mymA operon of Mycobacterium tuberculosis: its regulation and importance in the cell envelope

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Abstract

Mycobacterium tuberculosis faces various stressful conditions inside the host and responds to them through a coordinated regulation of gene expression. We had previously reported identification of the virS gene of M. tuberculosis (Rv3082c) belonging to the AraC family of transcriptional regulators. In the current study, we show that the seven genes (Rv3083–Rv3089) which are present divergently to virS (Rv3082c) constitute an operon designated the mymA operon. Further investigation on the regulation of this operon showed that transcription of the mymA operon is dependent on the presence of VirS protein. A four-fold induction of the mymA operon promoter occurs specifically in wild-type M. tuberculosis and not in the virS mutant of M. tuberculosis (MtbΔvirS) when exposed to acidic pH. Expression of the mymA operon was also induced in infected macrophages by 10-fold over a 6-day period. To gain an insight into the function of the proteins encoded by this operon, we carried out a bioinformatic analysis, which suggested the involvement of these proteins in the modification of fatty acids required for cell envelope. This was supported by altered colony morphology and cell envelope structure displayed by the virS mutant of M. tuberculosis (MtbΔvirS).

Keywords: Mycobacterium; Tuberculosis; Transcriptional regulation; Cell envelope

1. Introduction

Invasion of the host by Mycobacterium tuberculosis is a complex process involving both pathogen and host derived effector molecules. Interplay of these molecules leads to establishment, elimination or long term persistence of bacteria in the host. Mycobacteria reside preferentially in the host macrophages where they encounter various stressful conditions such as changes in pH, exposure to reactive oxygen, nitrogen intermediates and degradative enzymes and deprivation of essential nutrients [1]. In order to survive and multiply in face of a highly unfavorable intracellular environment, mycobacteria appear to have devised various strategies [2,3]. Some mycobacterial genes whose expression has been shown to be crucial for bacterial survival are katG, encoding catalase-peroxidase [4], hspX (acr), encoding a homologue of α-crystallin [5], and erp, encoding an exported protein [6].

It is being increasingly recognized that genes involved in fatty acid metabolism and modification of the cell wall of pathogenic mycobacteria play an important role in their virulence. Specific modifications of mycolic acids are essential for mycobacterial virulence. It has been observed that strains carrying a disrupted copy of hma, required for the biosynthesis of oxygenated mycolic acids [7], or of pca, a gene encoding a mycolic acid cyclopropane synthase [8], are attenuated in mice. By using signature tagged mutagenesis, several loci required for the biogenesis of mycobacterial cell envelope were found to be attenuated in mice [9,10]. Transposon insertions in the promoter of the pps operon, encoding a putative multisubunit polyketide synthase required for phthiocerol biosynthesis and in the open reading frame of fadD28, whose product is probably involved in acyl transfer of mycocerosic acid, abrogated PDIM synthesis, while disruption of mmpL7 affected PDIM transport to the cell wall [9]. Each of these give...
earlier. The reaction was performed in a 20 μl final volume with 1× reverse transcriptase buffer, 400 μM dNTPs, 100 μg ml⁻¹ bovine serum albumin, 30 units of ribonuclease inhibitor, 5 μg template RNA, 3 μg of random hexameric primers and 200 μl of Moloney Murine Leukemia virus reverse transcriptase (United States Biochemical Corporation, Cleveland, OH, USA) at 37°C for 60 min. This was followed by heat inactivation of the enzyme at 75°C for 5 min. Subsequently, 5 μl from this reaction was used as a template for PCR using 1× Taq DNA polymerase buffer, 200 μM dNTPs, gene specific primers and 1.5 units of TaqPlus® Long PCR systems (Stratagene, La Jolla, CA, USA). The amplification conditions comprised of an initial cycle of denaturation at 94°C for 5 min, 30 cycles of 94°C for 45 s, 65°C for 1 min, 72°C for 1 min and a final incubation for 10 min at 72°C. The different primers used in RT-PCR reactions were (i) Pmyf (5’-CCAGGGCTCCAAGCATCCGTG-3’) (ii) Poxr (5’-CGGTGATGACCGGACCTTGGC-3’) (iii) Poxf (5’-GGCCTGGCAATTCTTCCTCCC-3’) (iv) Punr1 (5’-CGTTGAGCTGCGCATCATCG-3’) (v) Punf1 (5’-TCTGGAACCACTTGGAAATGT-3’) (vi) Punr2 (5’-GTTTTCCAGCGCCGAAATCT-3’) (vii) Punf2 (5’-CCACCAGACGCTTACTCTTG-3’) and (viii) PacoA (5’-CTCAGCGACTTGGGCGATCAG-3’).

