International Journal of Medical Microbiology 309 (2019) 151353

Contents lists available at ScienceDirect

International Journal of Medical Microbiology

journal homepage: www.elsevier.com/locate/ijmm

Mycobacterium tuberculosis DosR regulon gene Rv2004c contributes to streptomycin resistance and intracellular survival

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ARTICLE INFO

Keywords:
Mycobacterium tuberculosis
DosR regulon
Rv2004c
MSMEG_3942
SM
Aminoglycoside phosphotransferase
Mycobacterium smegmatis
Escherichia coli

ABSTRACT

Tuberculosis (TB) is the deadly infectious disease challenging the public health globally and its impact is further aggravated by co-infection with HIV and the emergence of drug resistant strains of *Mycobacterium tuberculosis*. In this study, we attempted to characterise the Rv2004c encoded protein, a member of DosR regulon, for its role in drug resistance. *In silico* docking analysis revealed that Rv2004c binds with streptomycin (SM). Phosphotransferase assay demonstrated that Rv2004c possibly mediates SM resistance through the aminoglycoside phosphotransferase activity. Further, *E. coli* expressing Rv2004c conferred resistance to 100μM of SM in liquid broth cultures indicating a mild aminoglycoside phosphotransferase activity of Rv2004c. Moreover, we investigated the role of *MSMEG_3942* (an orthologous gene of Rv2004c) encoded protein in intracellular survival, its effect on *in-vitro* growth and its expression in different stress conditions by over expressing it in *Mycobacterium smegmatis* (*M. smegmatis*). MSMEG_3942 overexpressing recombinant *M. smegmatis* strains grew faster in acidic medium and also showed higher bacillary counts in infected macrophages when compared to *M. smegmatis* transformed with vector alone. Our results are likely to contribute to the better understanding of the involvement of Rv2004c in partial drug resistance, intracellular survival and adaptation of bacilli to stress conditions.

1. Introduction

Tuberculosis (TB) caused by the intracellular pathogen *Mycobacterium tuberculosis* (Mtb) is a global emergency and a major cause of mortality around the world. Despite strong efforts to control the disease, about 10.0 million new TB cases were reported and 1.6 million deaths occurred in the year 2017 (WHO, Global Tuberculosis Report 2018). During latent infection, Mtb enters into dormant state with a physiological shift of replicating bacilli towards non-replicating, non-virulent and persistent state (Rustad et al., 2008) or dormant state in response to hypoxia by using *dosRS* two component regulatory system (Park et al., 2003; Roberts et al., 2004). The *dosRS* system senses the environmental stress conditions such as hypoxia, high nitric oxide and low pH that prevail in granulomas and regulates the transcription of a regulon that comprises 48 genes called as the DosR regulon (Honaker et al., 2010; Voskuil et al., 2003; Yang et al., 2018). The individual contribution of each of the DosR regulon genes to the latency system is not well studied. It is critical to unravel the functional contributions of DosR regulon genes as this will shed light on understanding the biological significance of these class of genes in the survival and persistence of the bacilli.

One of the major problems of treating the latent tuberculosis infection is persistence of the bacilli as they respond poorly to the drugs when their metabolic processes are shutdown or down regulated since most of the current drugs act only on actively growing bacteria (Gengenbacher and Kaufmann, 2012; Kumar et al., 2012). Population heterogeneity in dormant bacilli might confer drug resistance, a phenotype that challenges the ability of current drugs to treat latent tuberculosis infection efficiently (Barry et al., 2009; Dhar and McKinney, 2007). Although it was reported that continuous, 6–9 months of treatment with isoniazid reduces the risk of latent infection, existing drugs are not effective in eliminating the latent tuberculosis (Spyridis

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https://doi.org/10.1016/j.ijmm.2019.151353
Received 16 April 2019; Received in revised form 26 July 2019; Accepted 29 August 2019

et al., 2007; World Health Organization, 1982). The increasing emergence of resistant strains of Mtb to anti-TB drugs created interest to understand the underlying mechanisms of drug resistance and substantial development has been achieved in this direction (Nguyen, 2016). Among several anti-TB drugs, aminoglycosides are the second line of antibiotics often administered for TB relapse cases. These antibiotics have an affinity for the 16S rRNA of 30S ribosomal subunit as they inhibit protein synthesis by interfering with the binding of aminoacyl-tRNA to small subunit of ribosome (Kotra et al., 2000). Aminoglycosides are also capable of damaging the integrity of cell wall by forming divalent cations with LPS (Vaara, 1992). Emergence of increasing number of resistant isolates of Mtb has limited the use of aminoglycosides, particularly streptomycin (SM) for the treatment of TB (Verma et al., 2014). Generally, resistance to aminoglycosides occurs either by mutations in the genes encoding 16S rRNA, S12 ribosomal proteins or decreased uptake of drugs due to changes in membrane permeability and/or over expression of active efflux pumps (Honoré and Cole, 1994; Spies et al., 2008; Springer et al., 2001). Nevertheless, one third of the resistant isolates of Mtb do not contain these types of mutations indicating the involvement of other possible mechanisms that confer aminoglycoside resistance (Draker et al., 2003; Wright, 1999). It was reported that chemical modification or detoxification of the aminoglycosides by bacterial enzymes could also confer resistance to these antibiotics (Ahn and Kim, 2013; Hegde et al., 2001; Kim et al., 2011). Recently, Rv3168 of Mtb was reported to mediate a mild kanamycin resistant phenotype when overexpressed in *E. coli* system (Ahn and Kim, 2013). Depending on the chemical group that is modified, the possible three types of enzymatic modifications of aminoglycosides include *N*-acetylation, *O*-phosphorylation and *O*-nucleotidylation. Aminoglycoside *O*-phosphotransferase (APH) catalyses ATP dependent phosphorylation of hydroxyl groups of aminoglycosides. Among different classes of APH enzymes, APH (3′)-IIIa is the well-studied enzyme (Draker et al., 2003; Hegde et al., 2001; Wright, 1999).

