 7H11 agar media supplemented with 25 μg ml^{-1} Kanamycin sulfate when necessary [9]. *Mtb* H37Rv was grown to an $O.D._{600nm}$ of 0.1–0.2 and subjected to continued growth in the presence of Mitomycin C (MMC) at 0.2 μg ml^{-1} 100 μl total volumes. IC_{50} was performed in triplicate and was defined as the concentration of drug required to reduce bacterial growth 50% after 7-days incubation [11]. Viability testing was performed at 1, 3, and 5 days post-treatment with 0.2 μg ml^{-1} or 0.1 μg ml^{-1} MMC by determining colony forming units via direct plating and outgrowth. *Rv2216* [EMBL accession no. **CCP44993**] was cloned into the mycobacterial vector pVV16 and transformed into *M. smegmatis* and *Mtb* as described elsewhere [9]. For ultrastructural analysis, *M. smegmatis* cells overexpressing *madR1* were collected at mid-log growth then prepared and imaged as previously described [8]. The relative proportion of linear and V-form morphologies were tallied for 100 cells per field of view, and averaged for five different views per condition.

2.2. Bioinformatic and statistical analysis

Datasets of annotated SulA and MadR1 proteins were created from the UniProt and OMA Browser databases and aligned using the MafftWS global sequence alignment tool through Jalview V2.7 [12]. Aligned datasets were used to build Hidden Markov Models (HMMs) with HMMER tools and then used to search encoded *Mtb* proteins containing SulA-like motifs. BLASTX, BLASTP, and TBLASTN analysis against searchable databases prepared from prototype protein datasets were also used. Alignment and dendogram construction performed through Jalview [12]. Alignment quality scores indicate probability that observed amino acids in an aligned column are the result of conservation; lower scores indicate good potential for mutation and higher scores posing less cost. Distance values are the sum of the BLOSUM62 scores for each residue pair in the original multialignment, and are minimized using a Neighbor Joining algorithm. Tools from the EMBOSS package were used to analyze the proteins and datasets [13]. Functional enrichment calculations were performed through the Tuberculosis Database (www.tbdb.org) using the *MadR1* overexpression dataset obtained by microarray.

2.3. Transcriptional profiling

Cells were harvested at mid-log, resuspended in TRIzol reagent (Invitrogen™), and total RNA was liberated by physical disruption [14]. cDNA was generated from total RNA using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen™). Primer sequences (Additional file 4). And quantitative real-time PCR was performed as previously described [8]. Quantification of each gene was determined relative to a time zero, normalized to *sigA* reference gene expression and log base 2 transformed. Microarray analysis was performed with labeled cDNA generated using direct labeling from 5 μg of total RNA [15]. The resulting fluorescence for each channel of the array (Cy3 and Cy5) was normalized to the mean channel intensity and analyzed using ANOVA single factor analysis. Significance was considered to be a $\geq$ 2-fold alteration in expression, with p-value of $\leq$0.05.

2.4. Fluorescent microscopy

An extrachromosomal *E. coli*-mycobacteria shuttle vector, pMCSU7, was developed for the inducible expression of fluorescently tagged protein using the *Streptomyces coelicolor tetO* promoter from tcp3 (Additional file 5). The *MadR1* coding sequence was PCR amplified from H37Rv genomic DNA and combined into pMCSU7 using Gateway technology. Constructs were screened using DNA sequencing prior to transformation into electrocompetent *M. smegmatis*. Mid-log grown transformants were diluted to $O.D._{600nm}$ 0.2 and expression was induced in the dark for 6 h using 50 ng ml^{-1} anhydrotetracycline (Clontech). Cells were stained with FM 4-64 Fx in HBSS (Invitrogen™), fixed in 4% paraformaldehyde, applied to glass slides, and coated with Vectashield Hard Set with DAPI (Vector Laboratories). Slides were stored at 4C for a maximum of 24 h before imaging at 1000× magnification using an inverted, oil-immersion Olympus I × 71 microscope with a Retiga 2000R camera (QImaging) and Slidebook software (Intelligent Imaging Innovations Inc.).

3. Results

3.1. Identification of the SulA-like MadR1 protein encoded by rv2216

A putative cell division regulatory protein in *Mtb* was identified using a reciprocal best-hit (RBH) strategy constructed from local and global Hidden Markov Models (HMMs) of annotated SulA proteins from 85 bacterial genomes built from the OMA 415268 dataset [16]. BLAST searches using the SulA consensus model against the H37Rv proteome identified a 301 amino acid protein, encoded by *rv2216,* annotated as a conserved hypothetical protein of unknown function. Domain mapping through Sanger-Pfam revealed a SulA-like epimerase/dehydratase domain [Pfam accession no. **PF01370**] toward the N-terminus, followed by a domain of unknown function [Pfam accession no. **PF08338**] not traditionally associated with SulA proteins (Figure 1A) [17]. In addition, there is a notable difference in length between SulA-family (135-170 amino acids in length) and Rv2216 (301 amino acids in length) and