2. Materials and methods

2.1. Bacterial strains and culture conditions

*Mycobacterium smegmatis* strain LR222 and *M. tuberculosis* were grown in Middlebrook (MB) 7H9 broth supplemented with 0.2% Tween 80 and growth enrichment ADC (albumin dextrose complex). *Escherichia coli* DH5α and HB101 were grown in Luria–Bertani broth. Bacteria were cultured at 37°C with shaking at 200 rpm and whenever appropriate, antibiotics were added at the following concentrations: ampicillin (50 μg ml⁻¹), kanamycin (25 μg ml⁻¹), hygromycin B (150 μg ml⁻¹ for *E. coli*; 50 μg ml⁻¹ for *M. tuberculosis*).

2.2. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

*M. tuberculosis* H37Rv was grown in liquid culture to an OD₆₀₀ of 1.5 and total RNA was isolated as described earlier[20]. The RT reaction was performed in a 20 μl final volume with 1× reverse transcriptase buffer, 400 μM

dNTPs, 100 μg ml⁻¹ bovine serum albumin, 30 units of ribonuclease inhibitor, 5 μg template RNA, 3 μg of random hexameric primers and 200 μl of Moloney Murine Leukemia virus reverse transcriptase (United States Biochemical Corporation, Cleveland, OH, USA) at 37°C for 60 min. This was followed by heat inactivation of the enzyme at 75°C for 5 min. Subsequently, 5 μl from this reaction was used as a template for PCR using 1× Taq DNA polymerase buffer, 200 μM dNTPs, gene specific primers and 1.5 units of TaqPlus® Long PCR systems (Stratagene, La Jolla, CA, USA). The amplification conditions comprised of an initial cycle of denaturation at 94°C for 5 min, 30 cycles of 94°C for 45 s, 65°C for 1 min, 72°C for 1 min and a final incubation for 10 min at 72°C. The different primers used in RT-PCR reactions were (i) Pmyf (5’-CCAGGGCTCCAAGCATCCGTG-3’) (ii) Poxr (5’-CGGTGATGACCGGACCTTGGC-3’) (iii) Poxf (5’-GGCCTGGCAATTCTTCCTCCC-3’) (iv) Punr1 (5’-CGTTGAGCTGCGCATCATCG-3’) (v) Punf1 (5’-TCTGGAACCACTTGGAAATGT-3’) (vi) Punr2 (5’-GTTTTCCAGCGCCGAAATCT-3’) (vii) Punf2 (5’-CCACCAGACGCTTACTCTTG-3’) and (viii) PacoA (5’-CTCAGCGACTTGGGCGATCAG-3’).

2.3. Disruption of the virS gene of *M. tuberculosis*

The parent plasmid pSG10[16,17] carrying the entire *virS* gene (*Rv3082c*) along with flanking sequences was used to construct non-replicative vector p10AVKH for the disruption of *virS*. pSG10 was digested with *Bam*HI and *Msc*I and a 190-bp DNA fragment internal to *virS* (*Rv3082c*) was replaced with the gene encoding resistance to hygromycin (hyg). The hyg gene for this purpose was excised as a 1.9-kb *Bam*HI–XbaI fragment from plasmid pLk28res-hyg-res (kind gift from Dr. S. Bardarov, Albert Einstein College of Medicine, NY, USA) and end repaired prior to cloning in pSG10. The resulting plasmid was called p10virShy. The construct was further modified by cloning in its *Dra*I site, an *Nhe*I–*Bst*EI fragment from pSD5 encoding resistance to kanamycin to generate p10AVKH. This plasmid was pretreated with UV[21,22] before electroporating it into *M. tuberculosis* Erdman. The transformants were selected on MB 7H10 agar plates supplemented with hygromycin. Loss of *virS* (*Rv3082c*) was confirmed by Southern blot and Western blot analysis using genomic DNA and cell free extracts, respectively, which were prepared using the *virS* mutant of *M. tuberculosis* (*MtbΔvirS*).

2.4. Study of the regulation of the promoters of *virS* (*Rv3082c*) and *mysA* operon (*Rv3083–Rv3089*)

For this purpose, pSD5B, an *E. coli*-mycobacteria shuttle vector carrying the promoterless lacZ gene encoding β-galactosidase was employed[23]. A 1.38-kb *Bam*HI–*Bst*I fragment from pSG10, which carries the promoters of *virS*...
and mymA operon located divergently to each other, was end repaired and ligated to XhoI digested and end repaired pSD5B in either orientation to obtain Pmym-lacZ and Pvir-lacZ. Pmym-lacZ and Pvir-lacZ carry 709 and 746 nucleotides upstream of the translational initiation codons of mymA (Rv3083) and virS (Rv3082c), respectively.