Rv2004c is a member of DosR regulon. In our previous work, we reported the binding and interaction of Rv2004c with TLR-2 surface receptor and its ability to induce pro-inflammatory cytokines *via* NF-κB pathway (Doddam et al., 2017). In another study, peptide fragments of Rv2004c were shown to interact with surface of macrophages (Forero et al., 2005). Rv2004c was reported to be upregulated under NO stress conditions (Voskuil et al., 2003) and this gene was shown to be essential for the survival of Mtb in murine macrophages (Rengarajan et al., 2005). In this study, we investigated the functional role of Rv2004c for mild aminoglycoside phosphotransferase activity and its effect on *in vitro* growth and intracellular survival of bacilli using *M. smegmatis* as a model organism.

2. Materials and Methods

2.1. Bacterial strains, plasmids, media and growth conditions

E. coli DH5α was used for cloning of *Rv2004c* gene into PET28a and pRSET-A vectors between *Bam* HI and *Hind* III restriction sites. The recombinant *E. coli* strains were cultured in LB broth at 37 °C and 200 rpm for overnight period; subsequently glycerol stocks were prepared and stored at −80 °C.

E. coli BL21 (DE3) pLysS strain (supplemented with 50 μg/ml kanamycin and 34 μg/ml chloramphenicol) was used to express recombinant Rv2004c protein. The recombinant bacterial culture was grown to an OD_{600} of 0.3 and induced with 0.3 mM IPTG (Sigma, USA) to express Rv2004c. *M. smegmatis* mc^26 (procured from IMTECH-MTCC, Chandigarh) was cultured upto an OD_{600} of 1 in Middlebrook 7H9 broth (Himedia, France) with 2% glycerol and 0.05% Tween80 and 10% OADC. Bacterial glycerol stocks were prepared and stored in −80 °C.

2.2. Protein sequence analysis of Rv2004c and docking study

The amino acid sequence of Rv2004c gene (hereafter referred as query protein) was set for BLASTP (Altschul et al., 1990) against the PDB database (Berman et al., 2000). Since suitable template for the selected query protein for homology modelling was unavailable, LOMETS (Wu and Zhang, 2007), a local protein threading server for prediction of the suitable three dimensional structure was used. The 10 different templates identified by LOMETS were subjected to MODELLER (Sali and Blundell, 1993). Out of the top 5 homology models generated by LOMETS, the first model was selected and set for Ramachandran Plot analysis using RAMPAGE server (Lovell et al., 2003).

2.3. Docking of modeled query protein with SM

Energy minimization was performed using GROMACS for the selected homology model of the query protein structure (Berendsen et al., 1995). The three dimensional structure of SM was downloaded from RCSB database [http://www.rcsb.org/ligand/SRY]. The energy minimized three dimensional structure of the query protein was taken as receptor and subjected for docking studies with SM as the ligand using Patch Dock (Schneidman-Duhovny et al., 2005). Hydrogen bonds in the docked complex were determined using Ligplus+ (Laskowski and Swindells, 2011) and visualized using Pymol (DeLano, 2002).

2.4. Aminoglycoside phosphotransferase assay

Previous studies have demonstrated that both Thin Layer Chromatography (TLC) and spectrophotometric assays give similar results in accuracy and precision (Baghdady et al., 2013). Hence, we have opted TLC method for performing aminoglycoside phosphotransferase assay. The assay was carried out in a 15 μl reaction mixture containing 50 mM Tris (pH 8.0), 5 mM ATP, 2.5 mM $MgCl_2$, 200 μg SM and 10 μg of Rv2004c which was incubated for overnight at 37 °C. 2 μl of reaction mixture was spotted on cellulose F-TLC sheet (Merck Millipore, Germany) and ascending thin layer chromatography was performed in aqueous solution of 1 M NaCl for 2 h. Standard ATP and ADP molecules were used as reference. For controls, reaction mixtures without SM or Rv2004c were used. The hydrolysis of ATP was visualized by exposing the TLC sheet under UV light.

2.5. Antibiotic resistance assay

The recombinant construct *Rv2004c* + pRSET-A was transformed into *E. coli* BL21 (DE3) pLys S strain. A single colony from the plate was inoculated and grown overnight in 10 ml of fresh LB broth (containing 1% glucose, ampicillin and chloramphenicol); 1% of the overnight grown culture was inoculated in 100 ml of LB broth and incubated in shaker incubator at 37 °C. When the O.D. reached to 0.35, the culture was washed twice with LB broth and then re-suspended in the same volume of fresh LB broth following induction with 0.3 mM IPTG for a period of 30 min. Later, different concentrations of SM (50 μM, 100 μM and 200 μM) were added to the IPTG induced cultures. OD at 600 nm was monitored spectrophotometrically at an interval of every 1 h, for 15 h after addition of SM. As a control, growth rate of *E. coli* cultures without IPTG induction was measured similarly. Growth curve of *E. coli* transformed with empty pRSET-A vector was measured as described above. An aliquot of each culture was plated on LB agar plate containing ampicillin to measure the viable cell count.

2.6. Cloning of MSMEG_3942 in pMV261vector

To study the role of Rv2004c in the growth of Mtb, we used *M. smegmatis* as a model organism in our study, which has been extensively employed to investigate the Mtb physiology in several studies (Cascioferro et al., 2007; Dheenadhayalan et al., 2006; Trauner et al.,

Table 1
List of primers used in this study.

S.No	Primer name	Sequence	Reference
1	MSMEG_3942	F-5'CG**GGATCC**ATGGAGGACAAGCCGGAAACG3' R-5'CC**AAGCTT**CTAGATCGCCAGACAGCAGACGC3'	This study
2	qRT_MSMEG_3942	F-5'GCGCCGGCTGGCTTCGCAGTA3' R-5'GACAGTCGAGAATGGCCGGTCCC3'	This study
3	qRT_MSMEG_SigA	F-5'CGCGCCTACCTCAAGCAGATCGGC3' R-5'GCCCTTCTCGGCGAGTTCGGCC3'	This study

2012). We have chosen to overexpress the *MSMEG_3942*, an orthologous gene of *Rv2004c* in *M. smegmatis* to study the *in vitro* and *in vivo* growth properties, as MSMEG_3942 shares 57% of homology and similar domains with Rv2004c (analysed by using NCBI online tool protein BLAST; Supplementary Fig. S2). Hence, MSMEG_3942 might exhibit similar function as that of Rv2004c. pMV261 vector is specifically used to overexpress mycobacterial genes (Liu et al., 2017). The plasmid has a strong constitutive gene promoter HSP 60 that drives higher gene expression levels (Stover et al., 1991). *MSMEG_3942* was amplified using the forward and reverse primers as listed in Table 1 and cloned into pMV261 shuttle vector at the *Bam* HI and *Hind* III restriction sites. The recombinant *MSMEG_3942* + pMV261construct and empty pMV261 vector were electroporated into *M. smegmatis*. Recombinant *M. smegmatis* strains harbouring MSMEG_3942 (MS_MSMEG_3942) and empty pMV261 (MS_Vec) were selected against kanamycin in Middlebrook 7H10 agar plates supplemented with Middlebrook 7H10 Agar (Himedia, India) containing 10% OADC and 0.05% Tween 80.

