Molecular and Functional Characterization of Rv3241c of *Mycobacterium tuberculosis*

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DOCTOR OF PHILOSOPHY

By

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DECLARATION

The research presented in the thesis entitled **"Molecular and Functional Characterization of Rv3241c of *Mycobacterium tuberculosis*"** has been carried out by me at the Department of Biotechnology and Bioinformatics, School of Life Sciences, University of Hyderabad, under the guidance of Prof. Niyaz Ahmed. I hear by declare that this work is original and has not been submitted in part or full for any other degree or diploma of any other university or institution

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CERTIFICATE

This is to certify that the thesis entitled "**Molecular and Functional Characterization of Rv3241c of *Mycobacterium tuberculosis***" submitted by **Ms. Priyadarshini Yerra** bearing registration number **12LTPH05** in partial fulfilment of the requirements for the award of Doctor of Philosophy in the Department of Biotechnology and Bioinformatics, School of Life Sciences is a bonafide work carried out by her under my supervision and guidance.

This thesis is free from plagiarism and has not been submitted previously in part or in full to this or any other University or Institution for the award of any degree or diploma.

Parts of this thesis has been poster presented in the following conference

1. DRILS Young Scholar's Science Cafe Meet – 2015 held on Aug 10, 2015
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Dedicated to

My beloved Family

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List of Abbreviations

ºC	Degree centigrade
Aa	amino acid
ATP	Adenosine-5'-triphosphate
BCG	Bacillus Calmette Guérin
BSA	bovine serum albumin
Bp	base pair
DNA	Deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DNAse	Deoxyribonuclease
DTT	1, 4-Dithiothreitol
E. coli	Escherichia coli
EtBr	Ethidium Bromide
HER	Enduring Hypoxic response
EDTA	Ethylenediaminetetraacetic acid
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethane sulfonic acid)
HBS	3-HYDROXYBUTAN-2-ONE
IPTG	Isopropyl-b-D-thiogalactopyranoside
Kb	Kilobase pair
kDa	Kilo Dalton(s)
KOH	potassium hydroxide
L	Litre
M	Mycobacterium
MCS	Multiple cloning site
Mg	Milligram (10-3 gram)
Min	Minute(s)
Ml	Millilitres (10-3 litres)
mM	Millimolar
Mmol	Millimoles (10-3 moles)
nM	Nanomoles (10-9 moles)
Mmol	Millimoles (10-3 moles)

Ng	Nanogram (10-9 gram)
NRP	Non replicating persistent stage
NaCl	Sodium Chloride
OD	Optical density
ORF	Open reading frame
Ori	origin of replication
OADC	Oleic Albumin Dextrose Catalase
PAGE	polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PMSF	phenylmethanesulphonyl fluoride
PPD	purified protein derivative
pM	pico mole
RNA	Ribonucleic acid
rRNA	ribosomal RNA
Rpm	rotations per minute
SDS	Sodium dodecyl sulfate
SODC	Superoxide dismutase
Sec	Seconds
TB	Tuberculosis
V	Volts
WHO	World Health Organization
Mg	Microgram (10-6 grams)
Ml	Microliter (10-6 liter)
μM	Micro molar (10-6 Molar)
IGRA	Interferon Gamma Release Assay

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Chapter 1

Preamble and Context

1.1 The genus *Mycobacterium* and its pathogenic members

The genus *Mycobacterium* (M) belongs to the phylum Actinobacteria which is a highly morphologically diverse prokaryote (Servin *et al.*, 2008). Actinobacteria secrete different metabolites under different habitats and lifestyles (Ventura *et al.*, 2007; Servin *et al.*, 2008). *Mycobacterium* genus falls under the Mycobacteriaceae family with more than 150 recognized species (King *et al.*, 2017). The prefix *myco* in Greek means "fungus" and refers to the fungus like appearance of mycobacterial colonies (L. and Barrett, no date). The mycobacteria are acid-fast, aerobic, somewhat curved rod shaped or straight bacilli measuring 0.2 and 0.6 μm wide and 1.0 and 10 μm long and they are non-motile except for *M. marinum* which shows motility within macrophages (Kenneth and Ray, 2004). They do not form endospores and encompass an outer membrane containing capsule (Niederweis *et al.*, 2010; Traag *et al.*, 2010). One of the discriminate features of all the mycobacteria is that their cell wall is thicker than other bacteria and hence it is often held as a potential drug target (Abrahams and Besra, 2018). The cell wall contains three components: cross-linked polymer peptidoglycan, arabinogalactan polysaccharide, rich mycolic acids/mycolates, and a hydrophobic waxy layer (Brennan and Nikaido, 1995). The optimum growth temperature for mycobacteria ranges from 25 °C to over 50 °C and it requires simple substrates such as glycerol as a carbon source and nitrogen source as ammonia and amino acids in presence of minerals. The mycobacteria requiring longer periods of growth are termed as slow growers for example *M. kansasii, M. simiae, M. genavense, M. xenopi, M. celatum, M. malmoense, M. terrae,* and *M. ulcerans* (Sykes and Gunn-Moore, 2013) and colonies which are visible on solid medium within 7 days are rapid growers such as *M. chelonae/M. abscessus* group *and M. fortuitum* group (Esteban and Ortiz-Perez, 2009). Pathogenic species of *M. tuberculosis* form a complex of at least 9 species*: M. tuberculosis sensu stricto, M. bovis, M. africanum, M. microti, M. cannetti, M. caprae, M. orygis, M. pinnipedii* and *M. mungi* (Brosch *et al.*, 2002; Alexander *et al.*, 2010; Huard *et al.*, 2006; Van Soolingen *et al.*, 1994; van Ingen *et al.*, 2012). Newly recognized additions to the *M. tuberculosis*

complex (MTC) include *M. caprae,* which affects goats and was originally classified as '*M. tuberculosis* subsp. *Caprae'* or *'M. bovis* subsp. *Caprae'* (Aranaz *et al.*, 2003) and *M. pinnipedii*, which affects pinnipeds (Cousins *et al.*, 2003). *M. leprae* and *M. tuberculosis* (Mtb) are the causative agents of leprosy and tuberculosis. *M. tuberculosis* is the most common cause of human TB.

1.2 Tuberculosis – a dreaded pestilence

M. tuberculosis (Mtb) is an intracellular obligate pathogen and the causative agent of tuberculosis (TB) (Martínez, Torello and Kolter, 1999; Kenneth and Ray, 2004) which is one of the dreaded diseases of mankind since millennia and has co-evolved with its mammalian hosts for thousands of years (Hirsh *et al.*, 2004). It was first detected in fossils that were radiocarbon and were recovered from 9000, year-old humans in Eastern Mediterranean (Hershkovitz *et al.*, 2008; Rothschild *et al.*, 2001). In the year 1689, Richard Morton identified the pulmonary form as 'tubercles' and until 1820 it was not identified as a single disease. Later in the year 1839, J. L. Schönlein finally gave the name 'Tuberculosis'. It is also known as the "White plague" which inflicted humans for millennia. (Rene and Dubos, 2011). It was first identified by Robert Koch in the year 1882 who received the Noble prize for this discovery (Sakula, 1982). It is the first and most remarkable infectious disease studied to date due to its high mortality rate.

TB ranks beside HIV (Human Immunodeficiency Virus) as the well-known causative factor of death worldwide due to infectious diseases and thus affects millions of people each year (WHO, 2017). TB has been shown to affect the central nervous system, bones, and other parts of the body or the systems. Mtb has a highly complex interaction with the human host which has been studied intensely and it is considered one of the most sophisticated and successful pathogens.

Despite the worldwide use of several antibiotics and live attenuated vaccine, it is still one of

the greatest killers among all infectious pathogens (Smith, 2003). It is among the top 10 killer infectious diseases, second only to HIV, worldwide. People are still suffering from the disease despite new diagnosis and treatments (Sandhu, 2011). It is crucial to understand the mechanism of physiology and genetics of Mtb and related mycobacteria, to develop the new anti-tubular agents. It is also very essential to study the Mtb and host interactions to determine how bacteria avoid the host defenses and cause the disease (Bloom, 1994; Grange, 1996; Rom, W. N., 1996; Friedman, 2001).

Early last century, Calmette and Guérin in Lille, isolated and named the newly developed *M. bovis* BCG vaccine by which TB could be conquered. Bacillus Calmette- Guerin (BCG) provides a diverse protection (0-80%) against pulmonary TB infection in adults. It mainly protects leprosy patients and young children from TB (Singh, Saraav and Sharma, 2014). The advent of the first anti-TB antibiotics, streptomycin which was seen as inhibitory to the growth of the TB bacilli (bacteriostatic), the treatment appeared highly effective. However, as the BCG vaccine came in to practice and gave complacency (for many decades), drug resistance rapidly developed (SA, 1964; Ottenhoff and Kaufmann, 2012). Renewed worldwide efforts were then directed to develop newer vaccines and drugs with enhanced efficacy. Roughly, a 10% of the people exposed to Mtb develop tuberculosis disease. Besides 5% of humans develop, the infection within 1-2 years, and others develop the disease at any point in their lifetime (Frieden *et al.*, 2003; Esmail *et al.*, 2014; H, 2016).

Human infection with Mtb results in two forms, one being an active form and the other is an asymptomatic form known as latent tuberculosis infection (LTBI) (Lin and Flynn, 2010; Colangeli *et al.*, 2020). Latent tuberculosis infection (LTBI) is defined as a state of persistent immune response for Mtb antigens, or it is stated when a person is infected with Mtb which is unaccompanied by symptoms, microbiological evidence, and radiological abnormalities. Active disease is the state of primary infection or reactivation of LTBI suggesting the

mycobacterial infection (Siddiqui., 2020). Active TB acts as a source of contact and has a great burden on TB bacillus when compared to LTBI (H, 2016). To eliminate the active tuberculosis Directly Observed Treatment Short course (DOTS) has been started all over India to reduce the tuberculosis burden but it failed to eradicate the LTBI. Despite the treatment, the active tuberculosis cases are still in a dangerous state. The major threat to the control of tuberculosis is mainly due to the sources from HIV and LTBI which are associated with TB. The infection with HIV endorses the activation of LTBI (Tahir *et al.*, 2006; Kolappan *et al.*, 2012; Cazabon *et al.*, 2017; Doddam *et al.,* 2017). Most humans have latent TB who does not show any signs of active disease, but they can develop the disease in the future (Frieden *et al.*, 2003; Esmail *et al.*, 2014; H, 2016). The prevalence of LTBI in high-income countries is 28% while in low- and middle-income countries are as high as 51%. It is estimated that about a one-third of the population worldwide is infected with TB (Dheda K, Barry CE, 2016). The main approach for eradicating TB is by Calmette–Guérin vaccination and early-stage detection and treatment through finding the strategies for active cases (H, 2016).

1.3 Global Report on *M. tuberculosis*

As mentioned in the report of the WHO for 2021, TB disease led to an approximate death toll of 1.3 million in people that were HIV-negative. Due to the COVID pandemic, there has been a decline in the mortality rate, and the total number of deaths occurring in 2020 is almost equal to 2017 (WHO, 2021). Globally, about 71% of people were confirmed with pulmonary TB in the year 2020, 61% in 2019, and were in rifampicin resistance. Among these 25681 cases were XDR-TB and 132 222 cases were MDR/RR-TB. High burden countries and those on the watchlist appear to have achieved the 'End TB strategy 2020' milestone with a reduction of 35% of TB deaths between 2015 and 2020 (**Figure 1**). The annual number of TB incidents relative to population size varies widely among countries.

High-income countries have less than 10 cases per 100 000 population. There were fewer than 10 incident cases per 100000 populations in most of the high income countries, and 150–400 in many of the high burden countries (about 30) (WHO, 2018). According to the WHO global report 2019, nearly 10.0 million (ranging from 9.0-11.1 million) people were ill and 8.6% (range, 7.4-10%) were among the people living with HIV in the year 2018. About 484000 range incident cases of MDR/RR have been reported in the year 2018 (WHO, 2019).

Resistance to drugs has been continuing to be a major public health issue. As per the previous report about 558000 people, ranging from 483000-639000 developed resistance towards the first line of a drug called Rifampicin (RR-TB) in the year 2017 and about 82% had developed multidrug-resistant TB (MDR-TB). Worldwide estimates of the best confidence point at about 10 million people (range, 9.0–11.1 million) developed TB disease in 2017 including about 1.0 million children. Overall, 90% of the patients were adults, 9% comprised those having HIV infection (72% in Africa) and about 2/3rd were concentrated in eight countries: Indonesia (8%), South Africa (3%), Nigeria (4%), the Philippines (6%), India (27%), Pakistan (5%), Bangladesh (4%) and China (9%). Apart from these countries, other 22 countries of 30 in WHO's list are estimated for 87% of cases worldwide. The global cases were 3% from the America region and 6% were in the region of Europe as estimated by WHO (WHO, 2018).

Some of the socioeconomic and biological factors responsible for the transmission of TB relate to the circulation of drug-resistant bacilli, poor living conditions (such as sanitation and high population density), HIV-related immunodeficiency, drug or alcohol abuse may coexist, and diabetes pandemics which may be strongly associated with an increase in TB cases (Russell, 2007).

Estimated TB incidence according to WHO report (2021)

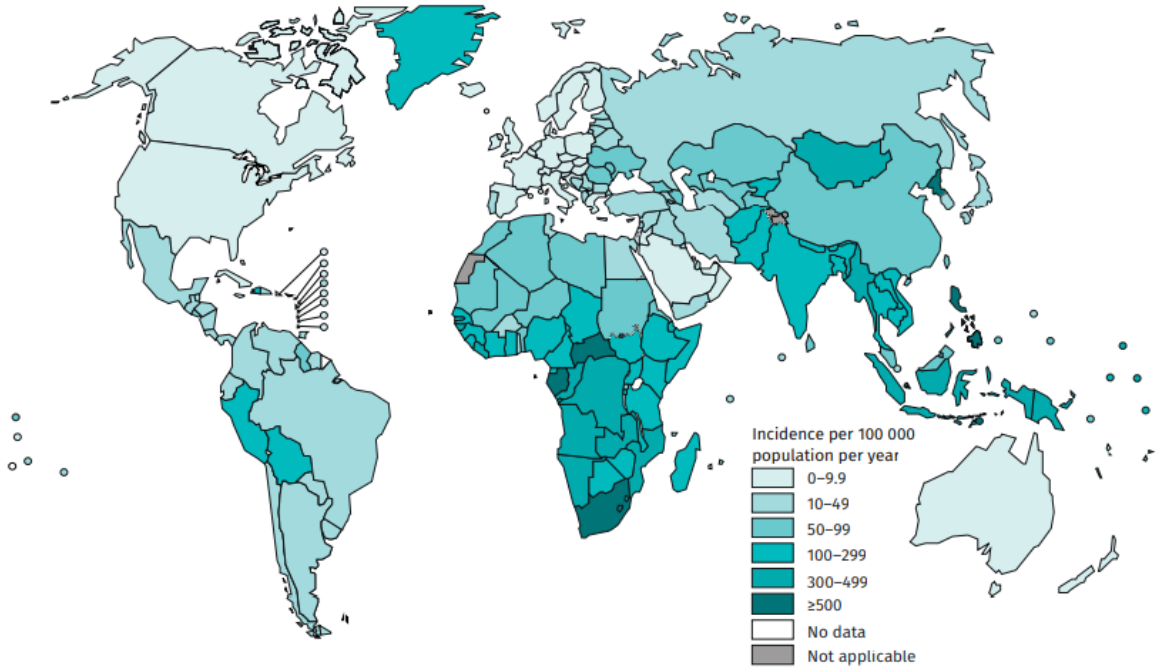

Figure 1: The severity of TB incidences cases varied widely among countries in 2020 (World Health Organization, 2021)

1.4 Characteristic features of tubercle bacilli

The characteristic features of Mtb are an acid-fast bacterium, neither Gram-positive nor negative, a facultative intracellular parasite, nonmotile rod-shaped, chemoorganotrophic, non-spore-forming, and of the size 0.2-0.5 (width) by 2-4 micrometers (length). It is an obligate aerobe, hence for this feature Mtb is always found in the well aerated upper lobes of the lungs. The physiological characteristic feature of tuberculosis is slow generation time i.e.., 15-20 hours this may contribute to its virulence (Cole and Cook, 1998a; Parish and Stoker, 1999; Fu and Fu-Liu, 2002; Kenneth and Ray, 2004) and under the laboratory conditions (37°C), it multiplies at every 24hrs and almost up to 3 weeks to form rough colonies and buff-colored on the agar plates. The Ziehl–Neelsen acid-fast stain is used to visualize the bacillus, whereby its waxy and thick cell, was retained by the carbol fuschin stain. Biochemical tests used to differentiate it from other mycobacteria are the ability to

reduce nitrate and positive niacin production, although PCR-based analysis of gene-specific loci has been used significantly for some time (Gordon and Parish, 2018).

The first Mycobacterium genome sequenced was *M. tuberculosis* H37Rv, containing 4.4Mb encoding 4018 genes and has high GC% content which is about 65.9% (Cole *et al.*, 1998). Studies on genome-wide mutagenesis have revealed that nearly 600 genes are essential for the *in vitro* growth of Mtb (Sassetti, Boyd and Rubin, 2003).

1.5 Pathogenesis

1.5.1 Transmission of tuberculosis:

One of the most important sources of infection is pulmonary TB (Narasimhan *et al.*, 2013). Progression of Mtb infection in a human occurs when Mtb enters via the aerosol route. These bacteria are suspended in the air for several hours depending upon the environment and the disease is not transmitted through surface contact. The main factors that determine the transmission of the bacillus are 1) the amount of the bacillus expelled into the air, i.e., in means of number, 2) concentration of organisms, which is determined by the volume of space and the breathing, 3) Time of exposure and 4) immune status of the individual (Peddireddy *et al.,* 2017). Modes of transmission have been outlined (**Figure 2)** (Gupta *et al.*, 2012).

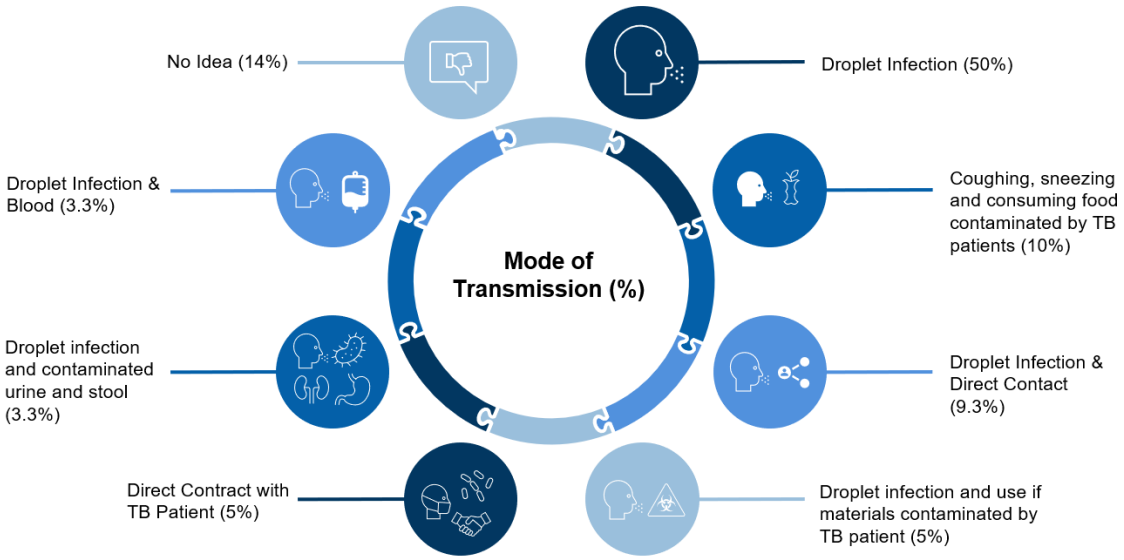

Figure 2: Modes of transmission of the diseases (Gupta *et al.*, 2012)

Infection occurs when a person inhales droplet nuclei particles of 1-5um in diameter originating from active pulmonary TB either by coughing or sneezing and these nuclei survive in the air for hours. (Cole and Cook, 1998b). The main site of infection is the lung. The inhaled droplets, due to their small size enter the terminal alveoli bypassing the bronchioles **(Figure 3)**. Immune phagocytic cells like macrophages and dendritic cells engulf these nuclei. M cells, epithelial cells of type 1 and type 2 and alveolar endothelial cells (non-phagocytic cells found inside the alveolar spaces) are infected by Mtb (Bermudez and Goodman, 1996; García-Pérez *et al.*, 2003; Teitelbaum *et al.*, 1999; Bermudez *et al.*, 2002; Kinhikar *et al.*, 2010; Peddireddy *et al.,* 2017). Mtb is up-taken by phagocytic immune cells at the start of the early steps of infection, where they are replicated intracellularly. The systemic dissemination occurs by the passage of immune cells across the alveolar barrier (Bermudez *et al.*, 2002; Teitelbaum *et al.*, 1999). The exceptional ability of Mtb to create a safe niche where they can stay for an extended period of time without being eliminated by the immune system is demonstrated by the intracellular multiplication and at the same time, their

diffusion to lymph glands and extra pulmonary locations (Chackerian *et al.*, 2002; Hingley-Wilson *et al.,* 2003).

The majority of infected persons experience an efficient early immune response (cell mediated) 2–8 weeks after infection, which further inhibits the growth of the bacilli. Activated macrophages and T lymphocytes, and other immune cells then create granulomas that prevent bacilli from replicating and spreading further. The progression of the disease is ceased when bacilli are killed in caseating granulomas. The granuloma can restrict the growth of bacteria as adaptive immune development. However, in some individuals, the pathogen is not completely eradicated and has an approach to avoid the immune response ensuring the persistence and survival of some bacilli in the host (Hingley-Wilson *et al.,* 2003; Tufariello *et al.,* 2003; Ahmad, 2011).

1.5.2 Formation of granuloma

Granuloma is the hallmark of the host response to TB infection and is the center of tuberculosis immune pathogenesis (Guirado and Schlesinger, 2013). Granuloma is defined as an innate inflammatory mononuclear cell infiltrate that has evolved into a complex structure. It provides a survival niche from which bacteria can disseminate by the capability of limiting their growth (Ehlers and Schaible, 2012). Granulomas are a hierarchical aggregation of immune cells in an organized manner. It is a compact structure consisting of epithelioid cells (uniquely differentiated macrophages), infected and uninfected macrophages derived from blood, foamy macrophages, multinucleated giant cells, and is surrounded by a ring of lymphocytes (Russell, 2007; Ramakrishnan, 2012). The main function of granuloma is to contain Mtb while compartmentalizing the immune response to its vicinity (Guirado and Schlesinger, 2013). Due to the chronic stimulation of immune cells, the granuloma is maintained in an infected host, persistently forming a base for the tuberculous lesion (Tufariello, Chan and Flynn, 2003).

Granuloma, in humans, shows high plasticity and can be divided into three types

1) Solid granuloma

2) Necrotic granuloma

3) Caseous granuloma

Solid granuloma mostly prevails during LTBI. It is typically surrounded by a fibrotic well which is separated from the neighboring tissues. Necrotic granuloma establishes the structure which occurs at an early stage of tuberculosis. In this, the organism starts to replicate and become active. Caseous granuloma occurhs when the infection is severe. It usually contributes the nutrients for promoting the growth of bacillus (Gengenbacher and Kaufmann, 2012).

The early advancement of a primary granuloma and the reactivation of an existing granuloma in patients having a latent infection are widely held as the potential causes of clinical TB. (Guirado and Schlesinger, 2013). Mtb-induced granulomas serve as a collection of organized immune cells that the bacteria uses to establish latency, and from the perspective of the host, granuloma formation limits the spread of infection. (Sasindran and Torrelles, 2011). Activated lymphocytes and macrophages follow the influx of neutrophils to the site of the infection that initiates granuloma development. (Ulrichs and Kaufmann, 2006; Russell, 2007). The established granuloma results from activated macrophages and epithelioid cells forming a necrotic central core composed of both Mtb and host factors providing nutritional support to the bacterium. Activated CD4+, CD8+, and necrotic macrophages surround a necrotic core to form a dense cellular wall that prevents bacterial spread. (Saunders and Cooper, 2000).