To study the effect of environmental stresses on the expression of mymA operon and virS, M. tuberculosis Erdman and MtbAvirS were individually transformed with Pmym-lacZ, Pvir-lacZ and Phsp-lacZ (carrying the lacZ gene under the transcriptional control of promoter of hsp60) and cultured as described above. Exponentially growing cultures of each of the transformants were divided into five parts. Parts 1 and 2 were incubated at 37°C (control) and 42°C (heat shock), respectively, and incubation was continued at 37°C. The other two parts were pelleted, resuspended in MB 7H9 medium at pH 6.9 and 5.0 (acidic stress), respectively, and incubation was continued at 37°C. After 12 h, the cells from all these cultures were pelleted and used to prepare cell free extracts. The promoter strength was determined by measuring the specific activity of β-galactosidase [24].

2.5. Construction of egfp (enhanced green fluorescent protein) based mycobacterial reporter strains

To study the expression of mymA operon in macrophages, an egfp based promoter probe vector pND4-egfp was constructed. The promoterless egfp was excised from pEGFP-1 (Clontech laboratories, Inc., CA, USA) by digestion with NcoI. This DNA fragment was end repaired and ligated to EcoRV digested plitmus-38 (New England Biolabs, Beverly, MA, USA) to generate plitmus-38-egfp. Next, the PstI–MluI fragment containing the entire egfp from plitmus-38-egfp was ligated with similarly digested pND4 (pSD5 is an E. coli-mycobacteria shuttle vector [25], pND4, a derivative of pSD5 has an additional BamHI site for promoter cloning) to create pND4-egfp. pSG10 was digested with BamHI and BglII to get a 1.38-kb DNA fragment (which includes 709 bp upstream of the translational start site of mymA (Rv3083)). This fragment was cloned into the BamHI promoter cloning site of pND4-egfp to generate Pmym-egfp. Finally, the PstI–MluI fragment containing egfp from plitmus-38-egfp was cloned into similarly digested pSD5shp [26] to obtain Phsp-egfp. This construct was employed as a control for constitutive gene expression in infected macrophages.

M. tuberculosis H37Ra was transformed with Pmym-egfp and Phsp-egfp individually. The transformants were selected on MB 7H10 agar plates containing kanamycin. A single transformant colony was inoculated in 5 ml of MB 7H9 medium. It was further subcultured in 50 ml of MB 7H9 medium and grown to an OD600 of 1.5. The bacterial clumps in the culture were removed by centrifugation at 200 × g for 5 min. The supernatant was passed through an insulin syringe (29G extra fine needle) 10 to 20 times to obtain a single cell suspension for macrophage infections. Bacterial viability and colony forming units were determined by plating serial dilutions of the mycobacterial suspension on MB 7H10 agar plates containing kanamycin.

2.6. Infection of macrophages to study the intracellular expression of the mymA operon

J774A.1 mouse macrophages were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (Gibco Invitrogen Corporation, NY, USA). Prior to bacterial infection, cells were washed thrice with 1 × Hank’s balanced salt solution (HBSS). Approximately 10⁶ cells were seeded per 90-mm-diameter tissue culture dish and allowed to attach overnight at 37°C. Next, the macrophages were infected at a MOI (multiplicity of infection) of 10–20 bacteria per macrophage. Six hours post-infection, the monolayers were washed thrice with pre-warmed 1 × HBSS to remove any extracellular bacteria and the incubation was continued in the presence of fresh medium supplemented with 50 μg ml⁻¹ of gentamicin. The infected macrophages could be maintained for 6 days without any apparent loss of monolayer viability. On days 0, 4 and 6, the monolayers were washed thrice with 1 × HBSS, the cells were scraped from the tissue culture plates and lysed by the addition of 0.1% Triton X-100. Cell debris was removed by centrifugation at 200 × g for 5 min at 4°C and the bacilli in the supernatant were pelleted at 2500 × g for 15 min at 4°C. The bacterial pellet was resuspended in 1 × phosphate buffered saline and passed through an insulin syringe to break cell clumps. A total of 20’000 bacteria were analyzed as a function of side scatter and GFP fluorescence by using FACScan (Becton Dickinson). Quantitative measurements were carried out with the FLOWJO software program (Becton Dickinson).

2.7. Computer analysis

The comparisons of genome sequences of Mycobacterium bovis, Mycobacterium leprae (http://www.sanger.ac.uk), M. tuberculosis CDC 1551, Mycobacterium avium and M. smegmatis (http://www.tigr.org) and comparisons with sequences in the non-redundant database (http://www.ncbi.nlm.nih.gov) were carried out by using BLAST algorithm to identify the homologues of genes present in the mymA operon. RPS-BLAST searches were performed for the identification of conserved domains. Primary sequence of VirS was analyzed by Net Phos 2.0 and PROSITE Biosoftwares for the presence of putative phosphorylation motifs.