2.7. MSMEG_3942 expression under different conditions

To see the fold of expression of *MSMEG_3942* gene in MS_MSMEG_3942 and MS_Vec strains, cultures were grown to late log phase in normal conditions. RNA was isolated by Trizol method. Two micrograms of RNA was subjected to DNase (Sigma) treatment and converted into cDNA using the first strand cDNA superscript III synthesis kit (Invitrogen, USA). To examine the expression of *MSMEG_3942* in stress conditions (low pH and micro-aerobic), *M. smegmatis* was grown until late log phase. For acidic stress, 25 ml of bacterial culture was washed twice with Middlebrook 7H9 broth (pH 5.3) and finally resuspended in 25 ml of 7H9 broth (pH 5.3) and incubated for 24 h at 37 °C with shaking. For micro-aerobic stress, cells from 25 ml culture were taken into 50 ml falcon tube and filled with 7H9 broth to the top of the tube and incubated at 37 °C without shaking for 24 h (Garg et al., 2015). RNA was isolated from 5 ml of the culture and converted to cDNA using the cDNA synthesis kit (Invitrogen, USA). For all cDNA samples, qRT PCR was performed to quantify the expression of *MSMEG_3942* in a real time PCR machine (Eppendorf, Germany). *M. smegmatis sig*A was used as internal control for normalization. The real time PCR reaction was carried out in a 96 well plate (Eppendorf) with a final reaction volume of 10 µl containing SYBR green master mix (Takara, Japan) and forward and reverse primers as listed in Table 1. Fold of expression of genes was analysed by using $2^{-\Delta\Delta\ CT}$ method as described earlier (Schmittgen and Livak, 2008).

2.8. Growth curve experiments with M. smegmatis strains

To obtain the growth curves under normal physiological conditions (stress free), Middlebrook 7H9 broth containing 25 µg/ml kanamycin was inoculated with late exponential cultures of MS_MSMEG_3942 and MS_Vec strains and O.D was adjusted to 0.03. For growth curve under acidic stress, cells were harvested, washed twice with 7H9 broth and inoculated with an initial OD of 0.05 in 7H9 broth (pH5.3) containing 10% OADC. In both growth curves, growth was measured at every 3 h interval for 72 h, spectrophotometrically, and CFUs were determined on Middlebrook 7H10 agar plates.

2.9. Intracellular survival assay

THP-1 cells were seeded at a concentration of one million per well in 12 well tissue culture plates (Corning, USA). Cells were treated with Phorbol 12-myristate 13-acetate (PMA) (Sigma, MO, USA) for 36 h to differentiate into macrophages. Fresh media was added to the cells and allowed for another 24 h to recover from PMA induced stress. Cells were infected with recombinant *M. smegmatis* strains at a multiplicity of infection (MOI) of 100:1 (*M. smegmatis* to THP-1 ratio; MOI 1:100 was chosen based on pilot infections of THP-1 cells with multiple MOIs.). After 4 h of infection, cells were washed twice with RPMI 1640 media and treated with 200 µg/ml gentamicin for 2 h to kill the extracellular bacteria. Later, cells were washed twice with RPMI-1640 media and fresh media with 10% FBS and 2 µg /ml of gentamicin was added to the cells. Cells were collected at 12, 24 and 48 h time points and lysed with sterile Milli Q water. The lysed macrophages were plated on MB7H10 agar plates containing kanamycin (25 µg/ml) and colony forming units were determined as a measure of intracellular growth.

2.10. Statistical analysis

Graph pad prism 5.01 software was used to perform statistical tests. Paired student's *t*-test was performed for relative gene expression analysis of real time data and two tailed student's *t*-test was performed for analysis of CFU counts of intracellular bacilli and growth curve experiments. p values < 0.05 were considered as statistically significant.

3. Results

3.1. Protein sequence analysis of Rv2004c and docking study with SM

A total of 5 best models from 10 different templates were generated by LOMETS. From the statistics generated by LOMETS, the normalized Z-score of all the alignments was found to be greater than 1 in all the cases, except for the template 3tdvA, which is almost near to 1 (the actual value was 0.97). Out of the 5 best models generated, the first 3D model of the query protein was selected (Fig. 1**A**). The Ramachandran plot, as generated by RAMPAGE server demonstrated that about 80.2% (398 amino acid residues) and 11.5% (57 amino acids residues) of the total amino acid residues were in favoured and allowed regions respectively. Docking results showed that the SM interacted with the modelled 3D protein (Rv2004c) at Asp 22 (Magenta), Arg 24 (Orange), Lys 103 (Green), Gln 104 (Blue), Ser 403 (Red) residues (Fig. 1B). The global binding energy of the ligand to the protein was found to be −24 kcal/mol.