Immunocompetent Mtb infections result in small, compact granulomas with a high concentration of IFN- γ CD4-T cells, whereas immunodeficient infections result in granulomas with a high concentration of large, richly activated macrophages and few

lymphocytes (Tufariello *et al.,* 2003; Ehlers and Schaible, 2012; Guirado and Schlesinger, 2013; Orme and Basaraba, 2014).

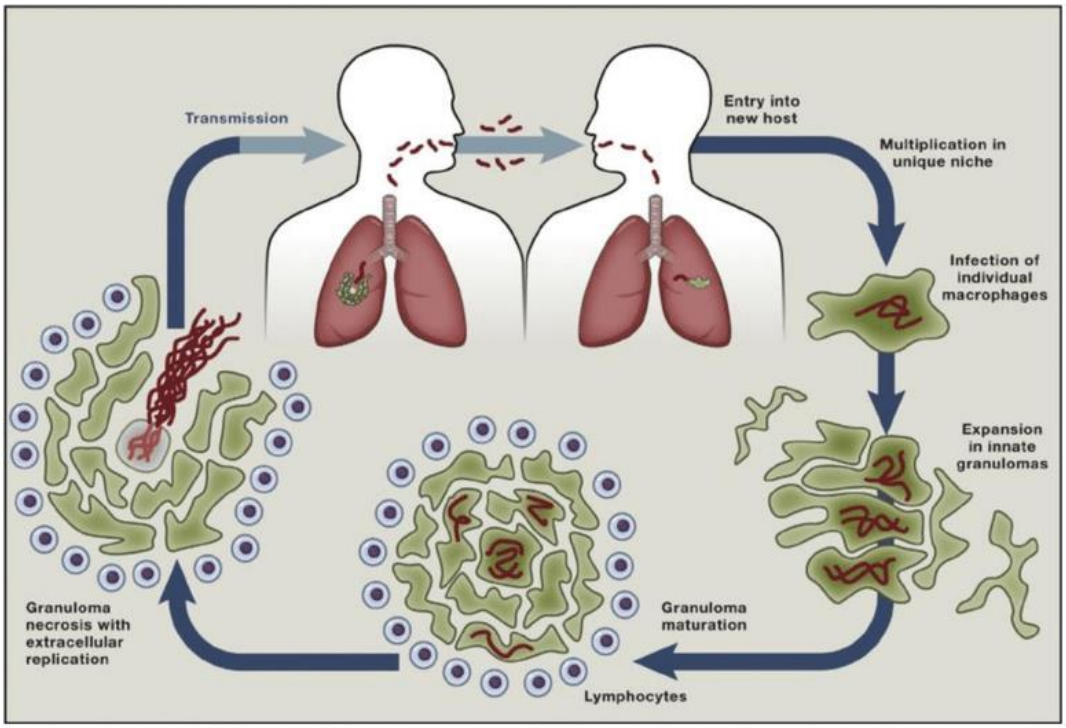

Figure 3: Pathogenic Life Cycle of *M. tuberculosis* (Cambier, Falkow and Ramakrishnan, 2014)

Granuloma limits bacterial growth by including nutrient deprivation, oxygen deprivation (hypoxia), production of host factors, and acidic pH. Among these, hypoxia is the best studied model. Years after exposure to hypoxia, bacilli remain alive and pathogenic, and their susceptibility to drugs is comparable to that of latent TB. (Rustad *et al.*, 2008). Hence, further studies are needed to understand the hypoxic adaptations of Mtb (Rustad *et al.*, 2008; Trauner *et al.*, 2012).

The diagnosis of a disease depends on how the host eradicates the bacillus. The infection may arise from a few problems to major health issues since the bacteria might adapt to live in a balanced immune response. A detailed study of the pathogenesis of the disease is essential to eradicate the disease at once. For which we need a good model to study to

understand the host and parasite relationship (Silva Miranda *et al.*, 2012).

1.6 Risk Factors

The risk of progression of a disease is a two-stage mechanism i.e.., exposure of the bacillus to the active disease, which is governed by exogenous and endogenous factors. Exogenous factors play a crucial role in the progression of disease where the bacillus is loaded in the sputum whereas the endogenous factors lead to the active disease. Exogenous factors include the closeness to contact and behavioral and social factors such as alcohol, smoking, air pollution, and overcrowding where chances of transmission are very high, and the endogenous factors that lead to the active disease which modify the immune response such as HIV co-infection, the most crucial factor of the disease. Co-infection with HIV develops 30% active disease when compared with people without HIV (Bruchfeld *et al.,* 2015). TB is essentially a disease leading to poverty by the transmission of disease, adverse medical expenses (Shete *et al.,* 2018) and the other endogenous factors include malnutrition, diabetes, tobacco smoke, alcohol, and indoor air pollution (CDC, 2000; Restrepo, 2007; Lawn and Zumla, 2011). The existence of genetic susceptibility remains undefined (Möller and Hoal, 2010; Lawn and Zumla, 2011). The risk of active infection is based on many factors including the inhaled bacillary inoculum, the host immune status, clinical illness of the source patient (contact case infectiousness), the vicinity or alignment of the contact etc. (Hill *et al.*, 2004; Mathema *et al.*, 2008; Kinhikar *et al.*, 2010; Ahmad, 2011). Efforts should be made in increasing the awareness through education, information, and communication and also through collecting the data during the surveillance of TB disease.

1.7 Mycobacterial stress, adaptation, and ribosomal stabilization

M. tuberculosis can become dormant, and little is known about the mechanism of how the bacilli persist in the host. According to Wayne and Sohaskey, latent physiological state referred as "non-replicating persistence" (NRP) where bacilli evade the immune system and

survive inside the macrophages and are responsible for the latent asymptomatic phase in tuberculosis (Alnimr, 2015; Wayne and Sohaskey, 2001; Flynn and Chan, 2001; Bacon *et al.*, 2014). The persistence mechanism has been widely studied using in-vitro models due to their rapidity, economy, and reproducible observations. The growth of stress resistant NRP cells is paramount inside the granuloma, under unfavorable conditions. This mechanism is necessary to decipher how actively growing mycobacteria become NRP and maintain their viability. Several factors are necessary for NRP viability such as maintenance of the proton motive force and ATP and NAD+ synthesis, whereas synthesis of protein is reduced significantly and sigma factor genes are upregulated, indicating that these genes might play a role in mediating adaptation to latency (Rustad *et al.*, 2009; Shiloh and DiGiuseppe Champion, 2010).

Hypoxia is significant condition of stress in the environment of granuloma which is faced by Mtb. The metabolic pathway used to generate energy needs to be deciphered. Many genes are involved in anaerobic respiration where some genes are repressed or induced. During persistence how these genes play a role in maintaining the viability of the bacillus need to be understood. The main regulons underlying the adaptation during hypoxia are EHR (enduring hypoxic response) and DosR (dormancy survival) (Rustad *et al.*, 2008; Leistikow *et al.*, 2010). The mycobacteria, namely, *M. tuberculosis, M. smegmatis,* and *M. bovis* need a critical dormancy survival regulon which is governed by a cognate response regulator, DosR, and is comprised of a total of 48 genes/proteins that might be essential for the survival of the bacillus amidst hypoxic conditions (Sherman *et al.*, 2001; Toole *et al.*, 2003; Park *et al.*, 2003; Voskuil, Visconti and Schoolnik, 2004; Leistikow *et al.*, 2010; Boon and Dick, 2012). In Mtb, the two-component response regulator DosR (also called DevR, Rv3133c) regulates the initial response to hypoxia. The DosR regulon is a two-component system chiefly made of DosS (Rv3132c), DosT (Rv2027c), and two sensor histidine kinases (of heme-containing type that bind NO and CO), and a cognate response regulator, DosR (Rv3133c) (Honaker

et al., 2009; Leistikow *et al.*, 2010; Chen *et al.*, 2013). It has also been observed that DosR induced during the nitric oxide condition suggests that DosR may not play a unique function in latency, as other factors may also be implicated. (Rustad *et al.*, 2009; Trauner *et al.*, 2012). The DosR regulon genes are widely conserved/distributed within mycobacterial genomes (Chen *et al.*, 2013) and also conserved in environmental mycobacteria (Park *et al.*, 2003).

Apart from DosR regulon, another regulon was identified based on the transcriptional analysis under hypoxia condition called enduring hypoxia response which comprises nearly 200 genes. EHR is largely independent, largely extensive, and better stable than the DosR regulon and it is important for long-term maintenance of hypoxia response. An additional study of EHR might give an important clue to the survival mechanisms of Mtb (Rustad *et al.*, 2009). The functional analysis of DosR and EHR genes might provide insight into the level of pathways occurring in Mtb under hypoxic stress conditions (Bose *et al.*, 2018). Hence, it is essential to improve the knowledge in understanding the genes involved in the pathogenesis and are associated with TB for developing more effective therapies and vaccines (Smith, 2003; Hunter, 2020).

During the persistence of Mtb, some of the DosR genes are upregulated which include nitroreductases, heat shock proteins, universal stress proteins, etc.., (Gerasimova *et al.*, 2011). Apart from the hypoxia mediated stress conditions, Mtb undergoes nutrient starvation in granulomas. The study of the mechanism of nutrient starvation and the metabolites involved in generating energy in bacteria are of significant interest. *In vitro* , nutrient-starved Mtb bacilli can endure for a very long time, and that the modulation (or slowing) of their transcription apparatus, biosynthesis and energy pathways and other genes functions might play a role in long term survival (Loebel *et al.,* 1933). Given this, it is pertinent to study further the aspects of translation level regulation since a critical changes have been documented in transcription under different stress conditions linked to dormancy. The ribosomes are described to

critically mediate the translation or protein bio-synthesis. Research on ribosomal functions and heterogeneity in bacteria and eukaryotes somewhat contradicts the assumption that translation and ribosomes comprise static components (Gilbert, 2011). One example of specialized ribosomes could be the stabilized ribosomes. An assembly of ribosomal subunits that makes them translationally silent or static is known as a stabilized ribosome. Ribosome stabilization is described to cause the emergence of 70S monomers and 100S dimers in *E. coli* (Yoshida *et al.*, 2002). Whereas, the stability of the 30S and the 50S subunits in mycobacteria results in the development of the 70S complexes. Stabilization, therefore, of ribosomes could be critically helpful in mycobacterial persistence and subsequently leads to a dormant infection with latency of the agent. Therefore, it is necessary to study the stability of mycobacterial ribosomes (Trauner *et al.*, 2012).

1.8 Working hypothesis and Objectives

Mtb is asymptomatic and can produce progressive disease or latent infection (Vynnycky and Fine, 1997). After initial infection, the acute tuberculosis is caused by the reactivation of latent infection (Vynnycky and Fine, 1997), which can persist in a host without replication (Wayne, 1994). During latent infection, Mtb bacilli are contained within the granulomas which offer a niche with absence of nutrients, low oxygen, and increased concentration of nitric oxide (Wayne and Sohaskey, 2001; Betts *et al.*, 2002; Tufariello, Chan and Flynn, 2003; Leistikow *et al.*, 2010). Under such unfavorable conditions, Mtb has a unique ability to survive and might have evolved a mechanism to endure stress environment.

The most important virulence factor of the pathogenic bacteria includes the protein secretion systems. In Mtb, five types of secretion systems have been identified, among them, the best characterized secretion system is the ESX1(Abdallah *et al.*, 2007). Another set of proteins that are under the control of a regulon called dormancy survival regulon (DosR) play an important role in the pathogenesis of the bacillus. Mtb under harsh conditions is

characterized by nutrient depletion of low oxygen in macrophages and granulomas and it responds by stimulating the dormant state. During which the bacillus stops multiplying, down-regulates the central metabolism, and activates the anaerobic metabolism. Upon induction of stress proteins, which provides Mtb with unique special immunological and biological features (Korch *et al.,* 2009; Delogu *et al.,* 2013). This metabolically active bacillus can persist for a long period *in vivo* without replication and can revert to the active state (Voskuil *et al.,* 2004; Korch *et al.,* 2009; Hett, Chao and Rubin, 2010; Kumar *et al.*, 2012).

The molecular mechanism for slow-growing bacillus which conserves the cellular energy needs to be deciphered. Bacteria may endure growth by slowing down at the level of replication, transcription, and translation (Kumar *et al.*, 2012). One of the most crucial structures are ribosomes which play an important role in the translation of genetic information into functional proteins and shift the bacteria into a stable state during the stress condition. In *E. coli* certain ribosomes are associated which induce conformational changes leading to the formation of stable and functionally inactive ribosomes to aid the survival of bacteria. But there is no experimental proof related to the ribosome stabilization in mycobacteria. During the stress condition, ribosome-associated proteins play an important role in the reactivation of bacteria. Ribosome-associated factors, which play a role in ribosome stability and function, were identified as S30AE proteins, which include the S30AE domain which is a ribosome-binding domain.

Among the mycobacterial genes, Rv3241c and Rv0079 are S30AE proteins containing the S30AE domain which is involved in the stability of ribosomes. Among these two genes Rv0079, one of the DosR regulon proteins (Rv0079) of Mtb has been characterized and shown that it is involved in the regulation of translation, and it can stimulate the peripheral blood mononuclear cells (PBMC) and macrophages. It has also been shown that it interacts with TLR2 (Kumar *et al.*, 2012; Kumar *et al.,* 2013)

Cellular pathways are also crucial for the survival of bacillus which might be a good candidate for designing rational drugs (Williams and Duncan, 2007; Balganesh and Furr, 2008; Ollinger *et al.*, 2012). Protein degradation is important in nurturing cellular homeostasis through the regulation of biological pathways and protein quality control (Goldberg, 2003; Ingmer and Brøndsted, 2009; Raju *et al.*, 2012). Most of the prokaryotes contain various ATP-dependent serine protease complexes such as Lon protease, Clp proteases (Frees *et al.*, 2007) and the serine protease which was first discovered and described in *E. coli* (Hwang *et al.*, 1987; Katayama-Fujimura *et al.,* 1987). These complexes mediate the degradation of abnormal and mutant proteins including short-lived regulatory proteins and antitoxins. *M. tuberculosis* encodes both proteasome and Clp protease. ClpP is a non-essential conserved serine protease that is involved in the stress response and contributes to the quality control of proteins(Ollinger *et al.*, 2012; Raju *et al.*, 2012). Most the bacteria have a single subunit of ClpP whereas mycobacteria have two subunits- clpP1and clpP2. These subunits are required for the removal of abnormal proteins. In the year 2014, Raju et. al, has predicted that r3241c serves as a Clp protease substrate (Raju *et al.*, 2014). Based on this, we have computationally evaluated the interaction of rRv3241c with ClpP.

Given the fact that for the mycobacterial survival under stress conditions is linked to ribosome associated proteins which could be important for the persistence of dormant bacilli, we pursued the research on Rv3241c to investigate its role in the regulation of translation and infection immune functions s with the following objectives:

OBJECTIVES

- **Identification and characterization of Rv3241c of *M. tuberculosis***
- **Studies on the possible role of Rv3241c in immune responses**
- **Molecular modeling of Rv3241c as a putative Clp protease substrate**

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Chapter 2

Identification and characterization of Rv3241c of *M. tuberculosis*

2. Introduction

M. tuberculosis (Mtb) is the etiological agent of tuberculosis (TB). It is the oldest known human bacterial pathogen. TB remains the leading cause of death from a single infectious agent, exceeding the death caused by HIV/AIDS (WHO, 2017; Leopold Wager, Arnett and Schlesinger, 2019). Mtb is successful as a pathogen and is associated with the ability to persist in the host for longer periods which is underlined by its subdued metabolic repertoire (Flynn and Chan, 2001; Sherman *et al.*, 2001; Park *et al.*, 2003; Wayne and Sohaskey, 2001; Boon and Dick, 2002; Voskuil, Visconti and Schoolnik, 2004; Leistikow *et al.*, 2010a). It infects alveolar macrophages, and these infected macrophages arrange in an ordered structure to form granuloma. The most important key and the best-studied model to stimulate dormancy and *in vivo* for host-induced stress limiting the growth of the bacteria is hypoxia and in Mtb, the granuloma will experience hypoxic conditions as they are necrotic, inflammatory, and avascular and its adaptation to the pathogen has a distinguished role in the pathogenesis of bacteria (Russel *et al.*, 1955; Canetti, 1956; Imboden and Schoolnik, 1998; Rustad *et al.*, 2009; Galagan *et al.*, 2013).

The hypoxic model of non-replicating persistence was developed by Wayne and co-workers to identify the state of Mtb in reduced oxygen (Wayne and Hayes, 1996) and other conditions such as nutrient models which may mimic the effect of nutrients that are available in granuloma for the metabolism of Mtb (Smeulders *et al.*, 1999; Betts *et al.*, 2002; Karakousis *et al.*, 2004). Bacteria undergo adaptive morphological changes and stop replication, in order to survive under these stress conditions, which can make them virulent for years. These bacilli are susceptible to drugs that have a similar profile to latent tuberculosis (LTB). Hence, future validation of the hypoxia model and determining the mechanism of how Mtb enter, persist and exist from the LTB state is necessary (Corper and Cohn, 1951; Wayne and Sramek, 1994; Herbert *et al.*, 1996; Wayne and Hayes, 1996; Stover *et al.*, 2000; Peh *et al.*,

2001).

Mtb shows a reduced growth rate in persistent or chronic infection which may be associated with an evolved resistance to antibiotics or may be entailing the host immune responses (Wayne and Sohaskey, 2001; Harries and Dye, 2006) and it has been observed to exist in a non-replicating style which is a signature function of latency. To survive under such conditions, the bacilli evolved a mechanism in which a few metabolic functions such as the protein bio-synthesis are greatly rationalized to economize on the resources of the cell (Kumar *et al.*, 2012; Trauner *et al.*, 2012). Consequently, such a situation has a bearing on the treatment of the infection with drugs (Gomez and McKinney, 2004). The ability of Mtb to survive is facilitated by a regulon called DosR/ devR (dormancy regulon) (Voskuil *et al.,* 2004) that consists of 48 genes and the majority of them are functionally characterized (Voskuil, Visconti and Schoolnik, 2004; Honaker *et al.*, 2009; Trauner *et al.*, 2012). This regulon is important for the survival of the various mycobacterial species such as *M. tuberculosis*, *M. bovis,* and *M. smegmatis*. The regulon is controlled DosS and DosT, the two response regulators functioning as sensor kinases, as described. Further, it is shown that DosR regulon is indispensable for the rapid regeneration of growth once bacteria exit the anaerobic state as it encodes a piece of novel metabolic machinery necessary for survival (Leistikow *et al.*, 2010b).

Besides the proteins encoded by the DosR regulon genes, several other proteins encoded by genes that are not part of this regulon are known to contribute to the survival strategies of Mtb during latency. Several studies have indicated that under latency, tubercle bacilli survive in granuloma in a non-replicating state and have evolved mechanisms to stabilize cellular components such as ribosomes by intense cellular reprogramming of many genes that do not belong to the DosR regulon (Kumar *et al.*, 2012). Most of the ribosomal genes are down-regulated suggesting that ribosomes play an important role in the stabilization process during

prolonged stasis (Trauner *et al.*, 2012). Mycobacterial ribosomes do not form the higher-order structures upon entry into the stasis when compared to the enteric bacteria (Trauner *et al.*, 2012). The proteomic analysis led to the identification of S30AE domain proteins as a ribosomal stabilizing factor. During stress conditions, the S30AE domain protein attaches to ribosomes and promotes the 70S ribosome formation leading to the stabilization of ribosomes (Vila-Sanjurjo *et al.*, 2004). Ribosomal stability has preliminarily been carried out in *E. coli*, during the transition from active to inactive state. In *E. coli*, when cells stop growing, a fraction of the ribosomal pool is degraded, and the other portion undergoes dimerization which leads to the accumulation of translationally inactive 100S complexes. This phenomenon is retained to prevent the ribosome turnover and serves as a reservoir and when the conditions become favorable the translation is recycled by allowing the hibernating ribosome to disassemble (Wada *et al.*, 1995; Yoshida *et al.*, 2002; Wilson and Nierhaus, 2004; Ueta *et al.*, 2008; Gohara and Yap, 2018; Sawyer, Grabowska and Cortes, 2018). Under hypoxic conditions, in contrast to *E. coli*, Mtb does not form the higher-order structures such as 100S dimers rather stabilization is associated with the 70S forms. This stabilization is mediated by RafH, a member of the DosR regulon (Trauner *et al.*, 2012). RafH is one of the two long hibernation-promoting factors (HPF) which has been described in Mtb and is related to the RMF and YfiA proteins of *E. coli* (Polikanov, Blaha and Steitz, 2012). By using the *E. coli* counterpart sequence, the presence of three *E. coli* proteins RMF, HPF, and PY in Mtb has been documented using BLASTP (Altschul, 1990) and it was shown that only one protein, Rv3241c, an hypothetical protein hitherto, shared the homology with the HPF.

The mycobacterial gene Rv3241c encodes a protein that contains the S30AE domain. It was detected by mass spectrometry in TB bacilli, in the Triton X-114 extracts, and was thus identified (Målen *et al.*, 2010). Its main predicted function leads to the dimerization of active 70S ribosomes into 100S complexes in the stationary phase

(https://www.uniprot.org/uniprot/O05886); however, the exact role of Rv3241c in latency function in Mtb is not yet clear. Due to the presence of the S30AE domain in Rv3241c protein, it might play a crucial role in the stabilization/ inactivation of ribosomes to favor Mtb survival in stress conditions. Given this, we characterized the functional role of the protein encoded by the DosR independent gene Rv3241c gene. We observed that the Rv3241c gene is conserved in most of the Mtb clinical isolates, and the protein encoded by this gene plays a role in ribosomal stabilization and function, thereby affecting protein synthesis *in vitro*.

2.1 Materials and Methods

2.1.1 Conservation of Rv3241c gene in different Mtb clinical isolates

Rv3241c gene (NCBI-GeneID: 888849) distribution was analyzed using the genomic DNA of clinical isolates representative of different geographical regions: *M. tuberculosis* H37Rv*, M. tuberculosis* Peru DM-148, *M. tuberculosis* Libya, *M. bovis* BCG*, M. tuberculosis* Agra 74-N, *M. tuberculosis* Holland 154, *M. tuberculosis* Austria 49, *M. tuberculosis* Turkey*, M. tuberculosis* Rajasthan R-426, *M. tuberculosis* H37Ra. PCR amplification was carried out by using the 100ng of genomic DNA, 10 pM of each primer (primers are listed in Table 1), 200 mM of each dNTP, and 1 unit of the DNA polymerase, Pfu (Fermentas, USA) with a PCR buffer of standard composition in a Gradient PCR machine (Eppendorf, Germany) suing the conditions as mentioned below: 94°C - 5mins, then 10 cycles of 94°C - 30sec, 50°C - 30sec, 72°C - 1min, 62.9°C - 30sec, 72°C - 1min and 72°C for 10mins. PCR products were analyzed and visualized on an agarose gel (1%) under UV light.

TABLE 1: Primers used in Conservation of gene and cloning

Primers	Sequence
Rv3241c Forward Primer	5'CGGGATCCATGTCAAGGCTAGCCGTG GATTCAG3'
Rv3241c Reverse Primer	5'CCCAAGCTTTCACGCCAGACGGATCAA GCC 3'
Rv3241c Forward Primer (BamHI)	5'CGGGATCCATGTCAAGGCTAGCCGTG GATTCAG3'
Rv3241c Reverse Primer (HindIII)	5'CCCAAGCTTTCACGCCAGACGGATCAA GCC 3'

2.1.2 Computational Analysis and Functional Characterization

The functional prediction of Rv3241c has shown that it is highly antigenic in nature (Lasergene software, DNA Star Inc, USA). The functional domains present in Rv3241c were identified using the Conserved Domain Search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) from the NCBI web resources and the Pfam resource (http://pfam.xfam.org) (Finn *et al.*, 2014; Marchler-Bauer *et al.*, 2017).