2.8. Electron microscopy

Cells were fixed for 24 h in 4% paraformaldehyde/2.5%
glutaraldehyde in 100 mM sodium cacodylate buffer, pH 7.4. Next, the cells were fixed in Karnovsky's solution containing 0.5% OsO4/0.8% K3Fe(CN)6 followed by 1% tannic acid and stained overnight en bloc in 1% uranyl acetate. Samples were dehydrated in a graded ethanol series and embedded in Spurr's resin. Thin sections were cut with an ultramicrotome (Leica; ultracut E), stained with 1% uranyl acetate and Reynold's lead citrate and observed at 80kV in a Philips CM-10 transmission electron microscope.

3. Results

We had earlier reported identification of a 38-kDa protein encoded by \textit{Rv3082c} gene of \textit{M. tuberculosis} [16,17]. This protein, here referred to as VirS based on the nomenclature adopted by Cole et al. [27], exhibits sequence similarity with proteins that regulate transcription of genes required for establishment of disease by \textit{Yersinia}, \textit{Shigella} and ETEC [18]. \textit{Rv3083} (referred to as \textit{mymA} in this article) is placed divergently to \textit{virS} (\textit{Rv3082c}) in \textit{M. tuberculosis} genome and is followed by six genes (\textit{Rv3084–Rv3089}) in a manner that suggests an operon-like arrangement. We questioned if \textit{virS} (\textit{Rv3082c}) regulates the transcription of this putative operon, as is the case in several pathogenic bacteria in which some transcriptional regulators are arranged divergently to the genes under their control [28,29]. In this study, we present further characterization of the locus comprised of \textit{Rv3083–Rv3089} and its regulation by VirS.

3.1. \textit{Rv3083–Rv3089 constitute an operon in \\textit{M. tuberculosis}}

The seven open reading frames, \textit{Rv3083–Rv3089}, are oriented in the same direction in the \textit{M. tuberculosis} genome, with a maximum distance of 38 bp between any two open reading frames. We wondered if these genes constitute a transcriptional unit. To investigate this, we carried out RT-PCR analysis using total RNA isolated from \textit{M. tuberculosis} and four primer sets (lane 1: \textit{Pmyf/Poxr}, lane 2: \textit{Poxf/Punr1}, lane 3: \textit{Punf1/Punr2}, and lane 4: \textit{Punf2/PacoA}) to confirm the arrangement of genes as an operon. PCR reactions were analyzed on a 1% agarose gel. Lane M represents a 100-bp DNA ladder.

![Diagram of operon](image-url)

**Fig. 1.** RT-PCR analysis of the \textit{mymA} operon. A: Schematic representation of the arrangement of open reading frames \textit{Rv3082c} to \textit{Rv3089} in \textit{M. tuberculosis} \textit{H}37\textit{Rv} genome (open arrows). Solid arrows indicate the location of primers used to perform RT-PCR. \textit{sadh} and ? represent the genes encoding short chain alcohol dehydrogenase and conserved hypothetical protein, respectively. B: RT-PCR analysis. Total RNA (5 μg) from \textit{M. tuberculosis} \textit{H}37\textit{Rv} was reverse transcribed using random primers and cDNA obtained was subjected to PCR amplification using four sets of primers (lane 1: \textit{Pmyf/Poxr}, lane 2: \textit{Poxf/Punr1}, lane 3: \textit{Punf1/Punr2}, and lane 4: \textit{Punf2/PacoA}) to confirm the arrangement of genes as an operon. PCR reactions were analyzed on a 1% agarose gel. Lane M represents a 100-bp DNA ladder.
for PCR confirming the specificity of the products of the RT-PCR reactions. These observations established that the genes Rv3083–Rv3089 are transcribed from a single promoter and constitute an operon referred to as ‘mymA operon’ (named after the first gene Rv 3083, which is a homologue of several monooxygenases present in the M. tuberculosis genome and thus designated mymA, i.e. mycobacterial monooxygenase).