3.2. Rv2004c acts as an aminoglycoside phosphotransferase

To determine whether Rv2004c mediates SM resistance through the possible aminoglycoside phosphotransferase activity, we performed phosphotransferase assay using recombinant Rv2004c as enzyme and SM as substrate. ATP hydrolysis was not observed when the reaction mixture was incubated for a period of 2 h. However, overnight incubation resulted in detectable levels of ATP hydrolysis. In the control samples, the reaction mixtures without SM or Rv2004c did not yield

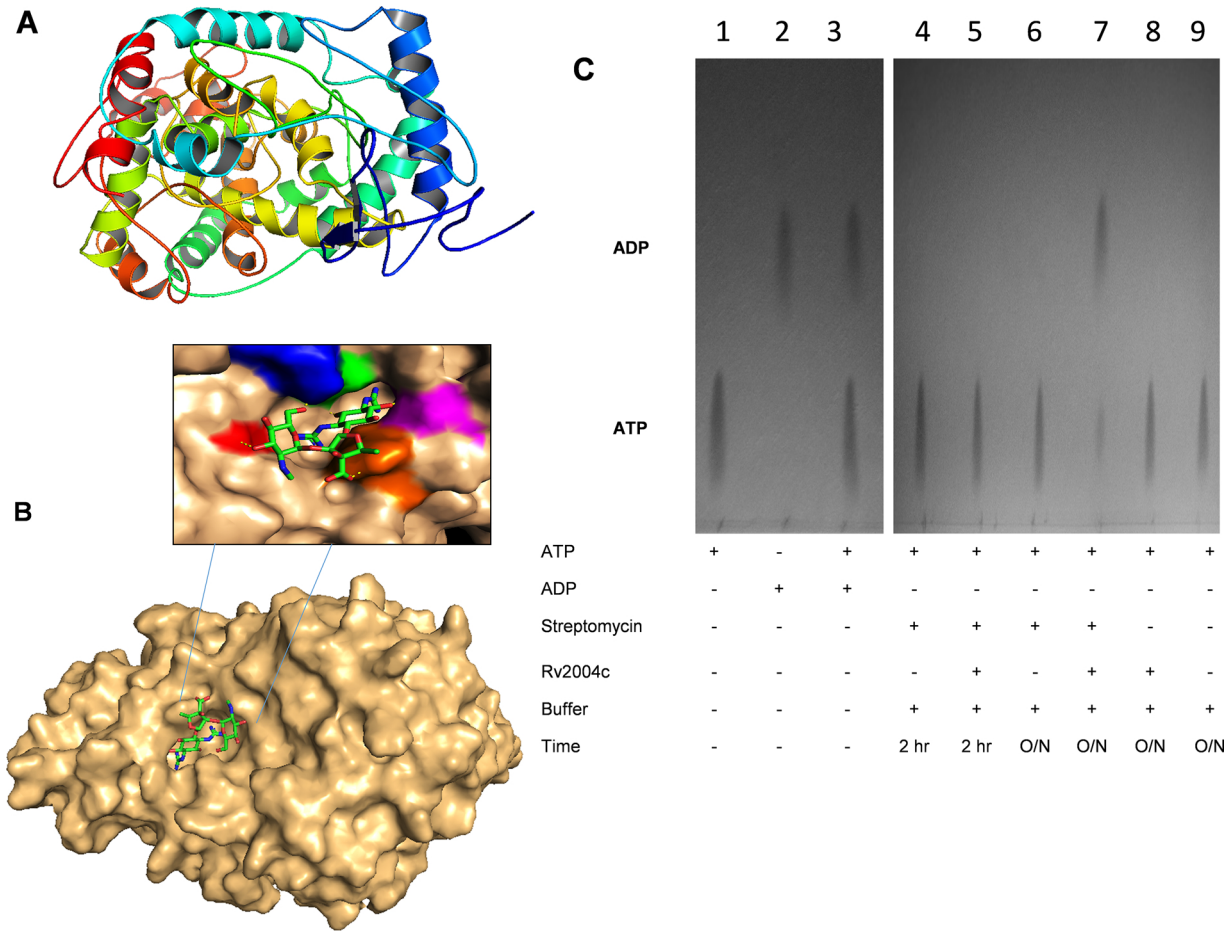

Fig. 1. Modelled 3D-structure of Rv2004c and its *in vitro* (or biochemical) interaction with SM.
A) Three dimensional structure of Rv2004c was determined using LOMETS modelling system and represented as a cartoon. **B)** Docking study of Rv2004c with SM: SM interacts with the Rv2004c at Asp 22 (Magenta), Arg 24 (Orange), Lys 103 (Green), Gln 104 (Blue), Ser 403 (Red) residues. **C)** Aminoglycoside phosphotransferase activity of Rv2004c was measured by incubating reaction mixture containing ATP, SM and Rv2004c at different time points. Reaction mixtures without Rv2004c or SM (or both) served as controls. ATP and ADP molecules spotted on the left side, served as standards. 2 µl of the reaction mixture was spotted on cellulose F TLC sheet and incubated in mobile phase for 2 h for the separation of ATP and ADP molecules. Right side of the figure contains spots of different reaction mixtures that are incubated for different time points. At the bottom of the figure, presence and absence of reaction contents was denoted with ˝+ ˝ and ˝-˝. O/N stands for overnight.

any detectable ATP hydrolysis even after an overnight incubation (Fig. 1**C**) indicating that Rv2004c mediates the phosphotransferase activity.

3.3. *E. coli harbouring Rv2004c confers resistance to SM in liquid broth cultures*

We studied antibiotic resistance effect of Rv2004c using *E. coli* as a model. *E. coli* harbouring inducible Rv2004c gene was exposed to different concentrations of SM. In the absence of SM in the media, *E. coli* harbouring Rv2004c did not show much difference in its growth pattern (Fig. 2**A**). In the presence of SM, *E. coli* strain expressing Rv2004c showed resistance up to 100 µM of SM (Fig. 2**B–C**) which is a growth inhibitory concentration for parental *E. coli* and *E. coli* harbouring an empty pRSET-A vector. The growth of *E. coli* expressing Rv2004c was decreased dramatically in the presence of 200 µM of SM (Fig. 2**D**); similarly, growth of *E. coli* harbouring empty pRSET-A vector was also diminished in the presence of 200 µM of SM (Fig. 2**E**). Viability of the *E. coli* strains grown under different concentrations of SM was confirmed by spreading the culture aliquots collected from each of the growth curves on LB Ampicillin agar plates. Similar kind of growth pattern, which was comparable to spectrophotometric growth measurements was observed on agar plates (Fig. 3).