The homology of Rv3241c in different mycobacterial species has been done using the BLASTP. As there was no structural homolog available with permissive similarity for Rv3241c, an iterative threading approach was implemented to predict the 3D model of the Rv3241c sequence using the I-TASSER server (Yang *et al.*, 2014). To compensate for a missing region (120-155 amino acid residues) from the top 10 threading templates obtained from I-Tasser, a region from 100- 200 amino acid residues was predicted ab-inito in QUARK (Xu and Zhang, 2013). The best model (model 1) prediction from QUARK was harnessed as a user template to remodel the protein in I-TASSER again. The best fit model having the highest c-Score was used as a template and homology modeling was performed in MODELLER (Webb and Sali, 2017). The model 2, with the lowest DOPE score, was

energy minimized using GROMOS 96 54a7 force field in Gromacs (Abraham *et al.*, 2015). This minimized structure was further refined at a higher resolution in ModRefiner (Xu and Zhang, 2011). The stereo-chemical quality of this final refined structure was analyzed in PROCHECK (Laskowski *et al.*, 1993). The biological function annotation based on the structure was determined using COFACTOR (Roy, Yang and Zhang, 2012). Based on the functional domain analysis, we investigated the interactions between Rv3241c protein and the 30S ribosomal subunit by docking studies in the PATCHDOCK server (Schneidman-Duhovny *et al.*, 2005), FIREDOCK server (Mashiach *et al.*, 2008) and HEX 8.0 (Ritchie and Venkatraman, 2010). The receptor, 30S subunit of the crystal structure of one 70S ribosome from *E. coli* (4V4Q – bundle1.pdb excluding chain 'V' and HETATM) was docked with the refined 3D model of Rv3241c as a ligand in the PATCHDOCK server and the best 10 solutions were refined in FIREDOCK server. Similarly docking the receptor with ligand in Hex was executed with parameters set to Shape+Electro+DARS for Correlation type, 3D for FFT mode, and post-processing was performed with DARS Minimization. For each run, 10000 docking solutions were estimated and clustered. Default settings were used for the remaining parameters. Docking analysis was carried out by modifying the Alpha, Beta, and Gamma scales of ligand orientation to best fit the receptor and obtain the lowest binding energy solution (best-docked solution). The lowest total binding energy solution in the top cluster from multiple runs was considered the best-docked solution of the 30S subunit of the ribosome with the Rv3241c protein model. This best-docked solution from Hex docking analysis was compared with the best-refined FIREDOCK solution (solution 9) and interactions were analyzed using web server PISA (Krissinel and Henrick, 2007) and visualized in Pymol (DeLano, 2002).

2.1.3 Cloning, Expression, and Purification of Rv3241c

The amplification of Rv3241c gene comprising 645bp length was done by using Mtb H37Rv

DNA. The amplified product obtained through primers listed in Table 1 was cloned into pRSET-A vector at the *BamHI* and *HindIII* restriction sites. The amplified product was confirmed by double digestion and sequencing was performed with T7 universal primers. The recombinant construct so obtained was transformed into BL21 (DE3) pLysS cells. One liter of the LB broth media that contained 34 mg/ml of chloramphenicol and 50 mg/ml of kanamycin was inoculated with overnight culture and allowed to grow to an optical density (OD) of 0.3 to 0.6 and the cells were centrifuged at 5000rpm for 5mins. After washing, resuspending, and inducing with 1 mM IPTG (Sigma, USA), the resulting pellet was retrieved after 4 hours at 37 °C. After being re-suspended, the pellet was sonicated in the lysis buffer (20 mM Tris, 300 mM NaCl), which also contained Triton X-5 percent, Superoxide Dismutase (SODC) 0.0025%, lysozyme 1 mg/ml, and 1 mM µl phenylmethanesulfonylfluoride (PMSF). At 4°C, the lysate was centrifuged for 30 minutes at 10,000 rpm. The supernatant was incubated with cobalt-based resin (TALON Metal Affinity Resins, Clontech) for 3 hours at 4°C while a rotor was spinning and then put onto a polypropylene column and twice washed using a buffer with various imidazole concentrations. Initially it was washed with the wash buffer 1 containing 20 mM imidazole in the lysis buffer. Then column was again washed with the wash buffer 2 containing the 100mM imidazole in the lysis buffer. The protein was finally eluted with elution buffer containing 200mM imidazole in the lysis buffer. On a 12% SDS-PAGE gel, the homogeneity of the protein was evaluated. The protein was quantified using Bradford's reagent (Bradford MM. 1976) and the protein was preserved at −80°C.

The Limulus amoebocyte lysate test was performed after being treated with polymyxin-B agarose beads and the levels of endotoxin levels were found to be low.

2.1.4 Ribosomal Interaction Studies

In silico analysis has shown that rRv3241c interacts with ribosomes. To identify its possible

role in interaction with ribosomes, we have done the *in vivo* and SPR (Surface Plasmon resonance) study.

2.1.4 a) *In vivo* interaction of rRv3241c and ribosomes:

To check the interaction of rRv3241c with ribosomes, we isolated the ribosomes as previously described (Kumar *et al.*, 2012). Briefly, empty vector (pRSETA) and clone (pRSETA+Rv3241c) were transformed into *E. coli* BL21 cells. Upon induction for 5hr with 1mM IPTG, the cells were pelleted and resuspended in 10 mM MgCl2, 4 mM 2-mercaptoethanol, 20 mM Hepes-KOH buffer having pH 7.8, 60 mM NH4Cl, 1 mg/ml lysozyme and 0.2 mM PMSF. The cells were centrifuged at 10,000 rpm for 30 minutes after being sonicated. At 4°C, the supernatant was collected and centrifuged for four hours at 40,000 rpm. The supernatant was discarded, and the pellet obtained was resuspended in the same buffer and centrifuged again at 40,000rpm for 2hr at 4ºC. The resultant ribosomes were collected and stored at -80°C. To find the presence of the rRv3241c protein, 6X His tag was probed with the help of anti-His antibodies used during Western blotting.

2.1.4 b) Surface Plasmon Resonance (SPR):

The documentation of interaction between *E. coli* ribosomes (New England Biolabs) and rRv3241c in a real time manner was performed using BIACORE T200 (GE Healthcare Ltd). In immobilization experiments, as running buffer, we harnessed HBS-EP+ buffer (10 mM HEPES, 3mM EDTA, 150 mM NaCl, 0.005% polysorbate 20, pH 7.4) at a flow rate of 5mL/min. Next, the immobilization of the protein on a sensor chip - CM5 was carried out by a standard protocol entailing an amine coupling approach (BiAcore, Uppsala, Sweden). Briefly, the surface of the biosensor was primed with a 1:1 solution (120 mL) of 200 mM 'N-ethyl-N0-(3-dimethylamino propyl) carbodiimide hydrochloride' and 50 mM 'N-hydroxy succinimide', and a 1 mg/mL protein solution diluted in 10 mM sodium citrate (pH 4.0) was injected onto it. This was followed by the injection of ethanolamine-HCl (pH 8.5) to

deactivate non reacted groups. The first cell served as a blank, while protein immobilisation was done in the other three flow cells. All steps were automated in order to monitor the interaction using the BIACORE instrument. The 40 mL of samples at a flow rate of 20 mL/min were injected and were regenerated by using a buffer of pH 7.6 (10 mm Tris-HCl, 1 m NaCl, and 5 mm EDTA). During the run, the analyte (ribosome) was diluted using the running buffer. The envisaged interaction was estimated by deducting the blank units from the response of rRv3241c units.

2.1.5 Translation Inhibition Assay

In-vitro transcription and translation, the assay was performed to identify the role of rRv3241c in protein synthesis inhibition by using circular DNA template of the pBEST luc ™ system encoding the firefly luciferase gene to express with the help of an *E. coli* S30 Extract System (Promega Corporation, USA). The reaction mixture contains template pBEST luc ™ (1ug/ul) 0.5µl, amino acid mixture (containing equal amounts of methionine and cysteine) 1.25µl, S30 extract circular 3.75µl, S30 premix without amino acids 5µl, and different concentration of rRv3241c (0.1µg, 0.5µg, 1µg, and 2µg) were added and the total mixture was made up to 12.5µl with nuclease-free water. A similar reaction was performed with the bovine serum albumin (BSA) as a control. The reaction was incubated at 37°C for 60mins. After incubation, an equal amount of luciferase reagent was added to the reaction mixture. The values were taken immediately after the addition of luciferase reagent using a luminometer.

2.1.6 Effect of Rv3241c on the growth of *Mycobacterium smegmatis*

To check the effect of Rv3241c on the growth of *M. smegmatis*, the growth curve was performed by using the recombinant *M. smegmatis* strain which overexpresses Rv3241c and a strain with an empty pMV261 vector. The recombinant clone (pMV261 + Rv3241c) and empty vector (pMV261) were electroporated into *M. smegmatis* to generate the Rv3241c

overexpressing strain and a vector control strain (with empty pMV261 vector). The single colony obtained was inoculated in Middlebrook 7H9 media added with 10% oleic albumin dextrose catalase (OADC) (Sigma) containing 0.05% Tween-80, 0.4% glycerol, and 25 μg/ml Kanamycin. To induce pH stress conditions the recombinant strains were inoculated into the growth media containing pH 4.5. Similarly, since glycerol acts as a carbon source, the percentage of glycerol was reduced to 0.04% to induce carbon stress conditions. The culture's optical density (OD) was monitored at intervals of 6 hours up to 48 hours starting at an initial optical density (OD600) of 0.04.

2.2 RESULTS

2.2.1 Conservation and *in silico* analysis of Rv3241c

The presence of the Rv3241c gene in the clinical isolates of mycobacteria which were collected from different geographical regions and whose DNA extracts were available in collection of our lab was analyzed. It has been observed that the gene Rv3241c was conserved in all the isolates as shown in **Figure 4** from modern to ancestral lineages. The homologous of Rv3241c in different mycobacterial species were shown in **Figure 5**. Due the presence of the Rv3241c gene in almost all the clinical isolates, we have characterized gene which might play an important in mycobacteria.

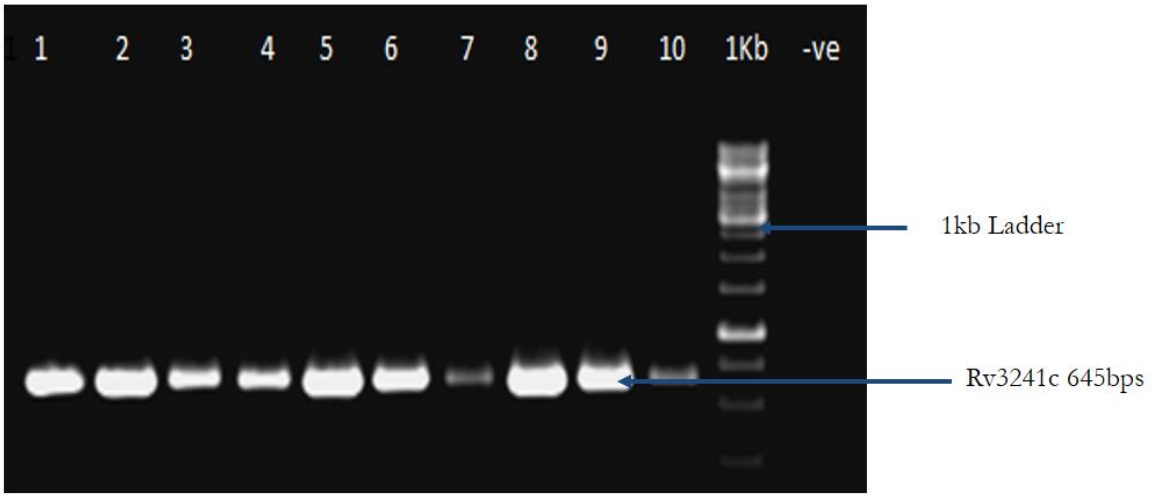


Figure 4: PCR results indicate the presence of conserved regions in almost all mycobacterium isolates. 1% Agarose gel showing PCR amplification of Rv3241c. Lane 1-10 denotes the different clinical isolates of Mtb such as *M. tuberculosis* Peru DM-148, *M. tuberculosis* Libya, *M. tuberculosis* Agra 74-N, *M. tuberculosis* Holland 154, *M. tuberculosis* Austria 49, *M. tuberculosis* Turkey, *M. tuberculosis* Rajasthan R-426, *M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra, *M. bovis* BCG.; 1kb ladder; -ve denotes the negative control.

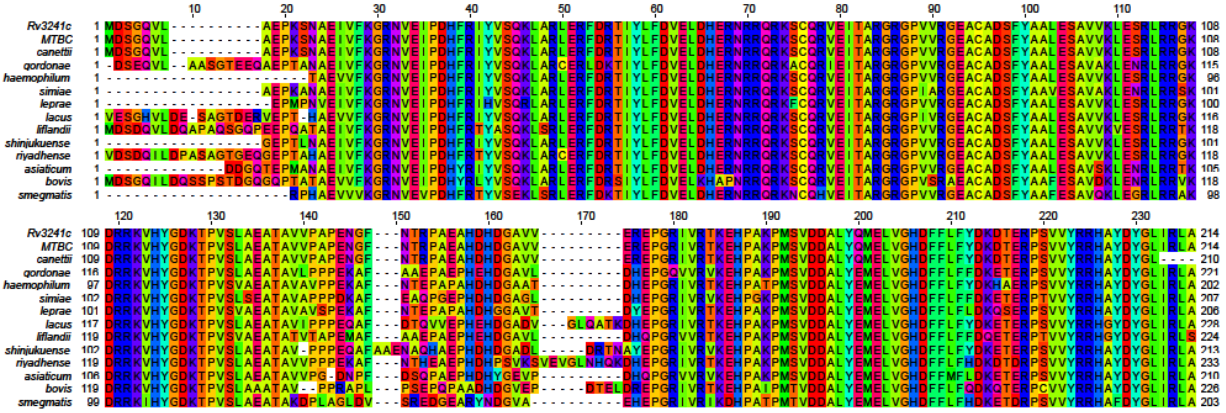


Figure 5: Homologs of Rv3241c in different mycobacterial species

2.2.2 Computational Analysis and Structure Prediction

Nearly 10 putative antigenic epitopes were identified which might play a role in drug development which was done by using DNA Star Software as shown in **Figure 6**. Based on the domain analysis, we observed that Rv3241c has two conserved domains, and it does not

contain either transmembrane domain or signal peptide as indicated in **Figure 7**. The first domain spans 14-114 amino acids, and functions like ribosome-associated inhibitor A (raiA) which might be involved in the arrest of translation by obliterating the binding of aminoacyl-tRNA to the ribosomal site A (Agafonov, Kolb and Spirin, 2001). The second domain ranges from 158-210 amino acids (S30AE ribosomal protein C) and this domain is predicted to be binding to a small subunit of 30s ribosome and dsRNA, and stabilize the ribosomes against dissociation when bacteria are under environmental stress (Ye *et al.*, 2002).

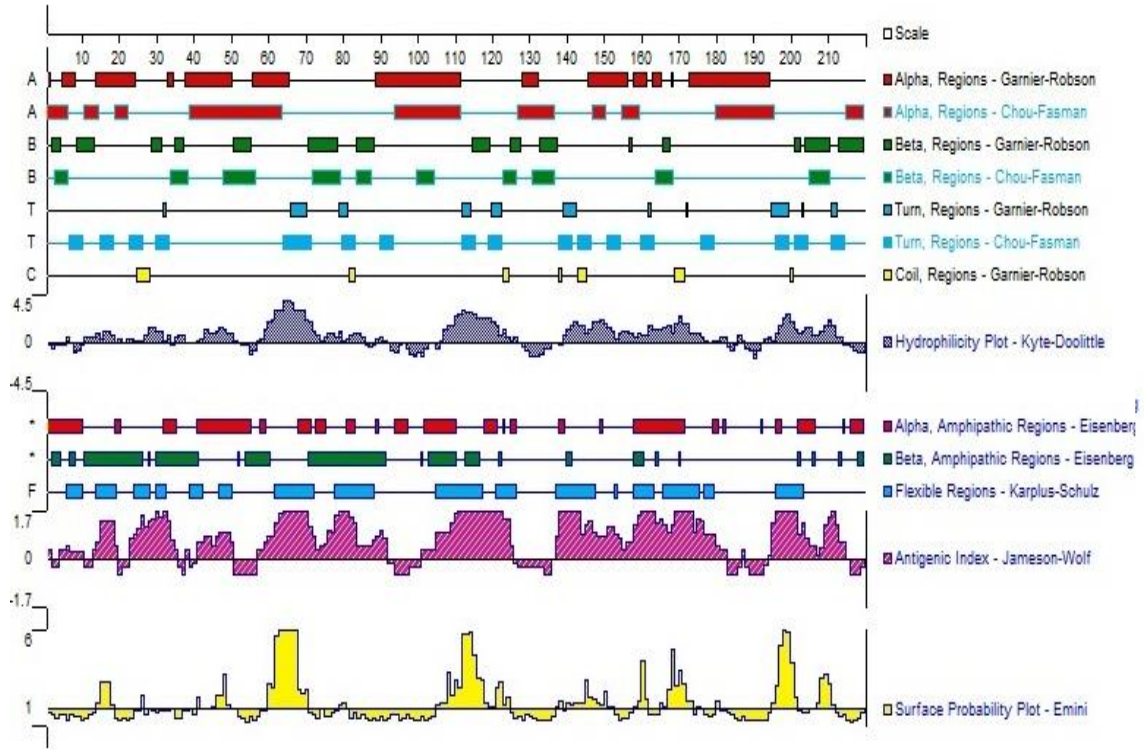


Figure 6: In Silico antigenic profile of Rv3241c using the DNA star Software

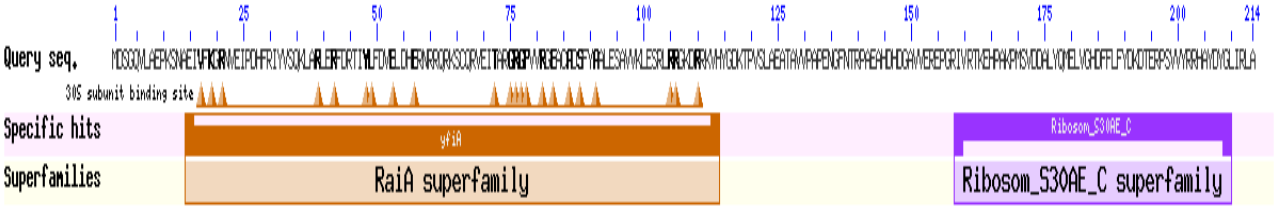


Figure 7: Rv3241c has one active conserve domain from amino acids 15 to 125 and a predicted domain, which functions like ribosome-associated inhibitor A; it possibly functions as a stress-response factor by binding to the ribosomal subunit interface and to arrest translation by interference with the alignment of aminoacyl-tRNA at the binding site A of the ribosome.

The 3D structure of rRv3241c was obtained by multiple threading approaches from the online available I-TASSER server as shown in **Figure 8**. Several programs were used to search for a structural template for rRv3241c, but there was no homolog available in the protein data bank (PDB). Hence, an I-TASSER server that identifies structural templates from the PDB by threading method was used which provided five models as possible structures. We chose the model with the lowest possible energy. The possible docking interaction between rRv3241c and 30S ribosomal subunit was performed by using several programs such as PatchDock, GRAMM-X, and HEX. HEX 8.0 servers were used for the docking study with rRv3241c and 30S ribosome as a ligand and receptor respectively. A total of 128 predictions were generated using HEX and were analyzed using PyMOL software to check the region of interaction, bond clashes, bond distances, etc. After analysis, five structures were selected and subjected to a PISA server to determine the solvent accessible area and solvation energy. It was found that all five predictions have different numbers of residues in the interface; further solvation area and energy were also diverse. However, we have selected the most appropriate complex as shown the **Figure 9** based on maximum surface interaction area and lowest solvation energy. The predicted amino acids involved in the interaction has shown in Table 2.

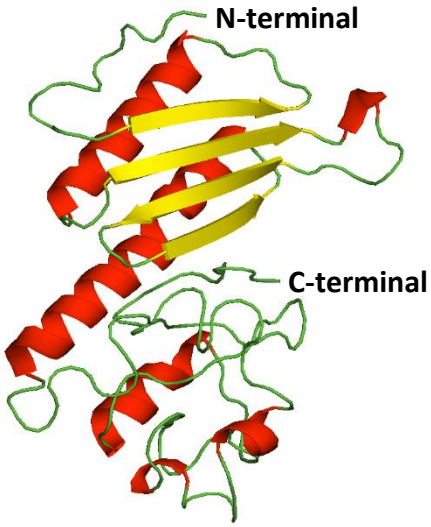


Figure 8: Homology modeling of Rv3241c: The helices and sheets of protein secondary structure were colored in red and yellow respectively.

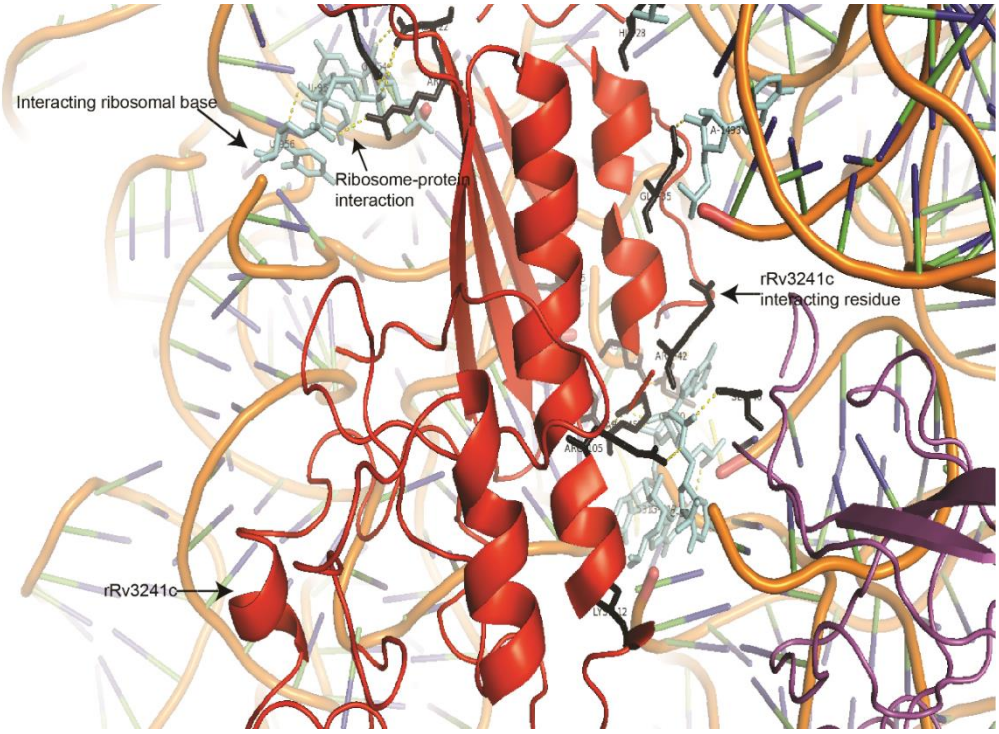


Figure 9: The interaction of Rv3241c with 30s ribosomal subunit: The interaction of rRv3241c with 30s ribosomal proteins was done by using Patch Dock and FireDock. The 30s ribosome's protein chains and rR3241c's protein chains are shown as electrostatic

surfaces and are coloured in green and blue, respectively.

Table 2: Predicted amino acids involved in the interaction

Residue no	RNA	Distance
GLU 15	A:G1053	3.41
ARG 21	A:G 954	2.57
ARG 21	A:U 956	3.44
ASN 22	A:G 954	3.57
ASN 22	A:U 955	3.49
HIS 28	A:U 1495	3.11
GLN 35	A:A1 493	2.02
ARG 45	A:G 530	2.08
ARG 45	A:U 531	3.73
TYR 48	A:U 531	3.6
ARG 61	A:G 954	3.43
ARG 105	A:C 519	3.73
LYS 112	A:G 517	3.44
ARG 42	K:SER 46	3.77
ASN 13	B:GLU 160	2.1

2.2.3 Molecular Cloning and Purification of Rv3241c

The Rv3241c gene fragment was amplified and analyzed on 1% agarose gel by using specific primers as mentioned in Table 1. The product was about 645bp in length and was confirmed by double digestion as shown in **Figure 10** and by using sequencing with T7 universal primers. The His-tagged recombinant protein Rv3241c was expressed by using *E. coli* BL21 pLys S strain and the purified protein was observed on 12% SDS gel at 28 kDa position by using coomassie brillant blue as shown in **Figure 11**.