3.2. Regulation of the mymA operon by virS (Rv3082c)

To investigate if virS (Rv3082c), which is located diver-
gerately to mymA (Rv3083), regulates the expression of the mymA operon, we created a virS (Rv3082c) insertion-deletion mutant of \( M. \) tuberculosis. This was achieved by the allelic exchange of the wild-type virS (Rv3082c) gene on the \( M. \) tuberculosis genome with a mutant copy marked with the gene encoding resistance to hygromycin (Fig. 2A). The hygromycin resistance gene, which replaced the 190 bp of virS open reading frame, was inserted in this open reading frame in the same orientation as virS at a location far away from the mymA operon (709 bp upstream to the start codon of mymA and in orientation opposite to that of the mymA operon) to avoid any polar effect. The resulting mutants were selected by their ability to grow in the presence of hygromycin. The disruption of virS (Rv3082c) was confirmed by Southern blot analysis using the genomic DNA from \( M. \) tuberculosis and its virS mutant strain (Mt\( b \Delta \)virS) which was digested with the restriction enzyme \( Pvu \)II. As shown in Fig. 2B, a 3.5-kb \( Pvu \)II DNA fragment in the wild-type \( M. \) tuberculosis (lane 1) was replaced with a 5.2-kb fragment in Mt\( b \Delta \)virS (lane 2), which is consistent with the disruption of virS (Rv3082c) in the mutant. This was further confirmed by immunoblot analysis using polyclonal antibodies raised against VirS (Fig. 2C). The wild-type strain showed a specific protein band of 38 kDa corresponding to VirS (lane 2, Fig. 2C), which was absent in the mutant strain (lane 1, Fig. 2C).

Further, to investigate regulation of the mymA operon by virS (Rv3082c), we constructed reporter plasmids Pmym-lacZ and Phsp-lacZ carrying the lacZ gene under the transcriptional control of promoters of the mymA operon and hsp60, respectively. Wild-type \( M. \) tuberculosis as well as Mt\( b \Delta \)virS were separately electroporated with these plasmids. The transformants were grown to an OD\( _{600} \) of 1.5 and \( \beta \)-galactosidase activity was measured in their cell free extracts. The wild-type \( M. \) tuberculosis harboring Pmym-lacZ supported a \( \beta \)-galactosidase activity of 783 ± 67 nmol min\(^{-1} \) mg\(^{-1} \) in contrast to Mt\( b \Delta \)virS harboring the same construct, which did not show any significant activity of \( \beta \)-galactosidase above the background levels (20 ± 2.5 nmol min\(^{-1} \) mg\(^{-1} \), Fig. 2D). These observations show that transcription from the promoter of the mymA operon is dependent on the presence of VirS as evident from the loss of \( \beta \)-galactosidase activity in the cell-free extract of Mt\( b \Delta \)virS carrying Pmym-lacZ. On the other hand, the wild-type \( M. \) tuberculosis and Mt\( b \Delta \)virS transformed with Phsp-lacZ (used as a control for constitutive gene expression) showed no difference in their enzyme activities and supported \( \beta \)-galactosidase activity of 16,000 ± 690 nmol min\(^{-1} \) mg\(^{-1} \) and 15,500 ± 540 nmol min\(^{-1} \) mg\(^{-1} \), respectively (Fig. 2D). This substantiated the specificity of regulation of the mymA operon’s promoter by VirS.

To test if virS (Rv3082c) autoregulates its own synthesis, we constructed Prvir-lacZ, which carries the lacZ gene under the transcriptional control of the virS (Rv3082c) promoter region as described in Section 2. It was then electroporated into the wild-type \( M. \) tuberculosis and Mt\( b \Delta \)virS separately. In wild-type \( M. \) tuberculosis, Prvir-lacZ supported a \( \beta \)-galactosidase activity of 1748 ± 107 nmol min\(^{-1} \) mg\(^{-1} \). However, the same construct in Mt\( b \Delta \)virS exhibited a \( \beta \)-galactosidase activity of 742 ± 210 nmol min\(^{-1} \) mg\(^{-1} \) suggesting that VirS can down-regulate its own synthesis (Fig. 2D).

3.3. mymA operon is induced under low pH and requires the presence of virS (Rv3082c)

\( M. \) tuberculosis experiences various environmental stresses such as oxygen radicals, nitrogenous compounds, degradative enzymes, change in pH and deprivation of nutrients during its residence in the host cells. By using microarray analysis, a recent study had demonstrated an up-regulation of expression of the genes Rv3083–Rv3089 of \( M. \) tuberculosis, under low pH conditions [30]. This was indeed found to be the case in our studies employing lacZ as the reporter gene. The wild-type \( M. \) tuberculosis transformed with either Pmym-lacZ or Phsp-lacZ was grown under different pH conditions followed by determination of \( \beta \)-galactosidase activity in the cell free extracts. We noticed a four-fold increase in the specific activity of \( \beta \)-galactosidase when \( M. \) tuberculosis harboring Pmym-lacZ was exposed to an acidic pH of 5.0 (3240 ± 230 nmol min\(^{-1} \) mg\(^{-1} \)) as compared to that at pH of 6.9 (783 ± 67 nmol min\(^{-1} \) mg\(^{-1} \)) (Fig. 2E). However, no significant change in the hsp60 promoter driven expression of lacZ was observed when \( M. \) tuberculosis transformed with Phsp-lacZ was grown at the neutral or acidic pH (data not shown). These results confirmed that the promoter of the mymA operon is specifically induced under the acidic environment.