3.4. *MSMEG_3942 was differentially expressed under stress conditions*

We determined the fold of expression of MSMEG_3942 gene in recombinant MS_MSMEG_3942 (overexpressing MSMEG_3942) strain under normal conditions by comparing with MS_Vec strain (Fig. 4**A**). A 11 fold increase in mRNA transcript levels of MSMEG_3942 gene was observed compared to that of MS_Vec strain. Further, we were also interested to determine the response of MSMEG_3942 gene during stress conditions like micro-aerobic and acidic stress which usually persists in human granulomatous lesions. For that, we investigated the response of MSMEG_3942 during *in vitro* stress conditions by using normal *M. smegmatis* cultures. We observed 2.5 and 4.7 fold of increase in mRNA transcript levels of MSMEG_3942 during micro-aerobic and pH stress (Fig. 4**B**).

3.5. *Overexpression of MSMEG_3942 influences the growth of M. smegmatis in acidic (pH 5.3) conditions*

Cell surface proteins are believed to play an important role in maintaining the cell integrity and in adaptation during stress conditions. Based on this, we tested the effect of MSMEG_3942 on the growth of *M. smegmatis* in normal and pH 5.3 adjusted growth media. Growth of MS_MSMEG_3942 strain was compared with that of MS_Vec strain. The growth pattern of MS_MSMEG_3942 was similar to that of MS_Vec strain during normal conditions (Fig. 4**C**) (since Rv2004c upregulates

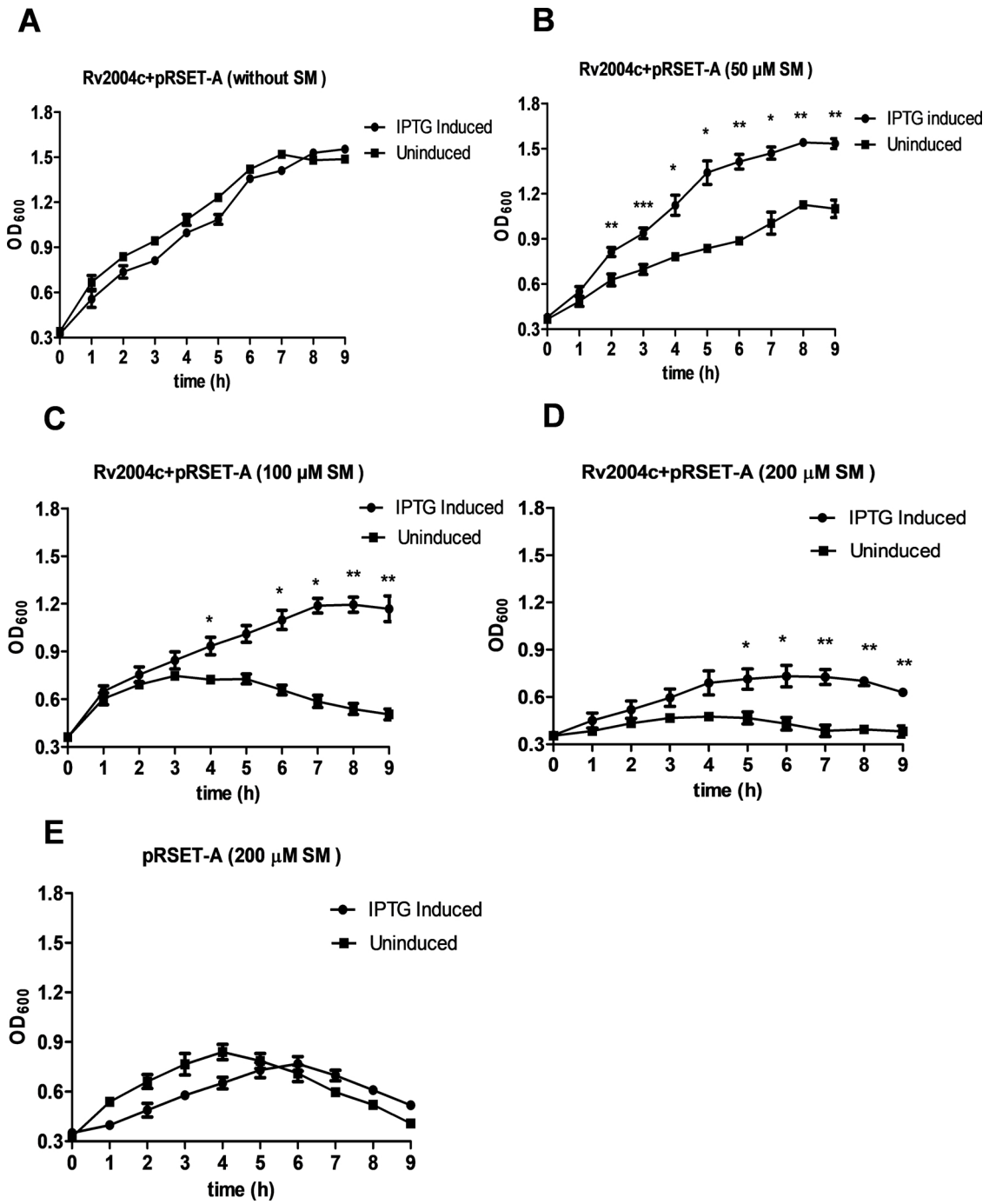

Fig. 2. Rv2004c expressing *E. coli* confer resistance to SM in liquid broth cultures.
E. coli strains transformed with recombinant Rv2004c + pRSET-A and empty pRSET-A vectors were grown overnight. Secondary cultures were inoculated with 1% of overnight grown cultures and induced with IPTG (at OD ~0.35) for 30 min. Later, growth of *E. coli* cultures under different concentrations of SM was monitored spectrophotometrically at OD_{600nm}. Each growth curve was performed with and without induction of IPTG. **A)** Growth of *E. coli* harbouring Rv2004c + pRSET-A without SM. **B–D)** Growth of *E. coli* harbouring Rv2004c + pRSET-A in presence of 50 µM, 100 µM and 200 µM of SM. **E)** Growth of *E. coli* harbouring empty pRSET-A vector in presence of 200 µM of SM. Data from three independent experiments were represented as mean ± SD. *p* values were represented with asterisk symbol: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

during stress conditions, its expression during normal physiological conditions could be minimal which might be a reason for no growth difference during normal conditions). But, in acidic media, MS_MSMEG_3942 grew faster when compared to MS_Vec strain (Fig. 4**D)** indicating that MSMEG_3942 might aid the bacilli to adapt to acidic conditions.