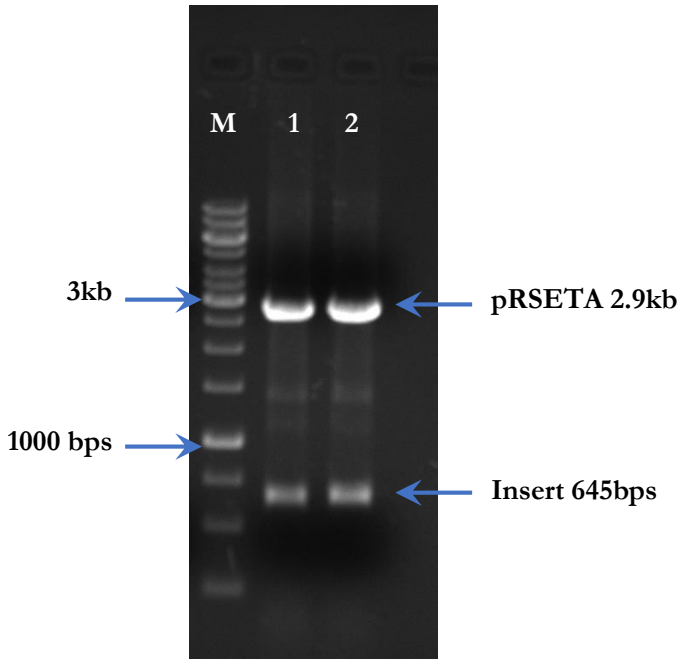


Figure 10: Double digestion was confirmed on 1% agarose gel to check the presence of Rv3241c insert in the pRSETA vector by using BamHI and Hind III restriction enzymes. Lane M–Marker 1kb ladder and Lane 1,2 – double digestion product

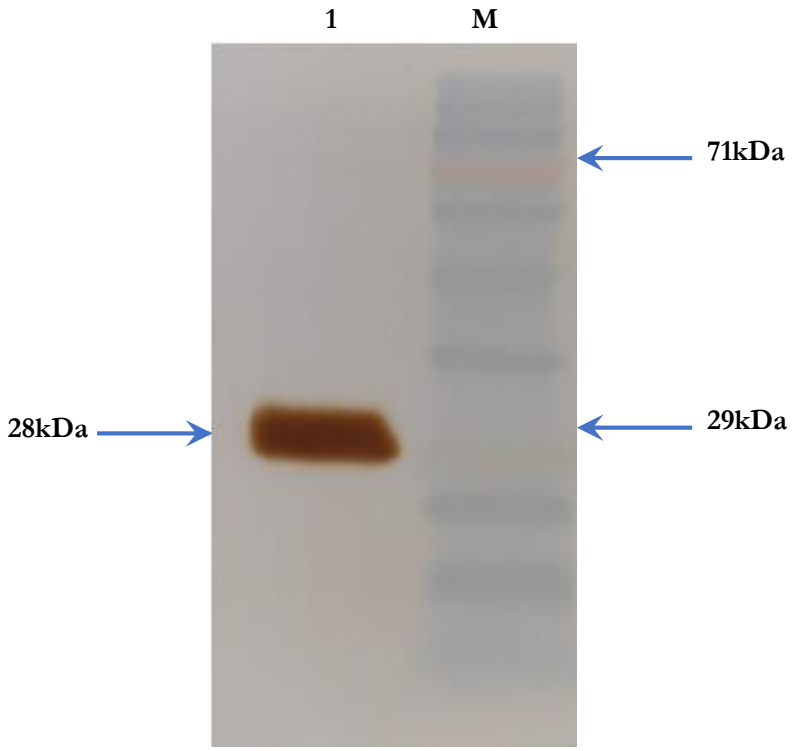
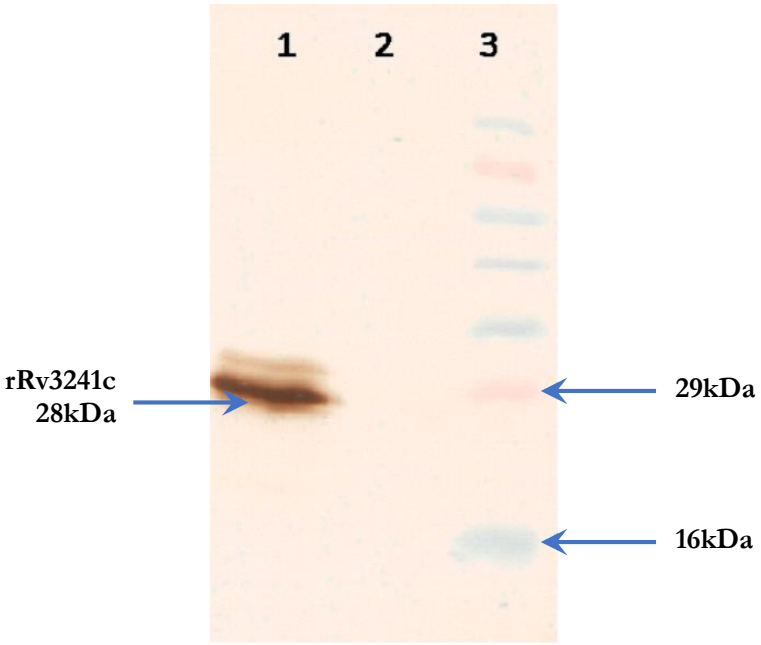


Figure 11: Purification of Rv3241c with a molecular mass of 28kDa was done by using affinity chromatography, as described in methods section. The protein purity was checked on 12% SDS gel. Lane 1: Purified protein and Lane M: Protein Ladder

2.2.4 Interaction of rRv3241c with ribosomes

The binding of rRv3241c to *E. coli* ribosomes was analyzed by western blotting. In contrast to the negative control, where the recombinant protein did not bind to the ribosomal fractions as shown in **Figure 12**, rRv3241c migrated with the ribosomes, showing that it stayed bound to ribosomes recovered from *E. coli* overexpressing rRv3241c.



Lane 1: Ribosomal Fraction
Lane 2: Negative Control
Lane 3: Protein Marker

Figure 12: *In vivo* rRv3241c and ribosome interaction: Western blot was used to detect the presence of rRv3241c with ribosomes. The blot indicated the migration of rRv3241c along with ribosomes when compared with the negative control which lacked any recombinant protein. Lane 1: Pure fraction of ribosomes from overexpressed rRv3241c in *E. coli* cells; Lane 2: Purified fraction of ribosome from *E. coli* transformed only with pRSETA vector Lane 3: protein molecular weight marker conveying different size indicators.

Further, analysis on the BIAcore® (Surface Plasmon Resonance) platform of the GE Healthcare, revealed that rRv3241c binds to the ribosomes (ka=6.611E+7) as shown in **Figure 13**. The complex obtained appeared to be highly stable as judged by the slow dissociation rate (kd= 0.005435). A nice binding fit (KD= 8.221E-11) was found confirming the above values of ka and kd.

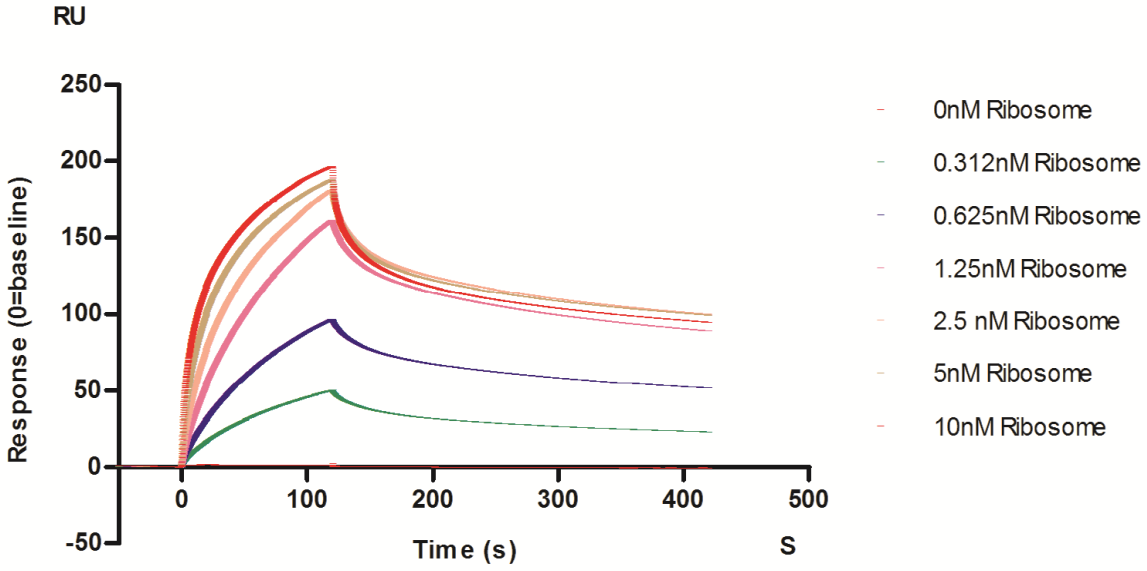


Figure 13: Interaction of rRv3241c with ribosome using Surface Plasma Resonance **(SPR):** rRv3241c was immobilized on a CM5 chip via amine coupling and different concentrations of ribosome were passed on immobilized rRv3241c.

2.2.5 *In-vitro* protein inhibition assay

Bioinformatics analyses revealed that rRv3241c contains a ribosome binding domain that may influence the translation of proteins on the ribosomes. Hence, we carried out *in vitro* protein synthesis using pBESTluc™ that contains the firefly luciferase gene as a template, in the presence of varying concentrations of rRv3241c protein. A dose-dependent decrease in luminescence was observed as shown in **Figure 14** suggesting that rRv3241c inhibits luciferase mRNA translation.

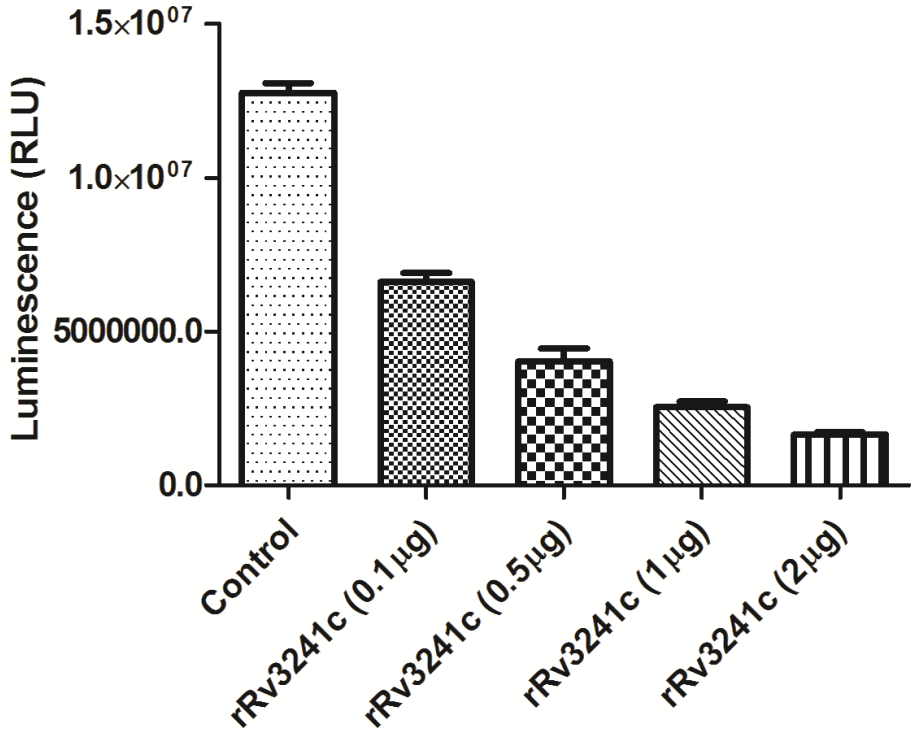


Figure 14: *In-vitro* protein synthesis inhibition: Assay was done by using pBESTlucTM as template DNA containing the luciferase gene. It has shown that there is a significant decrease in the synthesis of luciferase in a dose-dependent manner which suggests the inhibition of translation the rRv3241c might be involved in inhibition of translation.

2.2.6 Rv3241c does not affect the growth of *Mycobacterium smegmatis* under stress conditions.

Under pH stress conditions, no significant difference in the growth of *M. smegmatis* overexpressing Rv3241c was observed when compared with *M. smegmatis* transformed with the empty pMV261 vector (**Figure 15**). Under carbon stress conditions, growth appeared to be slow at the initial time points in Rv3241c overexpressing *M. smegmatis*, which recovered at later time points and was similar to the *M. smegmatis* harboring empty pMV261vector as shown in **Figure 16**. These results indicate that Rv3241c might not be involved in regulating the growth of *M. smegmatis* under normal and stress conditions.

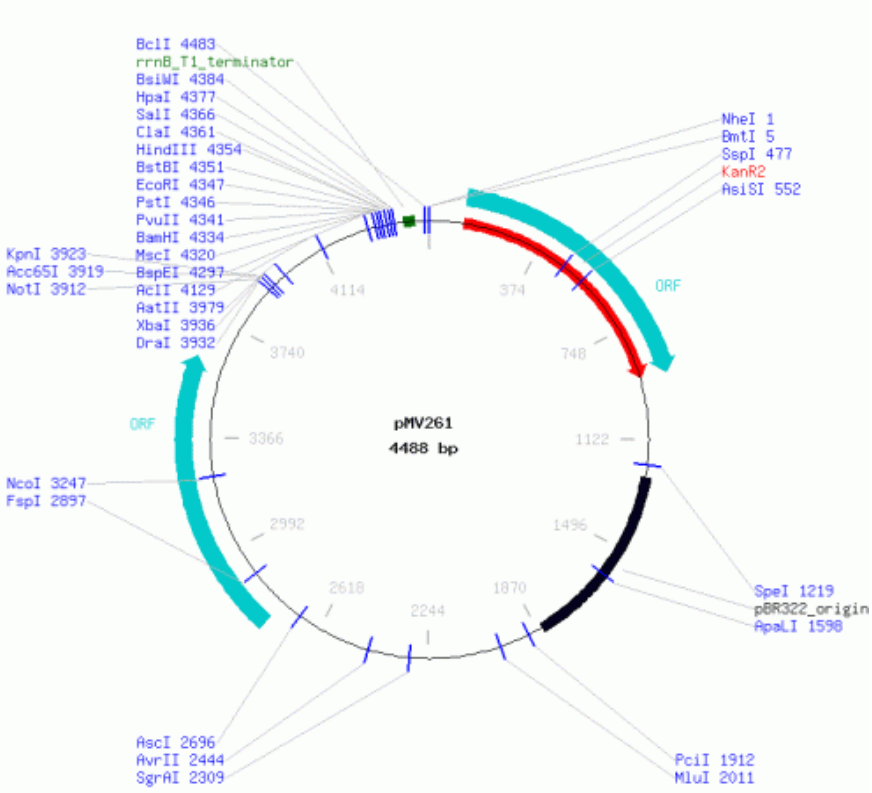


Figure 15: pMv261 vector map

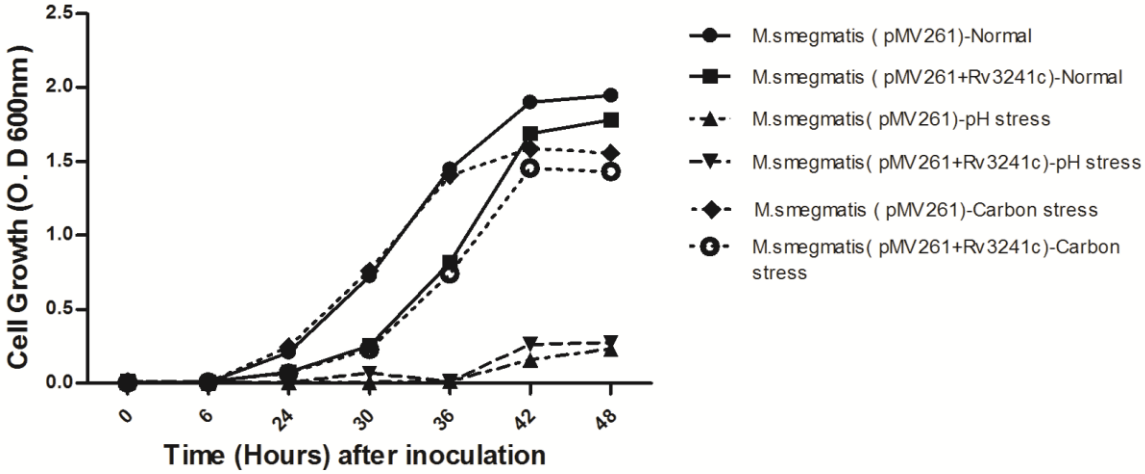


Figure 16: Growth of *Mycobacterium smegmatis* under different conditions: With an initial OD (600 nm) of 0.02 *M. smegmatis* (pMV261+Rv3241c) and *M. smegmatis* (pMV261) were inoculated into Middlebrook 7H9 broth. The infected cultures were kept in static 37°C conditions during incubation. By taking an OD reading at 600 nm, the growth curves of

strains under normal, pH-stressed, and carbon-reduced conditions were constructed. The growth of the *M. smegmatis* bacterium has not been found to differ significantly. The values in the graph are the mean and standard deviation of the three independent ODs/static cultures.

2.3 Discussion

M. tuberculosis is considered one of the oldest known chronic pathogens since it causes persistent infection in more than 2 billion people, yet largely without causing clinical symptoms (Lin and Ottenhoff, 2008; Gengenbacher and Kaufmann, 2012). TB remains a major cause of mortality and morbidity worldwide regardless of the effective drug treatment (Raviglione and Sulis, 2016) due to which there is an increase in the drug resistance to TB (Chao and Rubin, 2010; Law *et al.*, 2017). The antigens associated with latency are identified and the development of vaccines based on these antigens was pursued over several decades (Kaufmann, 2007; Smith, Wolff and Nguyen, 2013; Peddireddy *et al.*, 2016). However, understanding the ability of Mtb to adapt to adverse conditions within the host, its resuscitation and dormancy remain a challenge (Chao and Rubin, 2010; Kriel *et al.*, 2018). In recent years, it was identified that the ability of Mtb to adapt to the stress conditions of the host and to remain latent might involve maintaining the cellular structures and stabilization of the ribosomes (Kumar *et al.*, 2012). The role of Mtb proteins in inhibiting ribosome formation or stabilizing the ribosomes resulting in a slowdown of the protein synthesis was poorly reported. Some of the ribosomal binding proteins of Mtb have been previously characterized.

The phylogenetic analysis of mycobacterial proteins has shown two clusters, Group1 contains MSMEG_1878 a homolog of *M. tuberculosis* Rv3241c, and Group 2 contains MSMEG_3935 a homolog of Rv0079 of *M. tuberculosis* which have S30AE domains. MSMEG_3935 with homolog Rv0079 in Mtb has been implicated in the regulation or

inhibition of protein synthesis by interacting with ribosomal subunits (Trauner *et al.*, 2012).

The Rv3241c characterized in this study contains the S30AE domain that is responsible for maintaining the stability of ribosomes. Sequence and domain comparison analysis indicated that Rv3241c has two active conserved domains, namely ribosome-associated inhibitor A (raiA) and S30EA ribosomal protein C, both of them are involved in binding the ribosomes to affect protein translation (Ye *et al.*, 2002). In this study, docking simulations of rRv3241c with 30s ribosomes revealed that this protein interacts with the ribosomes, which is corroborated by the observations obtained using surface plasmon resonance analysis which showed that it binds to the ribosomes. Further, *in vitro* translation data conveyed that rRv3241c possibly inhibited translation indicating a role of rRv3241c in stabilizing the ribosomes to conserve cellular resources and preserve them after prolonged stasis.

Mycobacterial proteins are known to contribute to the survival strategies of Mtb when the pathogen encounters stress environments in the host such as extremes of pH, nutrient deprivation, and oxidative stress (Peddireddy, Doddam and Ahmed, 2017). Our results indicate that Rv3241c does not contribute to the growth patterns of *M. smegmatis* under pH stress and carbon starvation conditions. Mycobacterial proteins may function in multiple ways to enhance survival strategies. The absence of the effect on growth was observed in Mtb that expressed mutated universal stress proteins (USPs; Rv2028c, Rv2026c, Rv1996, and Rv2005c) (Hingley-Wilson *et al.*, 2010). Bacterial growth was not affected in *M. smegmatis* which expressed PpiA/PpiB protein (Pandey *et al.*, 2017). However, PpiA/PpiB protein affects the proinflammatory cytokine responses. The lack of effect on the growth patterns of *M. smegmatis* by Rv3241c (and by many other proteins reported previously) indicates that its function during latency is mostly limited to interacting with ribosomes and thereby inhibiting protein synthesis.

Therefore, in this study, we demonstrated the binding of rRv3241c to the ribosomes

suggesting that this protein might play a crucial role in ribosome stabilization and thereby latency and no influence on the growth of *M. smegmatis* has was observed.

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Chapter 3

Studies on the possible role of Rv3241c in immune responses

3.1 Introduction

Nearly one-third of the population is latently infected with Mtb, which serves as a reservoir of infection (Fenton, 1998; Hingley-Wilson *et al.,* 2003). To develop the new diagnostic kits, vaccines, or strategies for treatment, we need to better understand the host/ pathogen interaction (Fenton, 1998). For the long antibiotic treatment, persistent bacilli may be responsible for the efficient treatment of TB. Infection occurs when a person inhales the bacilli from airborne droplets. The bacilli then enter the alveolar macrophages where they survive and multiply. These infected macrophages form the granuloma and are the important hallmark of the immune response to Mtb. Granuloma is an organized structure that serves to protect the body by preventing the dissemination of bacilli to new sites, thus giving a chance to host immune cells to culminate the threat of developing the disease (Cosma *et al.*, 2008). Granuloma creates a micro-environment of high nitric oxide and low oxygen through which infection can be controlled, and it provides a niche where bacillus can survive by modulating the host immune response. Granuloma serves to protect the body by preventing the dissemination of bacilli to new sites, thus giving a chance to host immune cells to culminate the threat of developing the disease. Some of the systemic diseases have been characterized by using an innate immune inflammatory process where the macrophages and arms of innate immune response play an important role in this process (Petersen and Smith, 2013).

Hence, understanding the function and interaction between cytokines and chemokines is critical. The adaptive and innate responses play a crucial role in the eradication of microorganisms. Toll-like receptors, C-type lectins, Nucleotide Oligomerization Domain (NOD)-like receptors, and Pathogen Recognition Receptors are involved in the recognition of mycobacteria and initiation of cytokines responses (Romero-Adrian, 2015). Adaptive immunity is activated when bacillus evades the innate mechanism (Torrado and Cooper,

2013). During the early infection, bacterium resides in immune-privileged sites (Gallegos *et al.,* 2008; Urdahl *et al.,* 2011). Within macrophages, Mtb is phagocytized where its survival depends on phagosome processing (Manabe and Bishai, 2000). The infected macrophages secrets the TNF – α, which recruits CD4+ and CD8+ to the site of infection (Flynn and Chan, 2001) and further activation of macrophages is caused by Interferon-γ (IFN-γ) (Flynn *et al.*, 1993). Activated macrophages, TNF-α induced apoptosis of infected macrophages or cytotoxic functions of activated CD4+ and CD8+ T cells mainly kill the bacteria (Romero-Adrian, 2015).

The outcome or progression of infection in the granulomas is dependent on various specific factors of both the host and the pathogen that might be involved in the progression of the disease (Singh *et al.,* 2014; Doddam *et al.,*). Pattern recognition receptors (PRRs) are crucial in controlling the early Mtb infection as they interact with the cell wall components of the bacilli and induce the cytokines response (Doddam, Peddireddy and Ahmed, 2017). Toll-like receptors (TLR) play an important role in the reorganization of a wide variety of conserved ligands among the PRRs (Kawai and Akira, 2010). Pro and anti-inflammatory cytokines are secreted as a result of the interaction of TLRs with Mtb antigens, which triggers a downstream signalling pathway. These cytokines are essential for attracting macrophages and immune cells, as well as being involved in the maintenance of the granuloma (Nair *et al.*, 2009; Kumar *et al.*, 2012).