When Mt\( b \Delta \)virS was transformed with Pmym-lacZ, no difference in \( \beta \)-galactosidase activity in the cultures grown at neutral or acidic pH was observed. The enzyme activity in both these conditions was 20 ± 2.5 nmol min\(^{-1} \) mg\(^{-1} \) (Fig. 2E), indicating that the presence of a functional copy of virS (Rv3082c) was necessary for the mymA operon’s promoter to respond to low pH conditions. We did not observe any changes in the mymA operon’s promoter activity in the presence of H\( _2 \)O\(_2 \) or heat shock (data not shown).

3.4. Regulation of virS promoter under acidic conditions

We also investigated if the virS (Rv3082c) promoter is regulated differentially at low pH. For this, \( M. \) tuberculosis harboring Prvir-lacZ (carrying lacZ under the transcriptional control of the virS promoter) was grown at neutral pH (6.9) and acidic pH (5.0) as described above and the activity of \( \beta \)-galactosidase was determined in the cell free extracts. We observed a two-fold induction in \( \beta \)-galactosidase activity upon exposure to acidic pH (3650 ± 115 nmol min\(^{-1} \) mg\(^{-1} \) protein) as compared to neutral pH (1748 ±
107 nmol min$^{-1}$ mg$^{-1}$ protein) (Fig. 2E). This observation suggested that synthesis of VirS is induced under acidic conditions. When MtbΔvirS was transformed with Pvir-lacZ, no induction in β-galactosidase activity was observed in the cultures grown at acidic pH. The β-galactosidase activities at both neutral and acidic pH were 7428 ± 210 nmol min$^{-1}$ mg$^{-1}$ and 7420 ± 290 nmol min$^{-1}$ mg$^{-1}$, respectively (Fig. 2E).

3.5. Intracellular expression of the mymA operon

Having demonstrated that expression of the promoter of the mymA operon is induced under low pH conditions in broth cultures, we wanted to examine if its expression in the macrophages was altered. For this purpose, we constructed Pmym-gfp and Phsp-gfp, which carry the egfp reporter gene (encoding EGFP), under the transcriptional control of the mymA operon’s promoter and hsp60 promoter, respectively, as described in Section 2. The plasmid Pmym-gfp was used to transform the wild-type M. tuberculosis and the resulting strain was used to infect the macrophage cell line J774A.1. Macrophages were also infected separately with an M. tuberculosis strain transformed with Phsp-gfp construct to use as a control for constitutive gene expression. The macrophages were infected with M. tuberculosis harboring Pmym-gfp or Phsp-gfp at days 0, 4 and 6 post-infection was assessed by flow cytometry (white histogram) and compared to the mean fluorescence intensity of bacteria cultured in MB 7H9 broth (gray histogram) as described in Section 2.

Fig. 3. Induction of the promoter of the mymA operon of M. tuberculosis in macrophages. J774A.1 macrophages cell line was infected with M. tuberculosis harboring Pmym-gfp (mymA operon promoter transcriptionally fused to promoterless egfp gene) or with Phsp-gfp (hsp60 promoter transcriptionally fused to promoterless egfp gene). Macrophages infected with M. tuberculosis harboring Pmym-gfp were visualized at days 0, 4 and 6 under fluorescent microscope (A). The mean fluorescence intensity of bacteria recovered by the lysis of macrophages infected with M. tuberculosis harboring Pmym-gfp (B) and Phsp-gfp (C) at days 0, 4 and 6 post-infection was assessed by flow cytometry (white histogram) and compared to the mean fluorescence intensity of bacteria cultured in MB 7H9 broth (gray histogram) as described in Section 2.
expression. In parallel, these strains were also grown in liquid medium and the samples were collected at the indicated times. To detect any changes in the fluorescence of M. tuberculosis transformed with Pmym-gfp during the course of macrophage infection, infected macrophages were subjected to fluorescence microscopy. We observed a significant enhancement in the fluorescence of the macrophages infected with the recombinant M. tuberculosis at 4 and 6 days after infection (Fig. 3A). To quantify the changes in the observed fluorescence, M. tuberculosis transformed with either Pmym-gfp or Phsp-gfp was subjected to flow cytometric analysis at days 0 (immediately after infection), 4 and 6 post-infection. M. tuberculosis harboring Pmym-gfp showed a 0.5 and 1 log unit increase in the fluorescence intensity 4 and 6 days after infection, respectively, as compared to that exhibited by the bacteria growing in broth culture at 37°C (Fig. 3B). In contrast, the fluorescence intensity exhibited by M. tuberculosis harboring Phsp-gfp remained virtually constant throughout the course of infection (Fig. 3C). We also incubated the bacilli harboring Pmym-gfp or Phsp-gfp in DMEM medium alone to exclude any contribution of the cell culture medium in the observed changes in fluorescence intensity. The fluorescence of these bacilli remained low and relatively stable under these conditions (data not shown), thus establishing that the increase in the fluorescence intensity of the intracellular M. tuberculosis carrying Pmym-gfp was a result of specific up-regulation of the promoter of the mymA operon in the macrophage environment.