3.6. Overexpression of MSMEG_3942 enhances the intracellular survival of M. smegmatis

With the help of TrasH mutagenesis method, it has been reported that Rv2004c is required for the survival of Mtb in murine macrophages (Rengarajan et al., 2005). To reinforce the role of Rv2004c in intracellular survival, THP-1 cells were infected with recombinant MS_MSMEG_3942 and MS_Vec strains. MS_MSMEG_3942 strain showed

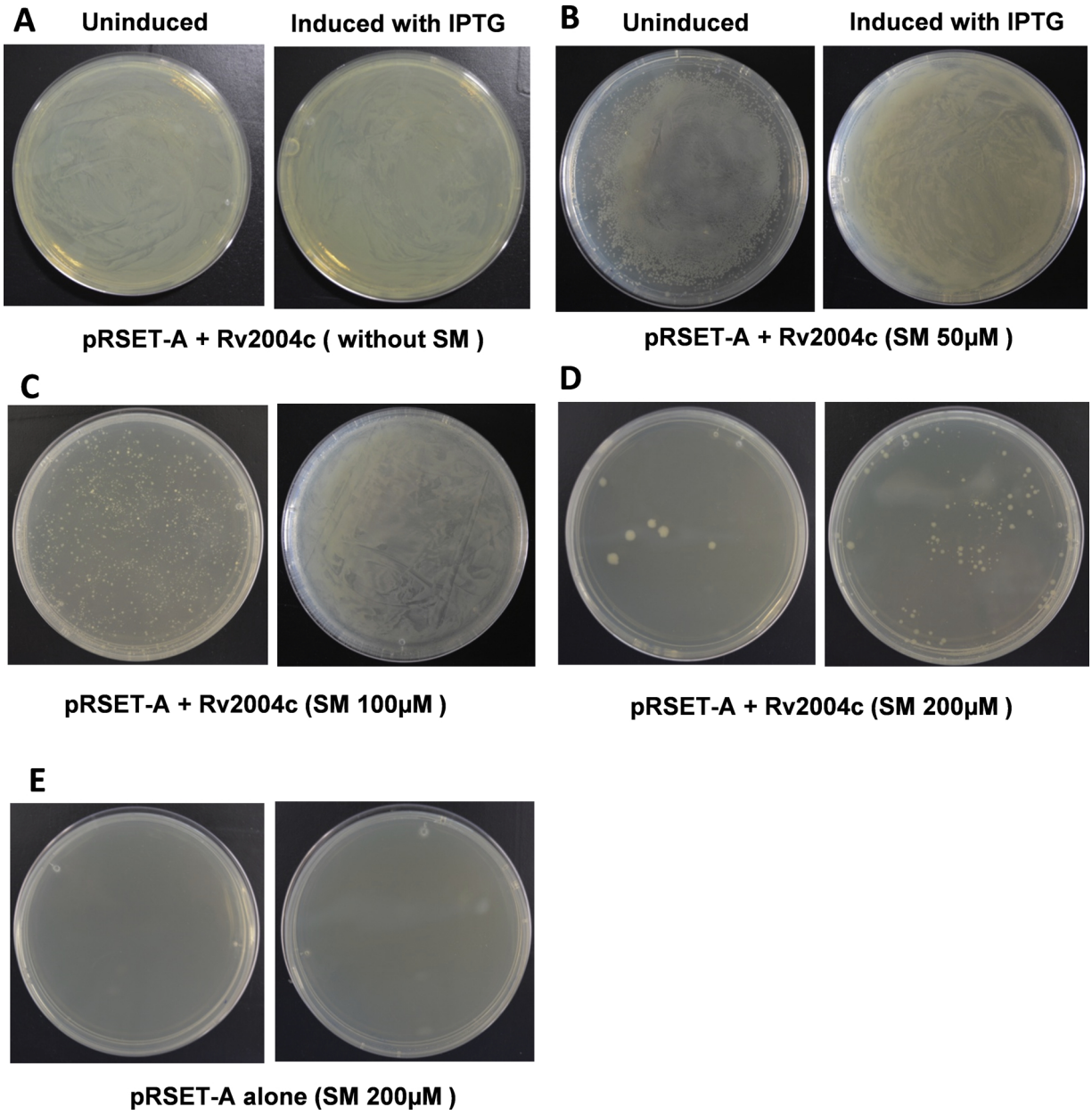

Fig. 3. Streptomycin resistance effect of Rv2004c on viability of bacteria.
The aliquots of *E. coli* cultures of each growth curve presented in Fig. 2 were collected after 2 h of induction with IPTG and were spread on LB agar plates containing ampicillin and incubated overnight at 37 °C. **A)** The growth of *E. coli* harbouring Rv2004c + pRSET-A in the absence of SM. **B–D)** Growth of *E. coli* harbouring Rv2004c + pRSET-A in the presence of 50 μM, 100 μM and 200 μM of SM. **E)** Growth of *E. coli* containing an empty pRSET-A vector in presence of 200 μM of SM.

significantly higher bacilli counts at 12, 24, and 48 h when compared to those of MS_Vec strain **(**Fig. 4**E)**. This indicated that overexpression of MSMEG_3942 might possibly enhance the growth of *M. smegmatis* inside macrophages.

4. Discussion

TB baffles the concerted global efforts directed at its control and its impact is further compounded by dormancy, HIV/AIDS as well as the global emergence of drug resistant strains of Mtb (Zumla et al., 2013). Among several classes of antibiotics, SM is the second line anti tubercular drug used for the treatment of TB relapses. Resistance to SM has been reported in clinical isolates of Mtb, majorly due to mutations in genes coding for 16S rRNA or S12 ribosomal proteins (Cooksey et al., 1996; Spies et al., 2008). Nevertheless, some of the clinical isolates showed low level SM resistance with normal 16S rRNA genes and S12 ribosomal proteins hinting at the involvement of other mechanisms such as presence of drug modifying enzymes. Whole genome sequencing of the Mtb H_{37}Rv strain provided needed insights into the functional proteomics and revealed the putative protein sequences that have homology with aminoglycoside modifying enzymes (Philipp et al., 1996). It has been reported that an acetyl transferase (Rv0262c) from Mtb could acetylate therapeutic aminoglycoside antibiotics and confer low levels of resistance to aminoglycosides (Hegde et al., 2001).