In order to establish the infection, a balance of the pro-inflammatory and anti-inflammatory cytokines is crucial for constantly maintaining the homeostasis by managing the immune system of the host in the granuloma for its existence and controls the effectiveness of the tissue damage (Romero-Adrian, 2015; Cadena *et al.,* 2017) **(Figure 17)**. The immune system reacts to the response from the host and pathogen, balancing the steady state through continuous assessment. It is necessary to determine how the host recognizes the infection

and the signals that activate the immune system (Etna *et al.*, 2014; Cicchese *et al.*, 2018).

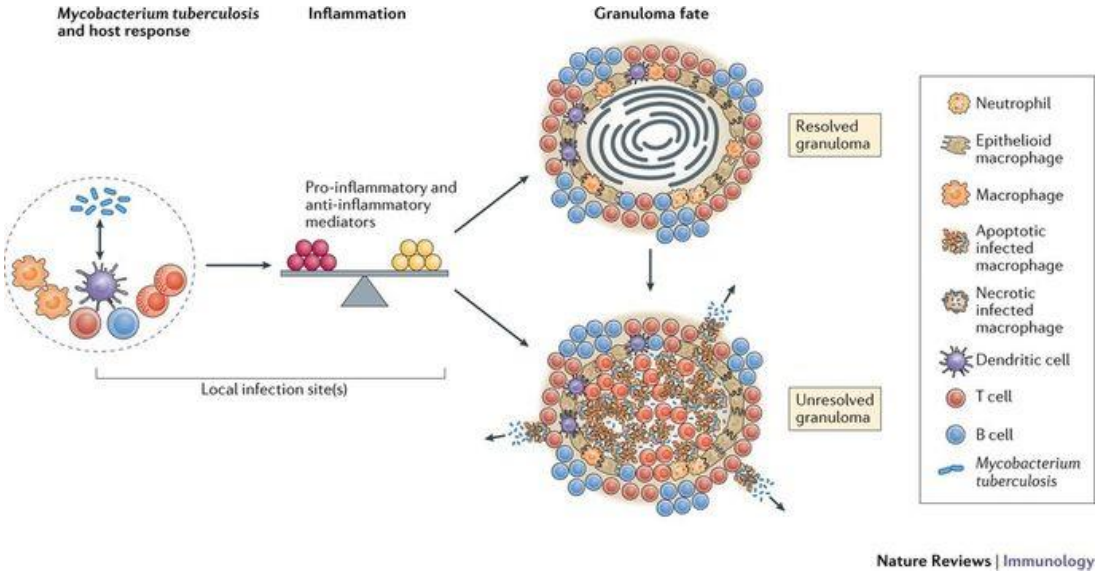


FIGURE 17: The interaction between the bacteria and the host immune cells plays a crucial role in influencing the inflammatory programs and the process is highly dynamic (Cadena *et al.,* 2017)**.**

Cytokines are essential in the progression of immunopathological symptoms, but the mechanism for the development of protective immunity and the pathogenesis of infection is less understood (Nair *et al.*, 2009; Kumar *et al.*, 2012). Understanding the host-pathogen interaction provides insights into the virulence mechanism and may provide new approaches to the immunological interface in tuberculosis (Cooper *et al .,* 2011).

The cytokines regulate bacterial proliferation. The pro-inflammatory cytokines such as TNF-α and IFN- γ are especially important in stimulating the formation and function of granuloma. The role of TNF-α has been extensively studied and is known to play an important role in the immune response to a variety of infectious pathogens (Champsi, Bermudez and Young, 1994; Roach *et al.*, 2002; Torrado and Cooper, 2013) while IL-10 of anti-inflammatory cytokines is one of the major negative regulators of immune response to the microbial antigens (Jo *et al.*, 2007; Kirschner, Young and Flynn, 2010; Cooper, Mayer-Barber and Sher, 2011b; Silva Miranda *et al.*, 2012; Rutz and Ouyang, 2016).

In this study, we have determined the ability of Rv3241c to elicit a pro-inflammatory cytokine. response.

3.2 Material and Method

3.2.1 Ethics Statement

The respective ethics committees of the participating institutes, namely the Bhagwan Mahavir Hospital and Research Centre (BMHRC) and the University of Hyderabad reviewed and approved the experiments involving the recombinant bacterial strains and human sera samples used in this study. A biosafety approval as well was obtained from the IBSC of the University of Hyderabad. These institutions are located in Hyderabad, Telangana, India. Immuno-compromised people, people under the age of 18, those with diabetes or any autoimmune disorders, and pregnant women were not included in the study before the collection of the human sera samples. Following the World Medical Association's code of ethics, written consent was obtained from each subject after written notification.

3.2.2 Characteristics of cohort and blood samples

The human cohort comprised of 121 people (32 with active TB, 52 with latent TB, and 37 Healthy) who reported at the BMHRC and Government Chest Hospital, Hyderabad. The serum was separated from 5 ml of blood collected from each subject and was stored at −80°C until further use. Active TB cases were determined by chest X-ray, acid-fast staining, and cultures before treatment with anti-TB drugs. For Latent tuberculosis-infected individuals (LTBI) household contacts with active TB cases, hospital staff from BMHRC and Government Chest Hospital and were recruited.

Positive cases of the IGRA (Interferon Gamma Release Assay) – a test based on (QuantiFERON-TB Gold (QFT®) commercially obtained from Cellestis (Australia), were held as LTBI, whereas negative cases were treated as healthy cohorts (HCS).

3.2.3 Cytokine assays

In a 24-well plate, approximately 0.2 million THP-1 cells were planted in each well. (TPP, Trasadingen, Switzerland), to differentiate into adherent macrophage-like phenotype. The cells were treated with Phorbol 12-myristate 13-acetate (PMA) (Sigma, MO, USA), overnight, at a concentration of 10ng/ml. The cells were washed twice with RPMI-1640 and then treated with varying concentrations of rRv3241c (10, 100, and 1,000 ng/ml) for measuring IL-8, IL-1β, and (10, 500, and 1,000 ng/ml) for measuring TNF-α for 24hr and 48 hr. LPS was used as a positive control. Untreated cells were used as a negative control. The plates were incubated at 37°C with 5%CO_2 and the supernatants were collected after 24hr and 48hr and stored at -80°C. A sandwich ELISA (eBiosciences, USA) was used to carry out the analysis of pro inflammatory cytokines, according to the instructions of the manufacturer.

3.2.4 Indirect ELISA active TB and latent cases sera samples

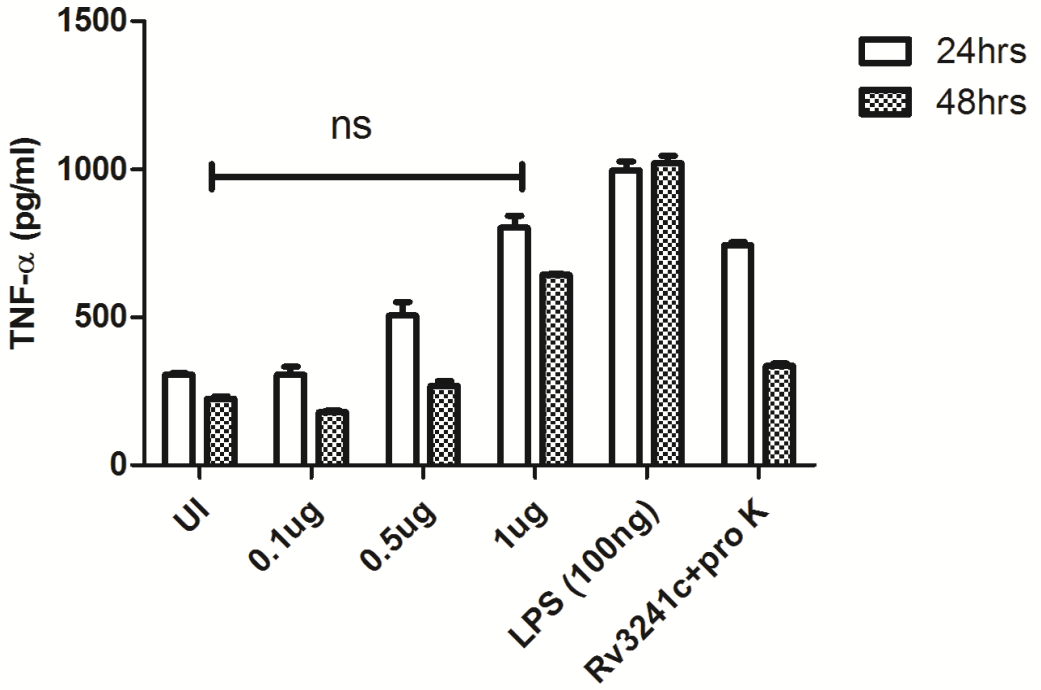
Indirect ELISA was performed as described earlier (Rani *et al.*, 2014; Doddam et al., 2017), to check the humoral immune responses of pulmonary tuberculosis cases (PTB), latent tuberculosis-infected individuals (LTBI), and healthy cohorts (HC) against the rRv3241c protein. Briefly, the purified recombinant protein rRv3241c in bicarbonate buffer, at 250 ng concentration, was used to coat the wells of the ELISA plates. The plates were kept night long in an incubator at 4°C. Subsequently, they were washed three times with 1X PBST (1 PBS with 0.05% Tween 20) and it was then blocked with BSA (0.2%) for 2 hr at 37°C. The plate was again washed five times by soaking the plate each time for 1min. The 1:250 diluted sera samples added in each well with 1XPBS of 100µl were incubated for 3 hr at 37°C. The plates were subjected to wash for five times with 1X PBST, after the completion of incubation. The plates were incubated with the HRP-conjugated, antihuman IgG (Sigma) used as secondary antibody (1:15,000 diluted), for 1 hr at 37°C followed by washing with 1X

PBST. It was developed with a substrate solution of '3,3',5,5'-tetramethylbenzidine', as described (eBiosciences, USA) for 30 mins. Reaction was then terminated with 2N H2SO4. By using the ELISA reader (TECAN Infinite M200) the intensity of color was read at 450 nm by tuning the wavelength at 570 nm.

3.3. Results

3.3.1 Role of rRv3241c in inducing cytokine response and its immunoreactivity against sera samples

To determine the ability of rRv3241c to elicit a pro-inflammatory cytokine response, IL-1β, TNF-α, and IL-8 levels were measured in PMA differentiated THP-1 cells. rRv3241c did not induce the production of the pro-inflammatory cytokines that we tested **(Figure 18)**. It was observed that there was no immunoreactivity to rRv3241c in LTBI and PTB sera sample cohorts indicating that rRv3241c might not be immunogenic **(Figure 19)**.



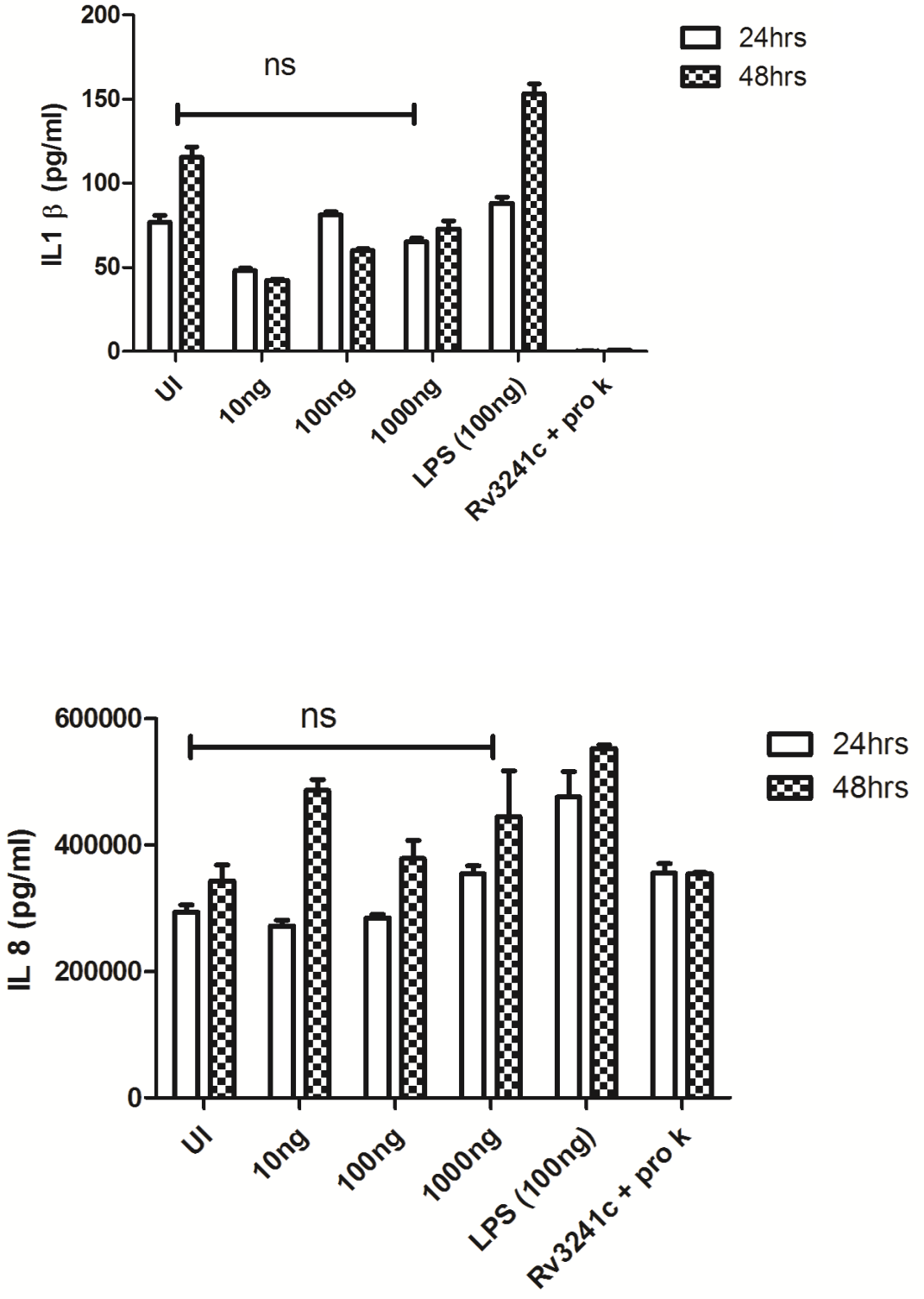


Figure 18: The release of pro-inflammatory cytokines by the THP1 cells: The cell-supernatants were subjected to treatment with varied concentrations of rRv3241c and the analysis of TNF-α, IL-1β and IL-8 was carried out in a time and dose dependent manner by

using sandwich ELISA. Cells having a treatment with proteinase-K or un-induced cells (UI) were taken as negative control and LPS (100 ng/ml) served as positive control.

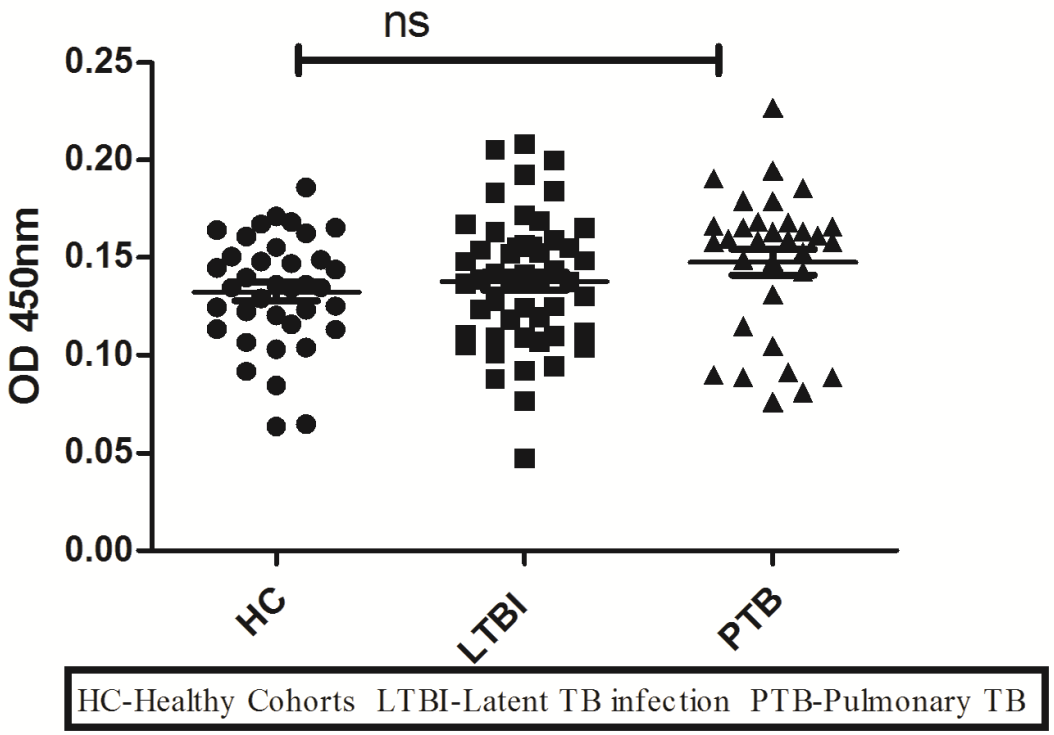


Figure 19: Humoral immune response of rRv3241c in HC, LTBI, and PTB groups: Indirect ELISA method was used to compare the humoral response against rRv3241c among HC, LTBI, and PTB. There was no significant difference observed in the immunoreactivity of rRv3241c among HC, LTBI, and PTB sera samples. Statistical analyses of different kind were carried out by using the Mann-Whitney U test and the data represent the three independent experiments. A horizontal line that included the ±SD served as a representation of the median values for each population.

3.4 Discussion

TB is held as one of the top 10 causes of death and an estimated 10 million people fall ill with it (WHO, 2019). Mtb infection is thought to result in two clinically defined stages -

latent infection defined as latent TB (LTB) and active disease. The main characteristic feature of LTB is immunological sensitivity to micro-bacterial antigens. It has been estimated that more than 2 billion individuals and about 90% of human infections are LTB (Corbett *et al.*, 2003; Getahun *et al.*, 2015; Cadena *et al.,* 2017). Hence, the identification of new markers and vaccine candidates is necessary to prevent LTB. Mtb causes the primary active infection and can persist in the host for years entering into a dormant state (Velayati *et al.*, 2016). It encounters the host's variable environment, leading to a complex immune response resulting in latent infection (de Martino *et al.*, 2019). One of the major causes of active tuberculosis is the reactivation of latent TB. Along with the formation of granulomas, the macrophages and CD4+ lymphocytes are considered as the pillars of an immune response against Mtb (de Martino *et al.*, 2019). It is crucial to understand the reactivation of latent tuberculosis with respect to both host and bacillus to control the disease (Flynn *et al.*, 1993).

Initially, infection with Mtb occurs in alveolar macrophages where bacteria replicate and induce the inflammatory cytokines response in the lungs. Macrophages and lymphocytes leave the site of infection leading to the formation of granuloma (Flynn *et al.*, 1993; Ehlers and Schaible, 2012; Dannenberg, Jr. and Rook, 2014) or aggregate in response to antigen. The main function of the granuloma is to isolate antigens such as fungi, bacteria, foreign bodies, and other immune complexes and serves to protect from dissemination to new sites (James, 2000; Ramakrishnan, 2012). Immune defects which particularly affect the innate immune system have shown the poor formation of granuloma. The lack of the cytokines such as interferon-gamma (IFNγ), tumor necrosing factor-alpha (TNFα) and Interleukin 12 (IL-12) leads to the poor formation of granuloma (Ramakrishnan, 2012; Petersen and Smith, 2013). Induction of pro-inflammatory cytokines by mycobacterial proteins is a hallmark of the system to survive and establish in the host by Mtb (Domingo-gonzalez *et al.*, 2016).

We have estimated the serodiagnostic potential of rRv3241c in LTBI and the results of our

study demonstrated that rRv3241c does not induce pro-inflammatory cytokines in PMA-activated cells *in vitro* . The inability of rRv3241 to induce pro-inflammatory cytokine responses might contribute to latency by targeting the ribosomes. It was observed that there was no immunoreactivity to rRv3241c in LTBI and PTB sera sample cohorts indicating that rRv3241c might not be immunogenic. These results might be significant in understanding the role of rRv3241c at the level of transcription and translation as it does not induce the cytokines.

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Chapter 4

Molecular modeling of Rv3241c as a putative Clp protease substrate

4.1 Introduction

M. tuberculosis is an organism of global importance and there is an urgent need to develop novel therapeutics as multidrug resistance strains are emerging significantly (Duncan, 2003; Ollinger *et al.*, 2012). Although new drug targets have been discovered, further efforts are needed for target validation. The main goal of TB chemotherapy is to develop highly active drugs and the duration of treatment should be short (Suresh *et al.*, 2020). During infection, cellular pathways are crucial for the survival of bacterium which might be a good candidate for designing rational drugs (Williams and Duncan, 2007; T. S. Balganesh and B. J. A. Furr, 2008; Ollinger *et al.*, 2012). Protein degradation constitutes the cellular process that is important in nurturing cellular homeostasis through the regulation of biological pathways and protein quality control (Goldberg, 2003; Ingmer and Brøndsted, 2009; Raju *et al.*, 2012). Most of the prokaryotes contain various ATP-dependent serine protease complexes such as Lon protease, Clp proteases (Frees *et al.*, 2007)and the serine protease (Hwang *et al.*, 1987; Katayama-Fujimura, Gottesman and Maurizi, 1987). These complexes were first described in *E. coli* whereas archaea and actinomycetes contain proteases such as threonine proteases. *M. tuberculosis* encodes both proteasome and Clp protease. The role of the Mtb proteasome has been explored while less is known about the mycobacterial Clp proteases (ClpP) (Darwin *et al.*, 2003; Burns, Pearce and Darwin, 2010; Cerda-Maira *et al.*, 2010).

ClpP is a non-essential conserved serine protease that is involved in the stress response and contributes to the quality control of proteins (Ollinger *et al.*, 2012; Raju *et al.*, 2012). Clp proteolytic complex is composed of proteolytic subunits and ATP adaptors such as clpX /clpA in gram-negative bacteria and clpX/clpC in gram-positive bacteria (Raju *et al.*, 2014). In *E. coli* ClpP is a tetradecamer, composed of identical ClpP subunits which are stacked heptameric rings that can form a shape of a hollow cylinder and having a total of 14 proteolytic sites located within its central chamber. Both the genes contain a Ser/His/Asp

catalytic triad characteristic of serine proteases (Wang, Hartling and Flanagan, 1997). This core is associated with the ATPase adaptors (such as ClpX and ClpC1 in mycobacteria) that catalyze ATP-dependent unfolding of globular proteins and are involved in substrate specificity (Wang, Hartling and Flanagan, 1997; Kenniston *et al.*, 2003; Mogk *et al.*, 2004). ClpP consists of 14 subunits with double ring peptidase and collaborates with ATPases (AAA+). ClpP is presumably needed for the physiological turnover entailing the mycobacterial proteins as their accumulation might lead to a damage to normal survival of the bacteria thereby affecting their growth. Proteolysis is a process of three steps, involving substrate recognition, unfolding of the protein substrate, and translocation by the clpC subunit **(Figure 20)**.