3.6. Characterization of the genes comprising the mymA operon

Computational analysis of the genes Rv3083–Rv3089 revealed that besides M. tuberculosis H37Rv, this operon is also present in M. tuberculosis CDC1551 and M. bovis. Interestingly, while the homologues of the genes comprising the mymA operon are present individually in M. leprae, M. avium and in the unfinished genomic sequence of M. smegmatis, they are not clustered or organized in an operon (data not shown). Our detailed inspection of the sequences of the proteins encoded by these genes revealed that Rv3089 (FadD13), which is an acyl-CoA synthase contain three domains (domains I, II and III), which are highly conserved among the various members of acyl-CoA synthases (data not shown). Domain I contains a consensus sequence SGXXGXPKG, glycine residues in which form the phosphate binding loop (p-loop) found in ATP and GTP binding proteins. This region is believed to bind the AMP moiety of the acyl-AMP intermediate [31]. Additionally, the domains II and III in acyl-CoA synthases are postulated to be involved in the formation of a hydrophobic pocket, which could accommodate the fatty acid moiety of the acyl-AMP intermediate [32]. Thus, it appeared that FadD13 could participate in fatty acid metabolism of M. tuberculosis.

In view of the above observation, we sought to examine the sequences of other proteins encoded by this operon for any motifs that would give further credibility to their role in fatty acid metabolism in M. tuberculosis. Our search revealed the presence of HHxxxDG motif in Rv3087 and Rv3088; this conserved motif is a common feature of the family of acyl transferases such as dihydrodiolipoyl transacylase [33] and wax ester synthase/acyl-CoA:diacylglycerol acyltransferase [34], and also of the condensation domain of NRPSs (non-ribosomal peptide synthetases) [33]. Thus, these proteins could be potentially involved in transferring an acylated product to an appropriate acceptor especially in view of their proximity to an acyl-CoA synthase (Rv3089). Such a functional association is known in several instances such as phospholipid synthesis in E. coli [35], mycocerosic acid synthesis in mycobacteria [36] and wax ester synthesis in Acinetobacter calcoaceticus ADP1 [34]. That these two acyl transferases (Rv3087 and Rv3088) and acyl-CoA synthase (Rv3089) could be functionally linked is further suggested by the overlap of the translational stop codons of Rv3087 and Rv3088 with the translational start codons of Rv3088 and Rv3089, respectively, indicating a translational fusion between these three proteins.

The first gene of mymA operon (Rv3083) encodes a monoxygenase homologue with 58% sequence similarity to the FMO (N,N-dimethylaniline monoxygenase) of humans [37]. It has NADPH and FAD binding domains placed in the same locations as found in the family of flavin containing monoxygenases. It is interesting that monoxygenases, in general, are capable of hydroxylating long chain fatty acids [38,39]. In addition, the proteins encoded by genes adjacent to the monoxygenase (Rv3083) namely, acetyl hydrolase esterase (Rv3084), short chain alcohol dehydrogenase (Rv3085) and zinc containing alcohol dehydrogenase (Rv3086) display similarities with enzymes involved in modification of the products generated by monoxygenases [40,41]. Thus, it appeared that the proteins expressed by this operon may participate in the modification of fatty acids and facilitate their transfer to the appropriate acceptor(s) in the cell wall of M. tuberculosis.

3.7. Colony morphology and the cell wall structure of the virS mutant of M. tuberculosis

Since the attributes of proteins encoded by the mymA operon suggested their involvement in the modification of fatty acids required for the cell wall of M. tuberculosis, as a next step, we compared the colony morphology and cell envelope structure of wild-type and virS mutant of M. tuberculosis. We observed that in comparison to the wild-type M. tuberculosis, its virS mutant displayed altered colony morphology, when grown on MB7H10 medium (Fig. 4A,B). Colonies of the wild-type M. tuberculosis were characteristically flat and appeared cored. However,
the colonies produced by MtbΔvirS formed rising structures rather than flat surface and appeared to be tubular in nature. These morphological changes in the appearance of M. tuberculosis colonies have been reported to be associated with the changes in the lipids on the surface of mycobacteria [9] and suggested a role for the mymA operon in the cell wall of M. tuberculosis. Further, to evaluate the effect of disruption of virS (Rv3082c) on the cell wall structure, M. tuberculosis and MtbΔvirS were analyzed by transmission electron microscopy. Both, the outer electron transparent layer (comprising primarily of mycolic acids) and the inner electron dense layer (plasmamembrane) [42] are visible in the cell wall of the wild-type M. tuberculosis (Fig. 4C). Appropriate staining of the cell surface is seen due to the normal permeability barrier. However, the cell wall of MtbΔvirS showed a drastic increase in its staining (Fig. 4D), which is indicative of altered cell wall permeability. The changes observed in the colony morphology as well as the cell wall structure in MtbΔvirS suggest that the mymA operon plays an important role in maintaining the appropriate cell wall structure.