In this study, we demonstrated the *in vitro* SM phosphotransferase activity of Rv2004c. BLAST analysis of Rv2004c showed a conserved aminoglycoside phosphotransferase domain (COG2187) and an ATPase domain (COG4639) which might be necessary to perform an enzymatic phosphotransferase function. In a relevant study, ATP binding proteome (121 proteins) of Mtb has been identified in which Rv2004c was found to be one of the ATP binding proteins in hypoxic exposed cultures (Wolfe et al., 2013). Interestingly, in an another proteomic analysis of

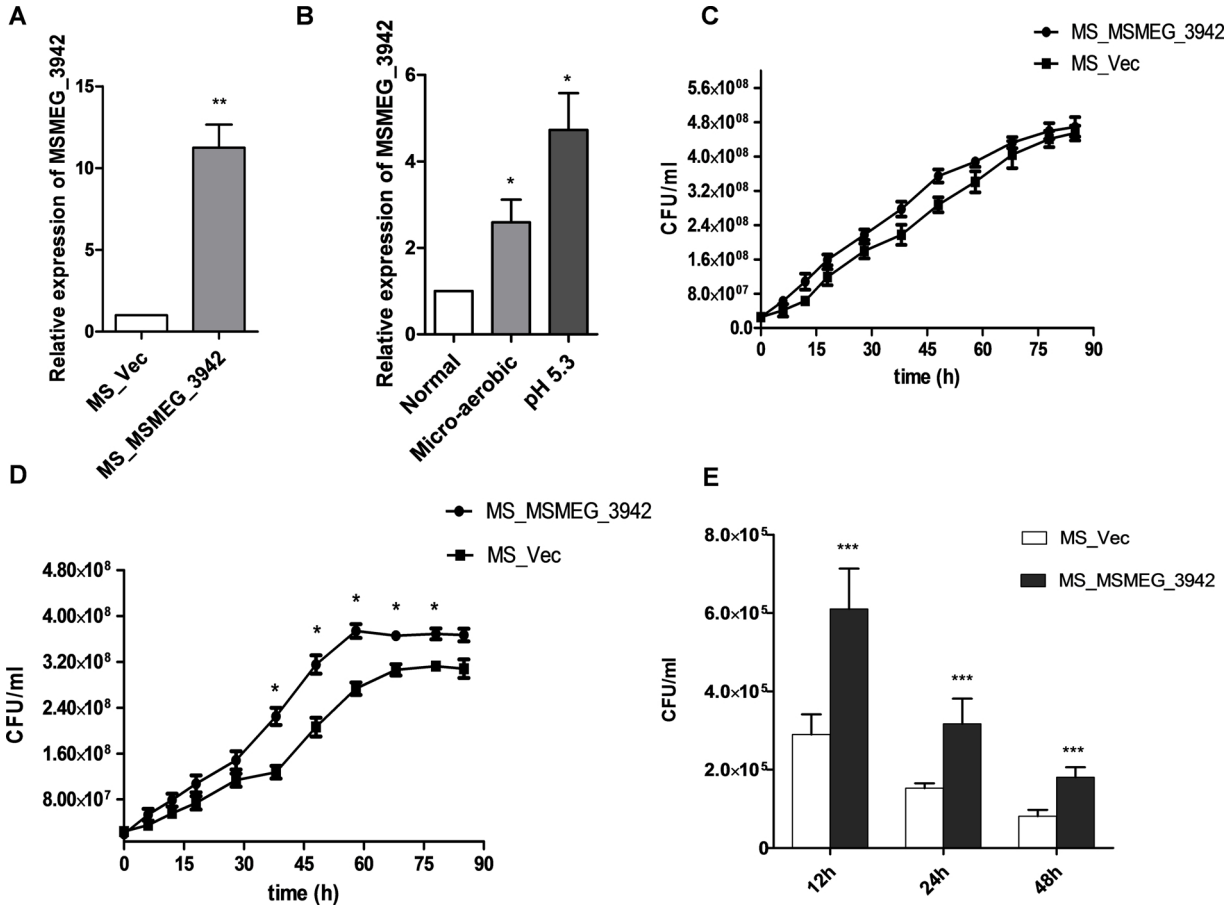

Fig. 4. Differential expression of Rv2004c in stress conditions and its effect on intracellular survival.
A) RNA was isolated from late log phase cultures of MS_MSMEG_3942 and MS_Vec strains (grown under normal conditions). Following cDNA synthesis, qRT PCR was carried out to quantify the expression of MSMEG_3942 in MS_MSMEG_3942 and MS_Vec strains. SigA was used for normalization of gene expression. Data were represented as mean ± SD. Unpaired student's *t*-test was performed for statistical analysis. **B)** Fold of expression of MSMEG_3942 was analysed by real time PCR in *M. smegmatis* late log phase cultures that were exposed to pH 5.3 and microaerobic stress for 24 h. C_T values of all samples were normalized with that of *sigA*. **C, D)** MS_MSMEG_3942 and MS_Vec strains were inoculated in Middle brook 7H9 broth with pH 7 (stress free conditions; Fig. 4 C) and pH 5.3 (acidic stress conditions; 4D) with an initial OD of 0.03 and growth of recombinant *M. smegmatis* strains was monitored by measuring CFU counts by plating 100 µl of diluted samples on Middlebrook 7H10 agar plates. Data were represented as mean ± SD of three independent experiments. **E)** Intracellular growth of MS_MSMEG_3942 and MS_Vec strains was measured by infecting the differentiated THP-1 cells at an MOI of 100. Cells were lysed at different time points and CFU was determined by plating 100 µl of diluted samples on Middlebrook 7H10 agar plates. Data represent the values of three independent experiments ± SD. In all graphs, p values were represented with an asterisk symbol:* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

four sequential isolates of Mtb from a single MDR-TB patient (from India), it was found that Rv2004c was one of the over expressed proteins in 3 out of 4 clinical isolates indicating that it may have some unknown functions related to drug resistance (Singh et al., 2015). With these clues, we investigated the possible phosphotransferase activity of Rv2004c. To begin with, we carried out computational modelling of Rv2004c with full length protein sequence (Fig. 1**A**); docking analysis of modelled protein with SM indicated a strong binding interaction between the two partners. SM finely fitted into the central cavity of the 3D protein (Fig. 1**B**). With these evidences from *in-silico* analysis, the envisaged SM phoshotransferase activity of Rv2004c was experimentally confirmed by *in vitro* phosphotransferase assay. The hydrolysis of ATP in the reactions was visualized by thin layer chromatography (TLC). Overnight incubation of the reaction mixture resulted in detectable levels of ATP hydrolysis. This pointed at the possibility that Rv2004c has low levels of phosphotransferase activity. We also confirmed that the resulting ATP hydrolysis was due to phosphate transfer to SM but not due to ATPase activity of Rv2004c (Rv2004c with ATP and with buffer alone did not show any ATP hydrolysis) (Fig. 1**C**).