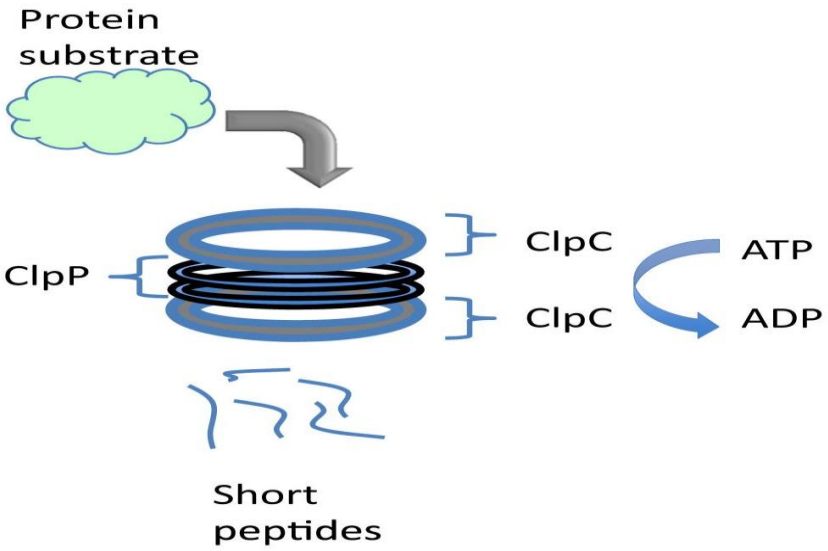

Figure 20: An illustration of the Clp Complex which comprises of the ClpP (proteolytic subunits), ClpC and an ATPase regulatory subunit. The mechanism involves substrate recognition, unfolding, and translocation (Parish, 2014).

Most of the bacteria have a single subunit of ClpP whereas mycobacteria have two subunits- ClpP1and ClpP2 which are required for the removal of abnormal proteins. Both subunits require an AAA+ partner and protein or a peptide substrate delivery to stabilize the active tetradecamer (Kress, Maglica and Weber-Ban, 2009; Schmitza *et al.*, 2014). In comparison to

other bacteria, ClpP is important in *M. tuberculosis* for its viability and virulence (Sassetti, Boyd and Rubin, 2003; Carroll, Faray-Kele and Parish, 2011; Raju *et al.*, 2012) and also plays an important role in proteostasis and as well in regulation of stress response. It is required for the turnover of incomplete products for degradation of SsrA tagged protein which arises due to the damaged mRNA (Barik *et al.*, 2010; Raju *et al.*, 2012; Personne *et al.*, 2013).

In the year 2014, Raju *et al.*, Rv3241c was predicted to serve as a Clp protease substrate. Based on this, we have computationally evaluated the interaction of rRv3241c with ClpP.

4.2 Material and methods

4.2.1 Computational interaction of Rv3241c protein with ClpP1

Rv3241c protein was modeled by the threading method using the I-TASSER tool (Yang *et al.*, 2014). The top 10 threading templates did not cover a region between 120 – 155 residues. To overcome this problem, a region from 100 – 200 residues of protein was modeled using ab initio in QUARK (Xu and Zhang, 2013). Taking the first QUARK model as a user template, I-TASSER (Yang *et al.*, 2014) was used to remodel the protein and the best model with the highest C-score and the lowest RMSD value was chosen for further analysis. This model was used as a template in Modeller (Webb and Sali, 2017). Energy minimization of model 3 with the lowest DOPE score was performed in Gromacs (Abraham *et al.*, 2015). This energy minimized model structure was further refined at an atomic level by ModRef (Xu and Zhang, 2011) which was further used for docking analysis. Docking of the refined Rv3241c protein with the clp1 hetero heptamers (PDB: 5E0S) was performed in PATCHDOCK (Schneidman-Duhovny *et al.*, 2005) server and the docked structures were further resolved in the FIREDOCK server (Mashiach *et al.*, 2008). The best 10 solutions were utilized after FIREDOCK server refinement. The interactions between the molecules were visualized in Pymol (DeLano, 2002) and interactions were checked using pp check.

4.3 Results

The 3D structure of Rv3241c was obtained by multiple threading approaches from the web based I-TASSER server. The best model was determined on the basis of a score of confidence generated by the server. To identify the possible interactions between the Rv3241c protein and Clp1 protein, we used the PATCHDOCK server. The best 10 solutions from the PATCHDOCK server analysis were utilized and then the FIREDOCK server for refinement. The interactions between the molecules were analyzed by pp check and visualized in Pymol. We observed the interaction of Rv3241c protein with the ClpP complex as shown in **Figure 21**. We have observed that Arg 58 peptide of Rv3241c protein forms a strong hydrogen bond between the residues Glnu47, Asp49 and Gln62 of clpP1 in **Figure 22**. We have identified similar interactions in two different poses with different chains of ClpP1 showing conservation of these interactions. Hence, Arg58 of Rv3241c protein might be playing a significant role in the binding interaction of ClpP1.

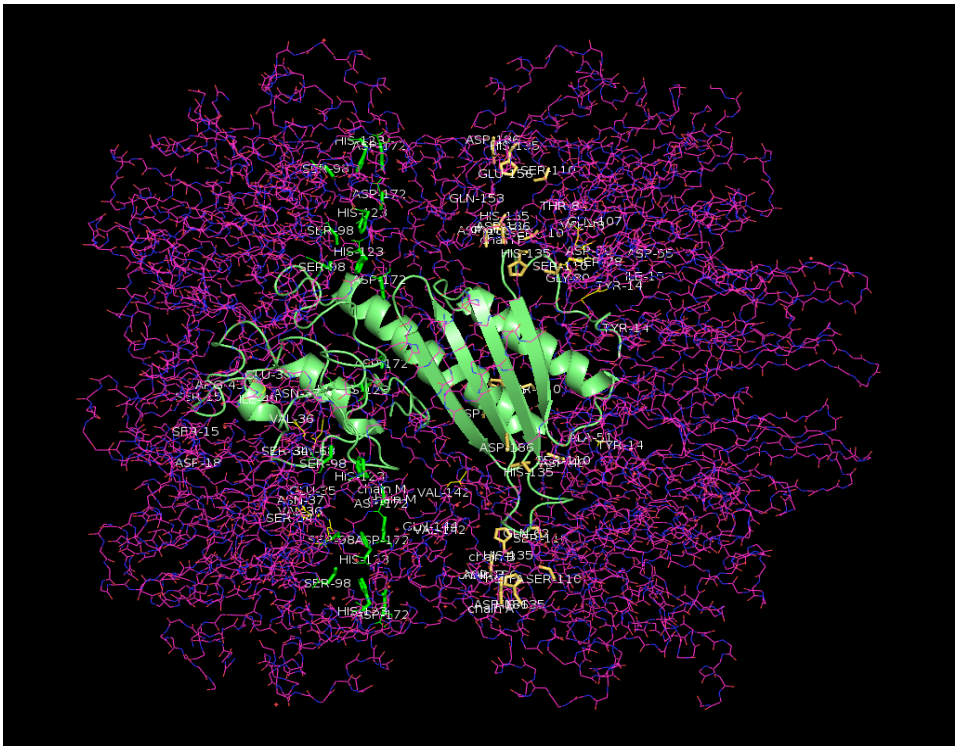

Figure 21: Interaction of rRv3241c with clpP complex

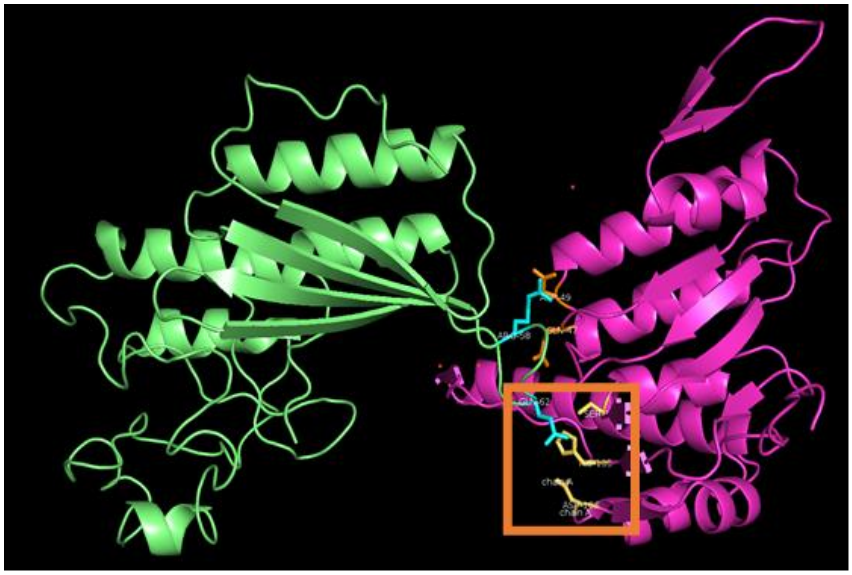

Figure 22 (a): Interaction of amino acid residues at the catalytic site: The interaction of Rv3241c protein with that of ClpP1

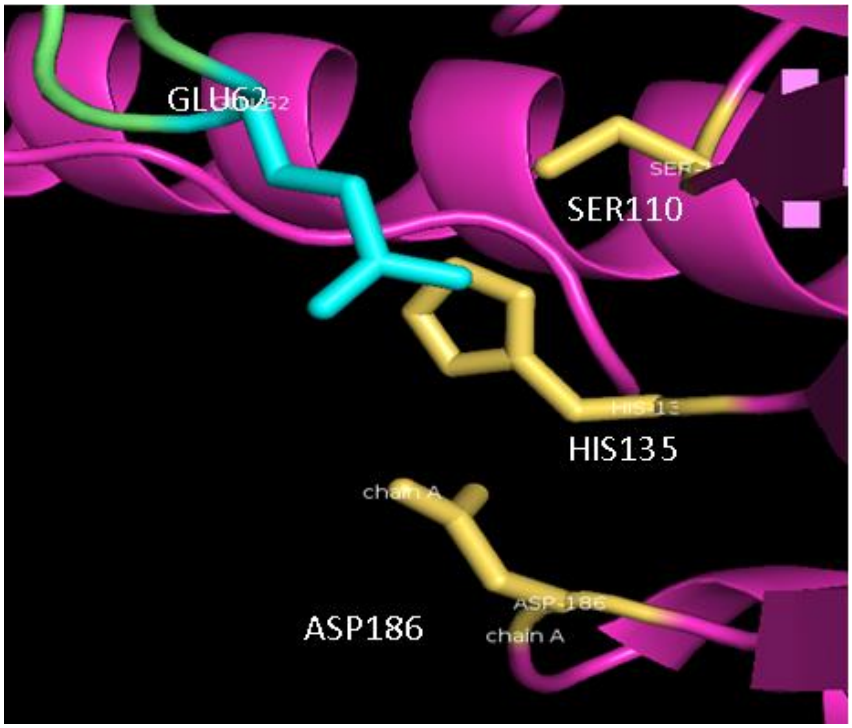

Figure 22 (b): Enlarge the view of the interacting residues: The above image shows the enlarge view of interaction.

4.4 Discussion

TB is a life-threatening disease inflicting a one-third of people worldwide and, most people

are infected with Mtb latently (Cohen *et al.*, 2019). The macromolecules such as proteins, DNA, and RNA control a huge array of cellular functions (Alhuwaider and Dougan, 2017). Among these, proteins play a critical part in ensuring the cell survival in stressful and normal conditions. This process is referred to as protein homeostasis.

Protein homeostasis has two important aspects i) folding and assembly of proteins and ii) removal of damaged and unwanted protein from cells. This is usually performed by proteases and chaperones. AAA+ are ATPases that are associated with different cellular activities and play an important role in protein homeostasis (Alhuwaider and Dougan, 2017). The structure and function of this mechanism are mainly studied in gram-negative bacteria such as *E. coli* (Alhuwaider and Dougan, 2017).

Bacterial pathogens harness a wide spectrum of factors that participate in virulence either directly or indirectly. The role of the proteases of conserved type such as Lon, HtrA, Clp, and FtsH to infection occurs in two ways. Firstly, as a part of the quality control process of the protein synthesis and secondly, the turnover of the regulatory proteins through a controlled proteolysis process (Ingmer and Brøndsted, 2009).

M. tuberculosis has various proteases such as Clp, FtsH, and HtrA which are essentially required for normal growth or cell survival suggesting its crucial role in bacterial physiology (Sassetti, Boyd and Rubin, 2003). In the year 2014, Raju et al. have identified some of the proteins that are degraded by Clp protease. One such protein is WhiB1 which acts as a Clp protease substrate and is essential for the regulation of transcription whereas CarD is the other substrate that is moderately upregulated and an essential mycobacterial protein that regulates the rRNA transcription (Stallings *et al.*, 2009).

During the proteomic screening, it was identified that Rv3241c protein act as a Clp substrate (Raju *et al.*, 2014). Hence, we have performed a computational study for the identification of the peptides of Rv3241c involved in the interaction with Clp proteases. Based on the

computational analysis, the interaction of Rv3241c protein with both Clp1 and Clp2 had shown that the peptide ARG 58 might be playing a significant role in Clp proteases. The role of Rv3241c protein might involve in either protein degradation or active structures transformation which needs to be deciphered. Further studies are required for understanding its role in the coordination of transcriptional and proteolytic regulations.

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Chapter 5

Summary and Conclusions

TB is a worldwide disease, and a leading cause of death among HIV-infected people (Sandhu, 2011). It can exist in two states: i) Active state and ii) Latent state. When bacteria reproduce and spread in the body and cause damage to the tissues, this results in active TB. Whereas the bacteria that remain in the body without causing illness to humans (dormant) lead to latent TB.

In a latent state, bacillus remains alive in the lungs after forming granuloma and without replication for several decades (Wayne, 1994; Lillebaek *et al.*, 2003; Kumar *et al.*, 2012; Trauner *et al.*, 2012). The latent infection can reactivate to cause active disease after the initial infection (Trauner *et al.*, 2012). The mechanism of Latent Tuberculosis (LTB) has been difficult to understand due to the unavailability of significant animal models (Kumar *et al.*, 2012).

The reason for the reactivation of LTB is partially understood, but it includes the host bacterial and environmental factors (Getahun *et al.*, 2015; Kiazyk and Ball, 2017). The dormant Mtb which persists in the micro-environment of granuloma is impassable for anti-TB drugs and also exists in a non-replicating state of bacilli. (Wayne and Sohaskey, 2001)

M. tuberculosis needs to maintain the macromolecular virtue to re-emerge from a non-replicating state. Bacteria are subject to various environmental changes, and they respond to these changes with different mechanisms for survival. Most antibiotics target cellular protein synthesis to inhibit bacterial growth or kill the bacteria. One of the important factors is the stabilization of ribosomes which is the key factor for the long-term survival of the bacteria during the stasis. The structure of ribosomes comprising RNA and proteins which are made of great genetic expense to cells and the macromolecular structure are involved in the cellular protein synthesis. The ribosomes play a crucial role in the survival of bacteria under normal and stressful conditions (Trauner *et al.*, 2012).

Mycobacteria become dormant in response to stress conditions and are regulated by a

regulon called DosR. This regulon consists of 48 genes that are important for the survival of the bacteria under hypoxia conditions. The first member of DosR regulon is a hypothetical protein Rv0079 which is involved in the regulation of transcription and translation for the survival of bacillus (Kumar *et al.*, 2012). Mycobacterial proteins which are not part of this regulon, are upregulated under different stress conditions. The upregulation of these regulon genes is associated with latency and increased drug resistance (Kumar *et al.*, 2012; Trauner *et al.*, 2012).

Mycobacteria promotes the balance between pro and anti-inflammatory cytokines to control or reduce the bacterial proliferation secreted by immune cells during the granulomatous stage to maintain its latency. IFN and TNF are important cytokines involved in promoting granuloma formation and its function, whereas IL- 10 is the main negative regulator of the immune response (Jo *et al.*, 2007; Kirschner, Young and Flynn, 2010; Cooper, Mayer-Barber and Sher, 2011) .

The mycobacterial gene Rv3241c contains the S30AE domain which is important for ribosomal stability. In this context, characterization, and functional analysis of Rv3241c is crucial to check its role in the translational process. Rv3241c is a hypothetical protein and DosR independent gene. According to the domain analysis, it has been predicted to contain a domain called the S30 AE domain which is important for ribosomal stability and function. These S30 AE domains have been mostly studied in *E. coli*. Some of the *E. coli* S30AE domains are shock protein Y which play a role in the stabilization of 70s ribosomes (Ueta *et al.*, 2005). HPF (hibernation promoting factor) is another *E. coli* protein that associates with the maturation of 90S ribosomal particles to 100S (Vila-Sanjurjo *et al.*, 2004). It has been reported that HPF shares homology with Rv3241c (Li *et al.*, 2015).

Given this, our study describes the functional role of Rv3241c in the inhibition of translation. To understand its role in the translation process, we initially performed the

computational analysis which has shown that rRv3241c binds to 30S ribosomes which indicates its possible role in the regulation of translation. Our *in-vivo* and surface plasmon resonance (SPR) analysis showed that rRv3241c binds to 30s ribosomal subunit indicating it might play a role in the stabilization of ribosomes. Further, we have observed that rRv3241c is involved in the inhibition of translation or inhibition of protein synthesis suggesting its role in conserving the cellular resources and preserving them for prolonged stasis. Similar DosR protein, Rv0079 has shown the same functional properties in the regulation of transcription and translation for the survival of bacillus (Kumar *et al.*, 2012). We have also examined the growth pattern of Rv3241c in *M. smegmatis* where it has shown that Rv3241c does not contribute to the growth of bacillus as it might indicate the arrest of cell growth due to starvation.

Understanding the role of the molecular mechanisms related to the cellular and immunogenic attributes of antigens is paramount. T cell and B cell-mediated immunity both contribute to immune protection against the Mtb infection. In this context, we have checked the immunogenic potential of rRv3241c in LTB in comparison with PTB and HC. Our results showed that there was no immunoreactivity against sera samples of LTB indicating that rRv3241c is not immunogenic. We have also examined the release of pro-inflammatory cytokines, which are essential for granuloma and other immune cell maintenance as well as the recruitment of macrophages. The results indicated that it does not induce the pro-inflammatory response and it might have a special contribution to targeting the ribosomes.

During infection, cellular pathways play an important role in the survival of the bacillus. Protein degradation is one such process that is involved in the regulation of biological pathways and protein quality control. Recently it has been shown that Rv3241c is a Clp protease substrate (Raju *et al.*, 2014). Clp protease is largely held as a serine protease of non-essential type and a conserved protease. It performs two crucial physiological functions. It

degrades endogenous proteins in addition to participating in protein quality control, like other proteases. Thus, Clp protease regulates the response to various stress conditions and pathogenic mechanisms.

M. tuberculosis contains two clp genes that are important for viability and might act as an attractive drug target. We have shown the computational modeling of Rv3241c with clp proteins of mycobacteria to understand its role in interaction and its role in proteases. We have found some of the antigens involved in the interaction might play a role in the regulation of the biological pathway.

Overall, our findings suggest that Rv3241c plays an important role in the regulation of translation by potentially inhibiting the protein synthesis and does not induce any inflammatory response indicating its role in the conservation of cellular processes by preserving the ribosomes. It has also been shown that Rv3241c is not immunogenic, and it does not induce a pro-inflammatory response indicating that Rv3241c might contribute to the survival by targeting the ribosomes. Hence, further studies need to be done to understand the exact function of Rv3241c, also to its possible role as a target protein for discovery efforts as regards to latent TB.

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

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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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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

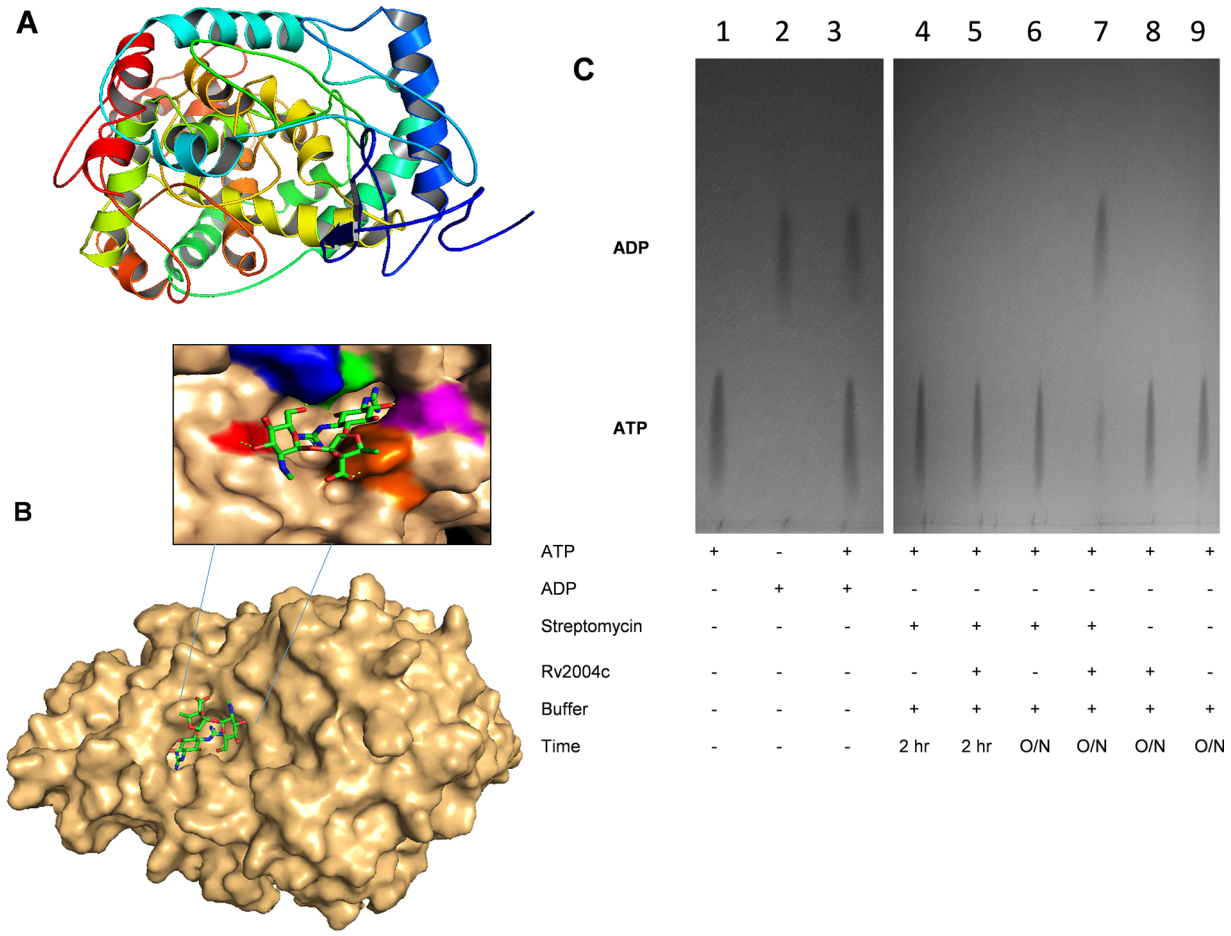

Fig. 1. Modelled 3D-structure of Rv2004c and its *in vitro* (or biochemical) interaction with SM.
A) Three dimensional structure of Rv2004c was determined using LOMETS modelling system and represented as a cartoon. **B)** Docking study of Rv2004c with SM: SM interacts with the Rv2004c at Asp 22 (Magenta), 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