4. Discussion

In this study, we have characterized the mymA operon (Rv3083–Rv3089) of M. tuberculosis, which is arranged in a divergent manner to virS (Rv3082c). Our investigation showed that the transcription of the mymA operon is dependent on the presence of VirS protein. To identify the environmental cues that might trigger an up-regulation of the mymA operon, we inspected its expression under various in vitro conditions that simulate those faced by M. tuberculosis in the host environment. We observed that VirS is essential for transcription from the mymA operon promoter. However, a four- to five-fold induction of the promoter of the mymA operon by VirS occurs specifically at acidic pH. This may be due to increased synthesis of VirS at acidic pH. Alternatively, the acidic pH might change the phosphorylation state of VirS, which could improve its affinity for the promoter region of the mymA operon. In this context, it is interesting that the primary sequence analysis of VirS shows the presence of nine putative protein kinase C phosphorylation motifs, [ST]-x-[RK]. Interestingly in our earlier publication on virS (Rv3082c) [17], we have reported the identification of a 12-bp indirect repeat which is present in between the open reading frames of virS (Rv3082c) and mymA (Rv3083) and overlaps with the −10 region of the virS promoter, suggesting that it may possibly play a role in the regulation of the mymA operon by VirS. Besides, at present it is not certain whether VirS directly regulates the promoter of the mymA operon or its action is mediated by another protein. However, induction of the mymA operon
at acidic pH and on infection of macrophages with *M. tuberculosis* underscores the importance of the encoded gene products in processes that are important during the mycobacterial residence in the host environment. In an effort to identify the phenotype of the *virS* mutant of *M. tuberculosis* inside the macrophages, we carried out survival assay of the *MtbΔvirS* mutant in J774A.1 murine macrophage cell lines. Interestingly, the *virS* mutant of *M. tuberculosis* showed a similar survival rate to the parental strain of *M. tuberculosis* in these cells (unpublished data). However, as macrophages do not necessarily mimic all conditions encountered by mycobacteria in vivo, we are currently initiating studies to investigate the phenotype of *virS* mutant in the guinea pig model of tuberculosis.

An extensive analysis of the conserved domains and the core motifs present in the gene products encoded by the *mymA* operon suggested that mycobacteria might use it for modification, activation and transfer of fatty acids to the appropriate acceptor(s) in their cell wall. *mymA*, a monoxygenase encoded by *Rv3083*, could potentially oxygenate mycobacterial fatty acids. The oxygenated fatty acids could be further modified by the acetyl hydrolase/esterase (*Rv3084*), short chain alcohol dehydrogenase (*Rv3085*) and zinc containing alcohol dehydrogenase (*Rv3086*). Finally, the acyl-CoA synthase homologue (*Rv3089*) could then activate the fatty acids (modified by the products of genes *Rv3083*- *Rv3086*), which could subsequently be transferred to an acceptor in the cell wall of mycobacteria by acyl transferases (*Rv3087* and *Rv3088*).

In a recent study involving the microarray analysis of *M. tuberculosis*, Fisher et al. [30] had demonstrated a significant up-regulation of genes *Rv3083*- *Rv3089* under acidic conditions along with a two-fold reduction in the expression of genes present in the FAS II operon. FAS II operon is responsible for the biosynthesis of meromycolic acids in *M. tuberculosis* by elongating long chain fatty acid precursors like C24 and C26 generated by the FAS I system [43]. Down-regulation of the FAS II system at low pH would be expected to decrease fatty acid elongation, leading to an accumulation of C24 and C26 fatty acids. However, since the *mymA* operon is up-regulated at acidic pH, it can utilize the C24 or C26 fatty acids and as suggested above, modify and transfer them to appropriate biological acceptor(s) on the mycobacterial cell wall. Thus, the induction of the *mymA* operon can play an important role in remodeling the envelope of intracellular *M. tuberculosis* under acidic conditions in the macrophages. Detailed biochemical characterization of the gene products of the *mymA* operon should provide a better understanding of their role in *M. tuberculosis*.

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