Determining MIC is an important parameter in studies that aim to develop antimicrobial agents (Dijkstra et al., 2018). In this study, we aimed to determine the ability of a trans-expressed protein to confer resistance. Hence, growth curve assays were performed to analyse the resistance conferred by Rv2004c.

We analysed resistance to SM in Rv2004c harbouring *E. coli* expression system wherein it conferred resistance up to 100 µM of SM (growth inhibitory concentration). This was evident by growth curve analysis at different concentrations of SM and viable cell measurements (Fig. 2 and 3**)**. The same growth curve experiments were also carried out in presence of gentamicin. *E. coli* harbouring Rv2004c did not show any resistance to gentamicin. Even in disc diffusion assay, *E.coli* harbouring Rv2004c did not show any resistace to gentamicin (Supplementry Fig. S1). Overexpression of Rv2004c was carried out in *E. coli* because of its faster doubling time. This allows the transfer of the ability conferring resistance over a larger number of generations, which is an advantage. Such a high transgenerational passage might not be possible in Mtb because of its very slow doubling time. Hence, streptomycin susceptibility tests were performed in *E. coli*. Recently, Kim et al reported the crystal structure of Rv3168, an aminoglycoside phosphotransferase of Mtb (Kim et al., 2011). Rv3168 expressing recombinant *E. coli* conferred resistance up to 100 µM of kanamycin which is a growth inhibitory concentration for parental *E. coli* (Ahn and Kim,

2013). Phosphotransferase activity of Rv2004c was comparable to Rv3168 which also hydrolysed ATP after an overnight period of incubation. In another study, Wright et al. demonstrated the aminoglycoside phosphotransferase activity of Rv3225c by ^{32}P-ATP phosphocellulose binding assay in which Rv3225c exhibited low levels of phosphotransferase activity, but expression of this protein in *E. coli* did not confer any antibiotic resistance (Draker et al., 2003). In the current study, the low phosphotransferase activity of Rv2004c with SM correlated with antibiotic resistance to low concentrations of SM in *E. coli* (growth curve experiments). It might also be possible that aminoglycoside phosphotransferase activity of Rv2004c demonstrated in this study might be fortuitous or a secondary function in Mtb.

DosR regulon antigens were not only proved to be immunogenic in nature, but also some of them were reported to be essential for intracellular survival of Mtb. Mutant/knockout strains of DosR regulon genes failed to grow in the human macrophage cell lines and in lungs of mice, indicating that these genes play an essential role in adaptation and survival of Mtb under granulomatous conditions (Garg et al., 2015; Hu and Coates, 2011). Roles of Rv0079 and Rv2623 in the regulation of bacillary growth and persistence during latency have been reported (Drumm et al., 2009; Trauner et al., 2012). In a recent study, it was demonstrated that mutants of DosR regulon failed to persist in hypoxic lesions and delayed the adaptive immune response in a well-accepted non-human primate macaque model (Mehra et al., 2015). In order to identify the role of a hypothetical protein in the survival of Mtb during stress conditions that prevail in granuloma or macrophages, it is conventional to perform intracellular survival assay, *in vitro* growth assays and identify transcriptional profile(s) under stress conditions (acidic pH, hypoxia) with over expressing *M. smegmatis* strains (Garg et al., 2015; Li et al., 2014; Tiwari et al., 2012). In our study, *M. smegmatis* over expressed the MSMEG_3942 gene in micro-aerobic and pH stress conditions and MSMEG_3942 over expressing *M. smegmatis* strain survived better than *M. smegmatis* harbouring empty pMV261 vector in THP-1 cells **(**Fig. 4**E)**. This indicated that Rv2004c might contribute towards adaptation of Mtb to adverse granulomatous conditions and may provide better intracellular survival advantage. As our preliminary work on Rv2004c was carried out using *E. coli* and *M. smegmatis* strains, further studies are warranted on overexpression of Rv2004c in an Mtb strain which may demonstrate more realistic view on streptomycin resistance conferred by Rv2004c. Deletion of Rv2004c gene in Mtb might reveal more details on the role of Rv2004c in the intracellular survival of bacilli during dormancy.

In conclusion, our study demonstrated that Rv2004c can confer low levels of resistance to SM by modest phosphotransferase activity. Further, its differential expression in stress conditions and its ability to enhance intracellular survival indicates the significance of Rv2004c in persistence of Mtb during dormant state.

Author contributions

S.N.D designed and performed wet-lab experiments and analysed the data and wrote the manuscript. M.A.Q helped in execution of the experiments and analysing the data. P.Y. helped during experiments and contributed to discussions of the observations. P.V.P.S.A. performed computational modeling and analyses. N.A. and V. P. conceptualized the study, provided/arranged funding, edited the manuscript and provided overall supervision throughout the study. S.N.D and V.P. are the custodians of all wet-lab data generated by them for this manuscript. R.B. and N.S. provided help in interpretation of the results and contributed to discussion and writing and final editing of the manuscript.

Acknowledgements

We would like to thank Dr. P. Sandhya Rani for her valuable scientific discussions and inputs to develop the manuscript. N.A., R.B. and N.S. are supported by core grants from icddr,b and would therefore like to acknowledge the core donors of icddr,b: the governments of Canada, Sweden, Bangladesh, and United Kingdom.

Funding

This work was funded by a grant from the Department of Biotechnology of the Indian Government (DBT; BT/PR12817/COE/34/23/2015). SD was supported by a CSIR fellowship (CSIR-SRF). VP was supported by UGC startup grant (9507309). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijmm.2019.151353.

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