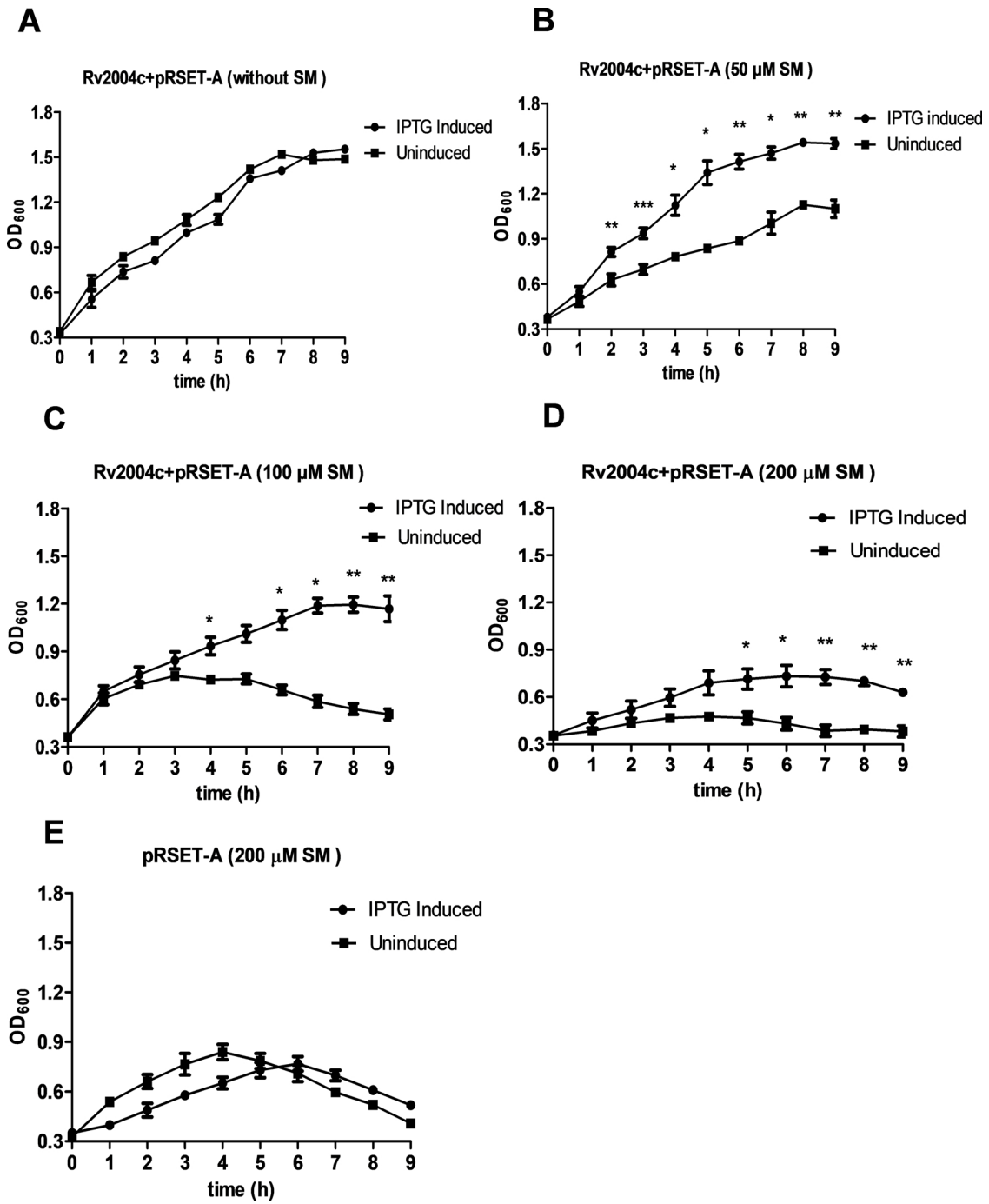

Fig. 2. Rv2004c expressing *E. coli* confer resistance to SM in liquid broth cultures.
E. coli strains transformed with recombinant Rv2004c + pRSET-A and empty pRSET-A vectors were grown overnight. Secondary cultures were inoculated with 1% of overnight grown cultures and induced with IPTG (at OD ~0.35) 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

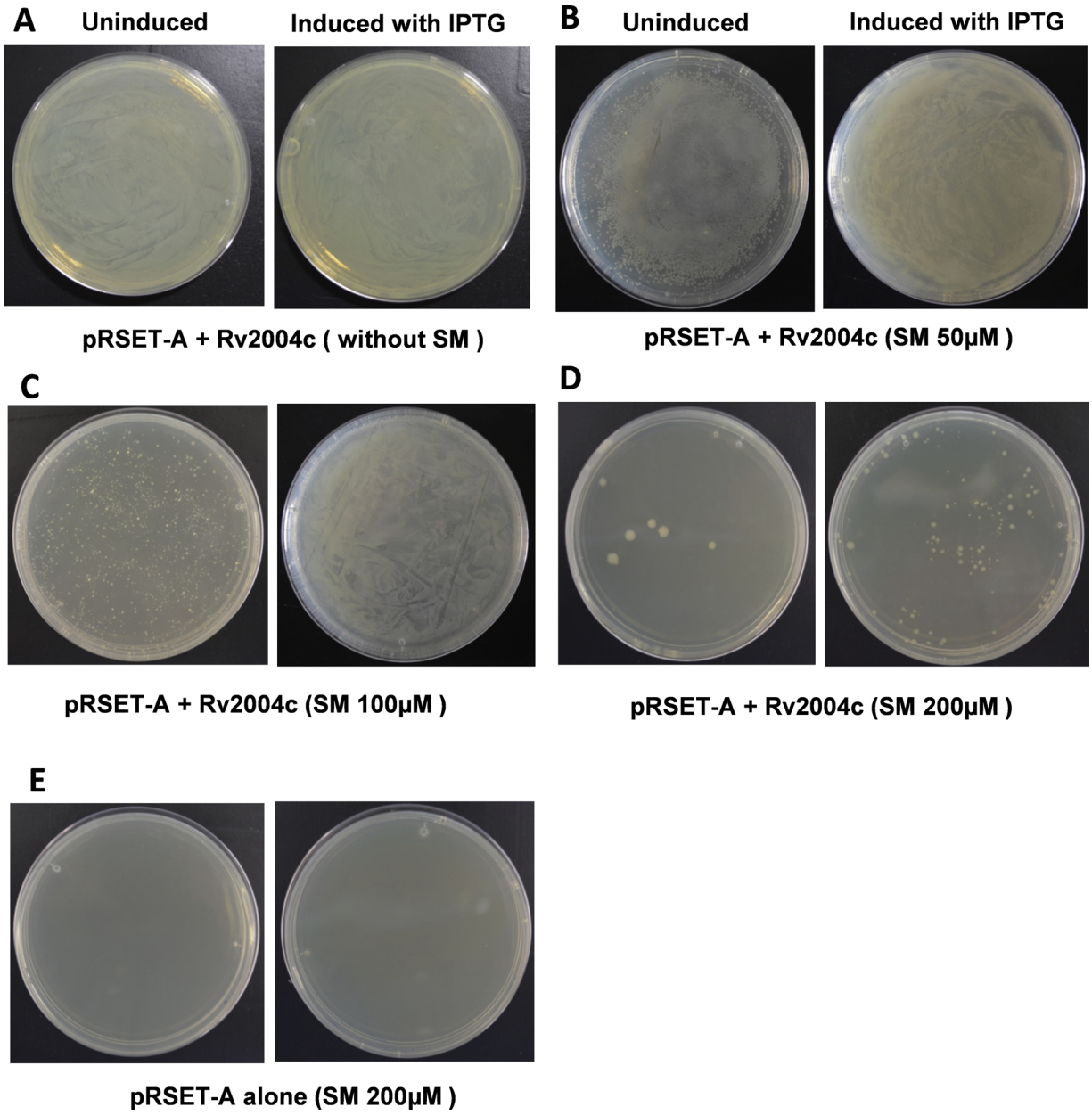

Fig. 3. Streptomycin resistance effect of Rv2004c on viability of bacteria.
The aliquots of *E. coli* cultures of each growth curve presented in Fig. 2 were collected after 2 h of induction with IPTG and were spread on LB agar plates containing ampicillin and incubated overnight at 37 °C. **A)** The 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

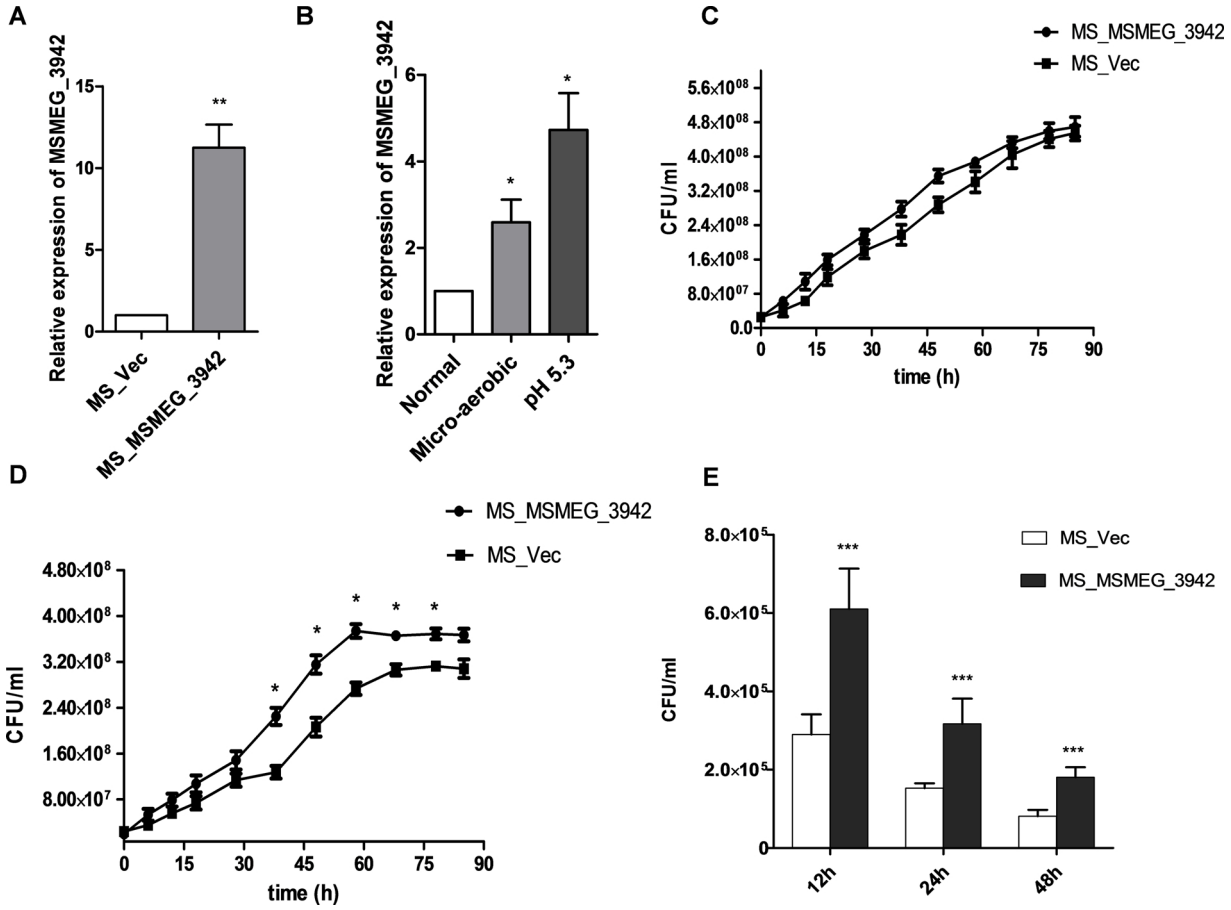

Fig. 4. Differential expression of Rv2004c in stress conditions and its effect on intracellular survival.
A) RNA was isolated from late log phase cultures of MS_MSMEG_3942 and MS_Vec strains (grown under normal conditions). Following cDNA synthesis, qRT PCR was carried out to quantify the expression 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.

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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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A putative nitroreductase from the DosR regulon of *Mycobacterium tuberculosis* induces pro-inflammatory cytokine expression *via* TLR2 signaling pathway

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Tuberculosis caused by *Mycobacterium tuberculosis* is a global encumbrance and it is estimated that nearly one third population of the world acts as a reservoir for this pathogen without any symptoms. In this study, we attempted to characterise one of the genes of DosR regulon, Rv3131, a FMN binding nitroreductase domain containing protein, for its ability to alter cytokine profile, an essential feature of *M. tuberculosis* latency. Recombinant Rv3131 stimulated pro-inflammatory cytokines in THP-1 cells and human peripheral blood mononuclear cells in a time and dose dependent manner. *In silico* analyses using docking and simulations indicated that Rv3131 could strongly interact with TLR2 *via* a non-covalent bonding which was further confirmed using cell based colorimetric assay. In THP-1 cells treated with Rv3131 protein, a significant upsurge in the surface expression, overall induction and expression of mRNA of TLR2 was observed when analysed by flow cytometry, western blotting and real time PCR, respectively. Activation of TLR2 by Rv3131 resulted in the phosphorylation of NF-κβ. Results of this study indicate a strong immunogenic capability of Rv3131 elicited *via* the activation of TLR2 signalling pathway. Therefore, it can be surmised that cytokine secretion induced by Rv3131 might contribute to establishment of *M. tuberculosis* in the granulomas.

Tuberculosis remains a global burden and is accountable for elevated morbidity and mortality. Though individuals remain asymptomatic, in most cases *M. tuberculosis* remains in a latent stage. Then, the bacilli unleash their responses inside a specialised structure called granuloma; the microenvironment of the granulomas is characterized by hypoxia and elevated nitric oxide[1]. *M. tuberculosis* survives in this unreceptive *milieu* by up-regulating a set of 48 genes of the dormancy survival regulon (DosR), whose protein products regulate a variety of physiological processes of the bacterium. A hallmark of *M. tuberculosis* infection is the organism's capability to rapidly acclimatise to altering environments since the cellular and biochemical dynamics of the granuloma are reliant on the oxygen tension[2]. Alternatively, during reactivation, *M. tuberculosis* adjusts to the aerobic conditions by switching on its metabolic activity. Under both these conditions, the DosR dependent regulation of DosR regulon member genes plays a critical role[3]. The protein products encoded by DosR genes can elicit humoral responses in the host, especially in patients with latent infection[4]. During latency, granuloma formation and its conservation requires production of pro-inflammatory cytokines and reactive oxygen intermediates[5]. These responses are instigated by the interaction of *M. tuberculosis* components with TLR1/TLR6 and TLR4 (Toll-like receptors) of macrophages[6].

TLRs, also known as pattern recognition receptors are responsible for the detection of specific molecular components on the surface of the pathogens to induce immunologic responses. The cell wall components of *M. tuberculosis*, namely peptidoglycans, interact with TLR2 and influence expression of CD25, CD69, NKp44 and IFN-γ production in NK cells[7]. The lipid fractions of three different strains of *M. tuberculosis* elicited TLR2, cytokines and MHC class II expression in human macrophages[8]. Further, mycobacterial lipoprotein interaction

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results in TLR2 mediated apoptosis of T-cells[9]. Mycobacterial lipoarabinomannan mediated interaction between TLR1 and TLR2 has been reported[10]. Other TLRs on the macrophage surface were also found to be recognising mycobacterial proteins and lipids[11]. Such recognition results in the production of pro and anti-inflammatory cytokines, which in turn dictates macrophage effector functions[12]. A majority of the studies hitherto remained focused on the interaction of mycobacterial cell wall components and TLRs. However, such interactions between the proteins encoded by the DosR genes and TLRs are not very well studied.

Proteins encoded by the DosR regulon genes elicit immunomodulatory responses in the host. Some of the DosR antigens that were found to be immunodominant include alpha-cystallin 2 (Acr2; *Rv0251*)[13], the alpha-crystallin homologue (also called 16-kDa protein; *Rv2031c*, *HspX*)[14], Ag85A (*Rv3804*)[15], *Hsp65 (Rv0440)*[16], *ESAT-6* (*Rv3875*)[17], and *CFP10* (*Rv3874*)[18]. Using mouse models and case controlled human studies in geographically diverse locations, the immunogenicity elicited by DosR regulon genes in latent infection was established[4,19–21]. Due to their immune-dominant nature they were considered to be potential candidates for the development of TB vaccines[22]. We previously reported and characterised the Dormancy Associated Translation Inhibitor (*Rv0079*/ DATIN) of *M. tuberculosis*, a protein coded by the DosR gene *Rv0079* and which was shown to be potentially immunogenic and leading to secretion of cytokines in macrophages and PBMCs; the immunomodulatory activity of DATIN was due to its interaction with TLR2[23,27]. Such interactions for other DosR antigens are not yet deciphered.

Rv3131, a hitherto uncharacterised member of the DosR regulon codes for a nitroreductase that might be involved in detoxification of nitrogen by-products in the host[24]. Nitroreductase genes, *acg* (*Rv2032*), *Rv3131* and *Rv3127* were predicted to guard against the nitrogen stress[25]. Rv3131, expressed robustly under stress, was found to contain two DevR/DosR binding sites[24]. The transcription of *Rv3131* encompasses binding of the DosR to the promoter in a cooperative manner[24]. Besides induction under hypoxic conditions, Rv3131 was also found to be antigenic[26]. In an earlier study comprising an African population, Rv3131 was found to be one of the most potent inducers of cytokine production among a set of seven classical and fifty one DosR regulon-encoded *M. tuberculosis* recombinant protein antigens[19].

Though the immunogenic property of most of the proteins encoded by the DosR genes was characterised extensively, the mechanisms by which they elicit this response are not yet fully understood. It is possible that the modulation of immune responses may be different for each of them or certain set of DosR antigens may use a common mechanism of action. Thus, it is essential to understand the molecular mechanisms involved in the immunogenic properties of the DosR regulon antigens. Except for the induction under stress conditions and the antigenic property, information on the role of Rv3131 in mycobacterial latency, manipulation of host responses and the molecular machinery used for its action was not yet deciphered. Characterising the antigenic nature of this protein and its contribution to the survival of *M. tuberculosis* under latency might facilitate novel strategies that can be directed in boosting the host's immune system or attenuation of survival of *M. tuberculosis* in the granulomas, respectively. In this study, we endeavoured to analyse the ability of recombinant Rv3131 to elicit cytokine response and its interaction with TLR2/4 and the ensuing downstream signalling.

Methodology

Molecular cloning, expression and purification of Rv3131 gene. *Rv3131* was cloned as described previously with slight modifications[27]. Briefly, *Rv3131* gene was amplified (supplementary information: Figure S1) using H37Rv DNA as a template and cloned into pET28a plasmid between BamHI and Hind III restriction enzyme sites. The plasmid was then transformed in to *E.coli* BL21 (DE3) and the recombinant protein was induced with 0.5 mM IPTG (Sigma Aldrich, USA) for 4 hours in LB broth containing Kanamycin 50 μg/ml. Protein extraction was carried out as described earlier[27]. The purity of recombinant Rv3131 (rRv3131) was checked by electrophoresing different fractions of the eluates on SDS gels. Rv3131 resolved as a single band and the purity of the protein as visualised was more than 95%. Endotoxin contamination was removed by incubating the protein with polymyxin-B agarose (Sigma, MO, USA). Limulus amebocyte lysate assay (Pierce™ LAL Chromogenic Endotoxin Quantitation Kit, ThermoFisher, USA) was used to measure the endotoxin content of the recombinant protein. Endotoxin contamination was undetectable in the protein fractions incubated with Polymyxin-B agarose.

Quantification of cytokines from culture supernatants of THP-1 cells and PBMCs. Isolation of peripheral blood mononuclear cells (PBMCs) from human blood was performed using Ficoll (GE Healthcare, USA) gradient method and 1 million PBMCs were seeded into each well suspended in 1 ml of RPMI (Invitrogen Life technologies, CA, USA) with 10% Fetal Bovine Serum (FBS) (Invitrogen Life technologies, CA, USA) and incubated overnight. Blood samples for isolation of PBMCs were collected from volunteers who appeared healthy and reported no history of tuberculosis. The study was carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. The study was approved by Ethics Committee of University of Hyderabad. An educated and written informed consent was obtained from all the subjects before obtaining the blood samples. THP-1 cells obtained from National Centre for Cell Science, Pune, India, were seeded (approximately 0.2 million per well) in 24 well plate and treated overnight with 10 ng/ml Phorbol 12-myristate 13-acetate (PMA) (Sigma, MO, USA) to allow differentiation into macrophage-like phenotype. THP-1 cells / PBMCs were treated with various concentrations of recombinant Rv3131 protein (rRv3131) (10, 100 and 1000 ng/ml) or LPS (100 ng/ml; positive control) (Sigma, USA). Cells treated with Proteinase K digested rRv3131 were used as negative control. After the treatment, supernatants were collected at 24 and 48 hrs time points and stored in −80 °C. Quantification of pro-inflammatory cytokines such as IL-1β, IL-8, TNF-α, and IFN-γ were performed using Elisa- Set Go eBiosciences (CA, USA) kit following manufacturer's instructions.

S.No	Gene Name	Primer Sequence	Ref.
1	*Rv3131*	F-5′-CG**GGATCC**ATGAACACCCATTTCCCG-3′	This study
		R- 5′-CC**AAGCTT**TCAGCACCGTTGTCGCAG-3′	
2	*TNF - α*	F-5′-TTC TCC TTC CTG ATC GTG GC-3′	28
		R-5′-ACT CGG GGT TCG AGA AGA TG-3′	
3	*IL - 8*	F-5′-CTG GCC GTG GCT CTC TTG-3′	53
		R-5′-CCT TGG CAA AAC TGC ACC TT-3′	
4	*Peptidylprolyl isomerase-B (PPIB)*	F-5′-ATG TAG GCC GGG TGA TCT TT-3′	28
		R-5′-TGA AGT TCT CAT CGG GGA AG-3′	
5	*TLR - 2*	F-5′-GGC CAG CAA ATT ACC TGT GTG-3′	53
		R-5′-AGG CGG ACA TCC TGA ACC T-3′	
6	*TLR - 4*	F-5′-CTG CAA TGG ATC AAG GAC CA -3′	53
		R-5′-TTA TCT GAA GGT GTT GCA CAT TCC-3′	
7	*Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH)*	F-5′-GGA AGG TGA AGG TCG GAG TC-3′	54
		R-5′-TGA GGT CAA TGA AGG GGT CA-3′	

Table 1. List of PCR and qRT-PCR primers.

Gene expression by reverse transcription PCR analysis. Differentiated THP-1 cells were treated with or without rRv3131 protein and total RNA was extracted using Trizol (Sigma, USA). 2 µg of DNase (sigma) treated RNA was converted into cDNA by SuperScriptIII reverse transcriptase (Invitrogen, USA) following recommendations of the manufacturer. Real-time PCR was carried out on a realplex machine (Eppendorf, Hamburg, Germany), using SYBR Green SuperMix (Clontech, USA) as described earlier[28]. Gene specific primers used in this study are mentioned in table 1. Mean fold changes in various cytokines were analysed using the ΔΔCT method. Peptidylprolyl isomerase B (PPIB) and Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) were used as housekeeping genes for cytokines and TLR2 / TLR4 respectively.

Protein sequence analysis of Rv3131 and docking study with TLR2. Amino acid sequence of Rv3131 (332 residues) was retrieved from *Mycobacterium tuberculosis* Database (http://tuberculist.epfl.ch/) and subjected to Pfam[29] and TOPCONS[30] for sequence analysis. The program BLAST-P has been used to detect similar crystallographic protein structures of Rv3131 and the template structures were formerly downloaded from RCSB Protein Data Bank (PDB). Nitroreductase proteins from *Mycobacterium smegmatis* (PDB ID: 2YMV) and *Streptococcus pneumonia* (PDB ID: 2B67) were used as templates to perform homology modelling using Modeller9.15[31]. Further, the geometry of generated model was checked and analysed using PROCHECK[32]. PyMOL program was used for molecular visualization and MetaPPISP[33] program was employed to predict the protein–protein interaction site. Simultaneously, for protein–protein docking studies, the web version of PatchDock[34] was executed and further improvement and ranking was done using FireDock[35]. Docking studies were done under default complex-type settings using Rv3131 model (ligand) and the crystal structure of TLR2 (PDB ID: 2Z82) was obtained from Protein Data Bank (receptor).

TLR2/TLR4 interaction assay. Interaction of Rv3131 with TLR2/TLR4 was analysed using HEK-Blue 293 hTLR-2 and hTLR-4 engineered cell lines (HEK-Blue™-hTLR-2/hTLR-4, InvivoGen, San Diego, US) which are co-transfected with TLR2/TLR4 and SEAP reporter gene (secreted embryonic alkaline phosphatase). Stimulation of TLR2/TLR4 ligands activates NF-κB and AP-1 which in turn induces the production of SEAP that can be readily assessed with HEK-Blue™ detection medium (with colour change from pink to purple) by measuring spectrophotometrically[36]. Different concentrations of rRv3131 (10–200 ng) was added to approximately 50,000 HEK-Blue 293 hTLR2 and 25,000 HEK-Blue 293 hTLR4 cells respectively. Another His-tagged recombinant protein, Rab5 was used as negative control and *Mycoplasma salivarium* derived synthetic lipoprotein FSL-1 (10 ng) (Sigma, USA) and LPS (100 ng) were used as positive controls. The intensity of colour formation was measured in an ELISA plate reader (Infinite® 200 PRO - Tecan) at 620 nm.

Flow Cytometry analysis. Surface expression of TLR2/TLR4 was determined by using differentiated THP-1 cells treated with various concentrations of rRv3131. Cells were harvested after a period of 24 hrs and incubated with goat anti-human TLR2 (2.5 µg/million cells) and goat anti-human TLR4 (R&D, MN, USA) or isotype-matched anti-goat mouse IgG antibodies for 60 min at 4 °C followed by incubation with donkey anti-goat IgG conjugated with FITC (0.5 µg/million cells) (Santa cruz, CA, USA) at 4 °C for another 45 min. Finally, cells were washed and re-suspended in 1× PBS containing 2% FBS. Fluorescence intensity was measured using BD FACS Canto II flow cytometer (BD Biosciences, NJ, USA). Untreated and stained cells were taken as negative control in both the assays. The data were analysed by Flow Jo software (Tree Star Inc., USA).

Western blot analysis. The total protein was extracted from rRv3131 (1000 ng/ml) treated and untreated THP-1 cells. Trichostatin A (TSA) (250 ng) and LPS (100 ng/ml) were included as positive controls for checking TLR2 and NF-κβ activities, respectively. Untreated cells served as negative control. In brief, 1×10^6 (TLR2) or 6×10^6 (NF-κβ) cells were washed with cold 1× PBS and pelleted by centrifugation at 400 g for 5 min. Cell pellets were treated with ice cold lysis buffer (0.1% NP-40 in 2 mM EDTA, 200 mM Tris–HCl, pH, 7.5, 250 mM

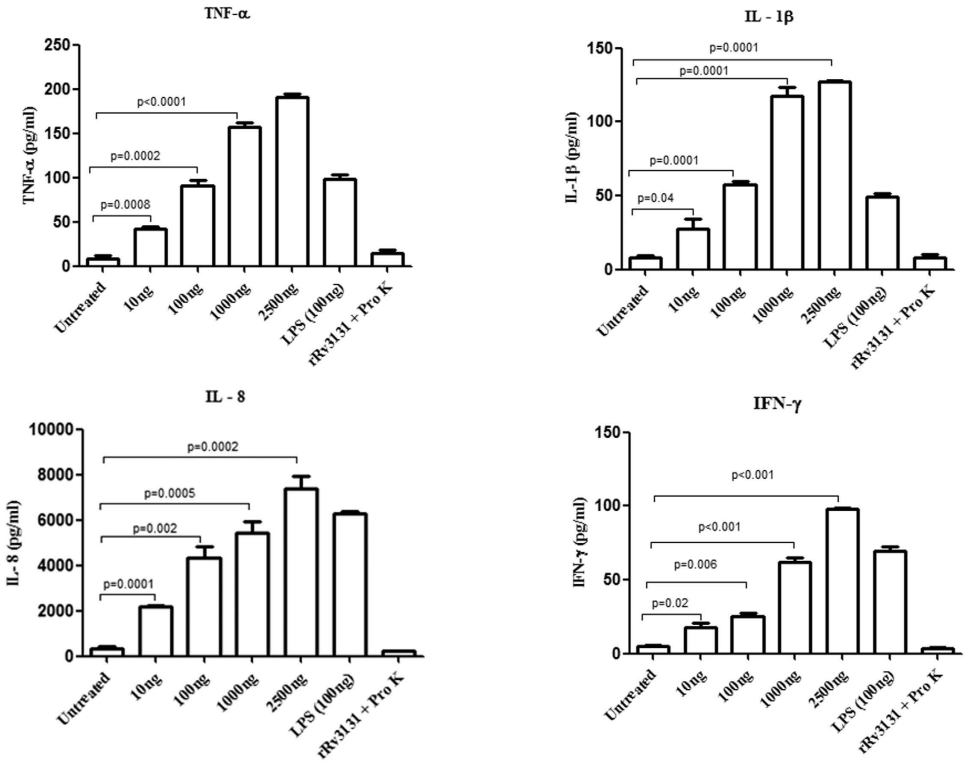

Figure 1. Rv3131 stimulates the secretion of pro-inflammatory cytokines in PBMCs. Isolated PBMCs from human blood were treated with 10–2500 ng/ml of rRv3131 or LPS (100 ng/ml) or proteinase K digested Rv3131 protein for 24 h. The levels of secreted cytokines like IL-1β, IFN-γ, TNF-α and IL-8 were quantified by ELISA. Data represent the mean ± SEM of three experimental replicates.

NaCl, 100 mM PMSF, 1 M DTT and protease inhibitor cocktail (Sigma, USA) by repeated pipetting at 4 °C. Supernatants, were collected after centrifugation at 13,000 g for 5 min and protein concentrations were analysed. Equal amounts of proteins were loaded and transferred on to a PVDF membrane at 4 °C. Subsequently, the membranes blocked with 4% BSA in TBST (137 mM NaCl, 20 mM Tris pH 7.6, and 0.05% Tween-20) for 3 h at room temperature and later incubated with primary antibodies against TLR2, p65 (NF-κβ) and β-actin antibodies (Santa Cruz Biotechnology Inc., USA). PVDF membranes were perceived by chemiluminescence (ECL). The band intensity in TLR2 assay was quantified densitometrically by Image J software[37] and normalized to β-actin.

Statistical analysis. For all the experiments, wherever required, Student's t-test and one way ANOVA was executed for the analysis of the results. The data were represented as the mean of triplicates ± SEM. $p < 0.05$ was considered as significant. Data analysis was carried out using GraphPad Prism 5 software.

Results

Rv3131 induces pro-inflammatory cytokines. To determine whether recombinant Rv3131can induce pro-inflammatory responses, THP-1 cells and human PBMCs were treated with varying concentrations of this protein and the secreted cytokines (IL-1β, IFN-γ, TNF-α and IL-8) were measured in the supernatants. The positive control, LPS (100 ng) induced the production of these cytokines at similar conditions tested. Treatment of PBMCs with rRv3131 resulted in increased production of IFN-γ, TNF-α, IL-1 β and IL-8 in a dose dependent manner (Fig. 1). Similarly in the THP-1 cells rRv3131 was observed to induce TNF-α, IL-1 β and IL-8 production in a time and dose dependent manner (Fig. 2A). The negative control (rRv3131 digested with proteinase K) did not affect the cytokine production. In order to analyse whether the increased production of pro-inflammatory cytokines in THP-1 cells after rRv3131 treatment was due to modulation at the transcriptional level, real-time PCR was performed. Significant increase in the mRNA levels of IL-8 and TNF-α was observed (Fig. 2B).

Protein sequence analysis of Rv3131 and docking study with TLR2. Computational analysis demonstrated that Rv3131 possessed conserved nitroreductase domain spanning 236–301 residues, while TOPCONS suggested the lack of signal peptide or transmembrane region in this protein. As crystallographic/solution structure of this protein is not available, BLASTp was used to find the structural templates and a template (PDB ID: 2YMV, nitroreductase from *Mycobacterium smegmatis*) with 37% identity was retrieved from PDB data bank. Modeller was used to generate structures using single template and the best structure was selected on the basis of different parameters including low DOPE score. To analyse the quality of structure, Ramachandran plot was generated and it revealed two residues (L327 and L328) in disallowed region. To improve quality of the structure, modelling was performed further with two templates i.e. PDB ID: 2YMV and 2B67. The quality of the

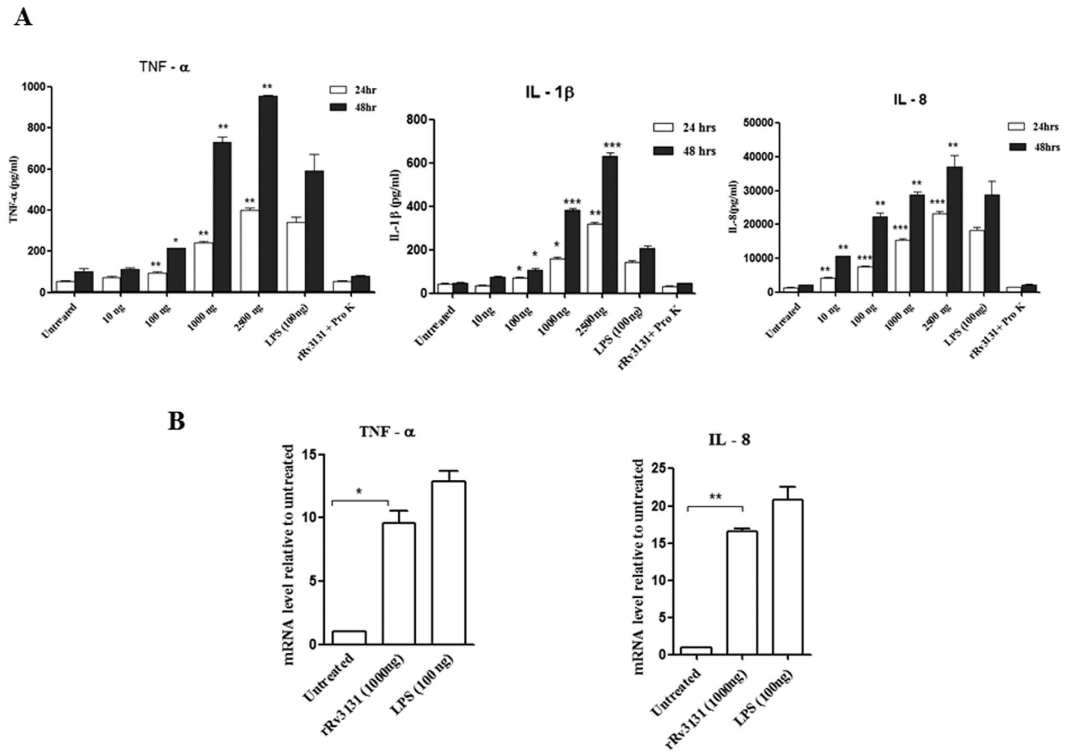

Figure 2. (**A**) Rv3131 stimulates the secretion of pro-inflammatory cytokines in THP-1 cells. THP-1 cells differentiated by PMA were stimulated with 10–2500 ng/ml of rRv3131 or LPS (100 ng/ml) or proteinase K digested rRv3131 for 24 and 48 h. Supernatants were collected and the levels of secreted cytokines were estimated by ELISA. Data represent the mean ± SEM of three technical replicates. *$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$. (**B**) Cytokine gene expression: relative expression of TNF-α and IL-8 in THP-1 cell lines treated with rRv3131 (1000 ng) and LPS (100 ng) was evaluated by quantitative real time-PCR (qRT-PCR). PPIB was used as endogenous control for normalization. The data represent mean ± SEM of triplicates.

structure was evaluated using Ramachandran plot acquired *via* Procheck and results of the validated model were as follows: most favoured regions contained 89.9%; additional allowed regions had 9.4%; generously allowed regions had 0.7% and disallowed regions contained 0.0% residues. Secondary structure of modelled Rv3131 protein revealed fourteen alpha helices and eight beta sheets (Fig. 3A). Identification of possible binding interface residues was performed using MetaPPISP programme. Protein-protein docking was performed with receptor as TLR2 and ligand as Rv3131 model using different online servers including GRAMM-X[38] and PatchDock. Among the used programmes, Patchdock provided several solutions based on shape complementarity criteria, where Rv3131 was placed besides TLR2. Out of obtained complexes, best 10 were further subjected to FireDock to refine the solutions based on global energy. However, complex 7 was evolved as the best solution for residues interaction between the two proteins. As shown in Fig. 3B, Rv3131 emanates close to TLR2 and shows strong interaction due to the presence of several non-covalent bonds between them.

Rv3131 interacts with TLR2. To lend further credence to the *in silico* observations, the interaction between Rv3131 and TLR2 was analysed using a cell based assay. In HEK-Blue 293 hTLR2 cells expressing TLR2 on their cell surface, alkaline phosphatase activity was found to be augmented with the increase in rRv3131 treatment. Similar increase in activity was observed in cells treated with the positive control FSL-1. The negative control, Rab5 did not cause any increase in alkaline phosphatase activity, thus validating the assay (Fig. 3C). Such an increase in activity was not observed in HEK-Blue 293 hTLR-4 cells expressing TLR4 on their cell surface when treated with Rv3131 protein, suggesting that the interaction of this protein was specific to TLR2 (data not shown).

Rv3131 induces TLR2 expression. The possible role of Rv3131 in modulating TLR2 expression, besides its interaction on the cell surface was analysed by real time PCR. In THP-1 cells treated with rRv3131 protein, TLR2 mRNA levels were significantly upregulated (Fig. 4A) similar to that of the positive control TSA. Besides the gene expression, TLR2 expression levels on THP-1 cell surface following rRv3131 treatment were also analysed using flow cytometry analysis. A significant increase in the expression of TLR2 (Fig. 4B) but not TLR4 (data not shown) was observed in THP-1 cells when treated with rRv3131. Western blot analyses also indicated an increased expression of TLR2 in rRv3131 treated THP-1 cells (Fig. 4C). To determine whether the interaction of Rv3131 with TLR2 initiates NF-κB signalling pathway, western blot was performed. Analysis of whole cell protein isolated from THP-1 cells revealed activated form of NF- κB (p65) in rRv3131 and LPS treated preparations, but not in untreated cells (Fig. 4D).

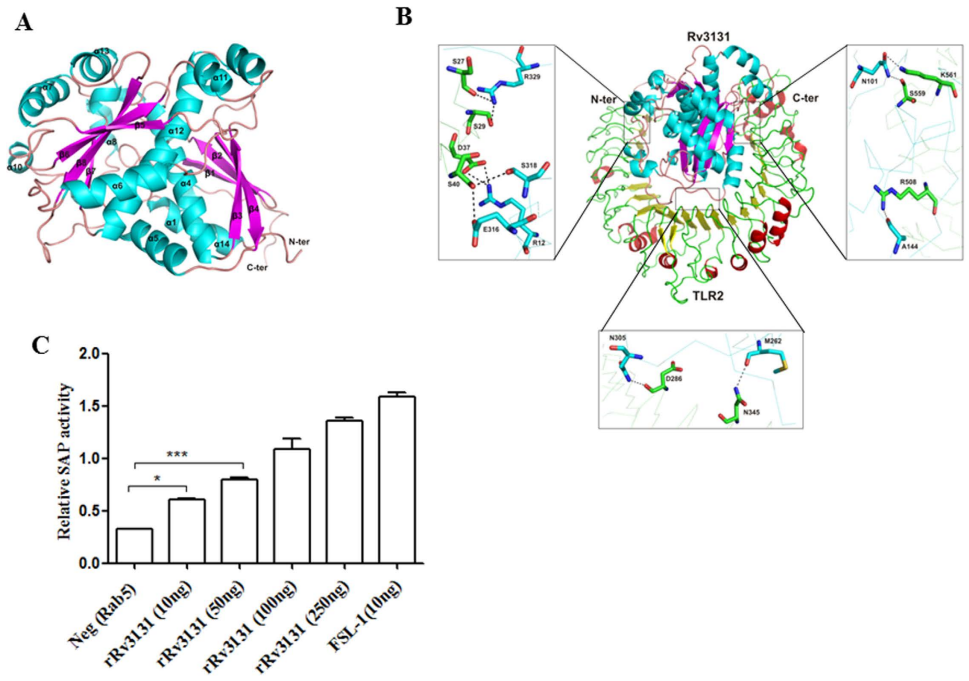

Figure 3. Modelled 3D-structure of Rv3131 and its interaction with TLR2. (**A**) Three dimensional structure of Rv3131 determined using homology modelling: the elements of protein secondary structure were coloured and labelled (helices and sheets displayed in cyan and pink, respectively). (**B**) Docking study of Rv3131 with TLR2: the residues of TLR2 and Rv3131 are coloured in green and cyan, respectively. The residues presenting interaction among both the proteins are labelled and shown as stick model in element colours (green/cyan colour represents carbon, blue represents nitrogen, and red represents oxygen). The black dashed lines represent hydrogen bonds. (**C**) Rv3131 interacts with TLR2: HEK-Blue 293 hTLR-2 cells were treated with various concentrations (10 ng–250 ng) of rRv3131 and interaction of Rv3131 with TLR2 was measured spectrophotometrically by the colour change read at 620 nm due to the activity of SEAP. *$p < 0.05$; ***$p < 0.0005$.

Discussion

Despite existence of tuberculosis infection worldwide, since ancient times, its pathology remained complicated even after extensive research. Majority of the tuberculosis patients experience latent phase infection before the active disease sets in. During this phase, immune responses to latency associated antigens are detected. The existing treatment approaches turn out to be ineffective due to a unique ability of this pathogen to remain dormant by vesting in granulomas, impermeable to the anti-tuberculosis drugs[39]. Adding to this is the lack of accurate *in vitro* or *in vivo* models that can mimic *M. tuberculosis* latency and/or resuscitation. Thus, development of effective vaccines using *M. tuberculosis* antigens is essential. Though classical antigens of *M. tuberculosis* were proposed to be potential vaccine candidates, because of the complicated life cycle of this pathogen, which includes latency, developing vaccines using antigens that are specifically expressed in latent stage is gaining prominence[40]. Recent studies identified that genes of DosR, upregulated during latency, also serve as antigens eliciting immune response[4,41]. Rv3131 was demonstrated to be significantly upregulated (up to 40 folds) under *in vitro* dormancy conditions[21]. Thus, such antigens were projected to be potential vaccine candidates due to their stage specific expression and immunogenicity.

We observed increased pro-inflammatory cytokine induction in THP-1 cells and human PBMCs with the treatment of recombinant rRv3131. Though a dose dependent increase in cytokine levels was observed in THP-1 cells and PBMCs treated with Rv3131, the highest dose (2500 ng) did not augment the release of IL-1β over 100 ng. This variation in IL-1β response could be due to physiological and phenotypic differences between the two cell types. The ability of Rv3131 to elicit immune response in *M. tuberculosis* specific T-cells obtained from tuberculin skin test positive patients was demonstrated[4]. In an Ugandan population, Rv3131 was demonstrated to be one of the most immunogenic antigens by virtue of its ability to induce interferon-γ[19]. TNF-α levels were found to be high in the lungs of mice with persistent tuberculosis[42] and is known to contribute to many immune regulatory functions such as macrophage activation and granuloma formation[5,43]. The ability of Rv3131 to induce pro-inflammatory cytokines suggests that it might activate macrophages during latency to govern the micro environment of the granuloma and favor the survival of latent *M. tuberculosis.* Recognition of *M. tuberculosis* in the host is executed by TLRs, especially TLR2 and to some extent by TLR4. The role of TLR2 in mycobacterial recognition and the downstream signalling during *M. tuberculosis* infection was well proven in a variety of TLR knock out animal models[44] and the susceptibility observed due to genetic polymorphisms in their genes[45] was demonstrated. Interaction of mycobacterial products with TLR2 leads to the secretion of pro-inflammatory[23,46] and anti-inflammatory cytokines[47]. It should be noted that *M. tuberculosis* is an intracellular pathogen and the

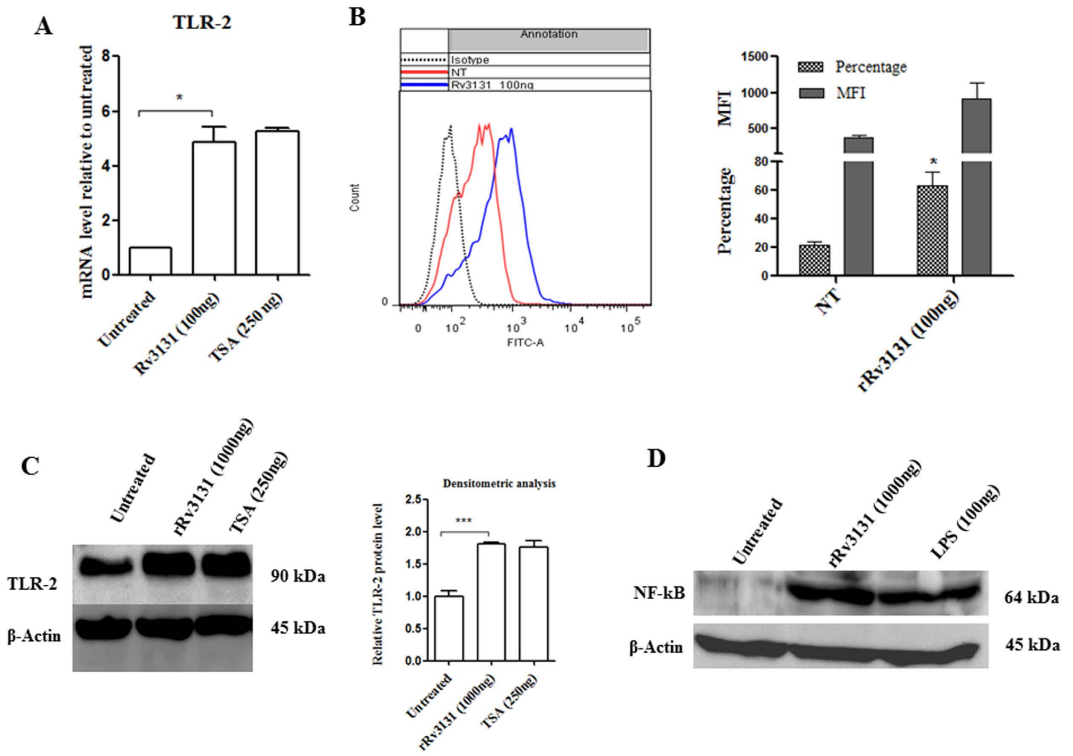

Figure 4. Rv3131 induces TLR2 expression and NF-κB activation. (**A**) Real time expression of TLR2: RNA isolated from differentiated THP-1 cells treated with rRv3131 (1000 ng) or TSA (250 ng) was reverse transcribed and real time PCR was carried out using gene specific primers. The expression of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as internal control. Data are presented as mean ± SEM of three independent experiments. (**B**) Surface expression of TLR2 by flow cytometry: PMA-differentiated THP-1 cells were treated with 100 ng of rRv3131 for 24 h followed by incubation with primary and secondary FITC labelled antibodies and analysed by flow cytometry. Data are expressed as mean ± SEM of percentage of cell population/MFI values from three independent experiments. (**C**) TLR2 protein expression: total protein isolated from differentiated THP-1 cells treated with rRv3131 (1000 ng) or LPS (100 ng) was electrophoresed on polyacrylamide gel, transferred onto PVDF membrane and probed with antibodies against TLR2 and β-actin (internal control). Signal corresponding to the intensity of the band was measured using chemiluminiscence. The graph represents relative TLR2 expression levels quantified densitometrically (**D**) NF-κB phosphorylation: the total protein isolated was analysed using western blot with antibodies specific to phosphorylated p65.

proteins secreted by it could also enter or be located in extracellular spaces during infections. We earlier characterized the mycobacterial Hsp65 and found it to have dual roles in autoimmunity and inflammation[48]. It is possible that Rv3131 could also reach the extracellular space and presented to immune cells to trigger cytokine secretion. Though our study proves that cytokine secretion induced by Rv3131 involves TLR2 and NF- κB, the possibility that cytokine secretion is also due to presentation of Rv3131 to immune cells cannot be ruled out. It is worthwhile to mention that cytokine response in isolated PBMCs could also be contributed by other cells such as dendritic cells, T and B cells, etc. Analysing cytokine expression using purified fractions of each of these cells would provide information on the individual cell type contribution and/or cell specificity, if any, in Rv3131 induced cytokine response.

Interaction of mycobacterial classical antigens such as peptidoglycan, with TLR2 marks the commencement of downstream signaling[7]. We previously demonstrated that the DosR regulon antigen DATIN can interact and activate TLR2 resulting in secretion of pro inflammatory cytokines[23]. Similarly, PE35 (*Rv3872*), a mycobacterial protein physically interacts with TLR2 resulting in a dose dependent increase in the secretion of pro-inflammatory cytokine secretion[49]. The interaction of Rv3131 predicted in this study was based on computational modeling that generates models with a high degree of confidence. However, detailed analyses of the binding pockets and the associated specificity need to be addressed using site directed mutagenesis of both the ligand and the receptor.

In the current study, besides interacting with TLR2, Rv3131 induced TLR2 mRNA and protein expression. Modulation of TLR2 expression by the classical *M. tuberculosis* antigens was reported[50]. For example, the lipid fractions of three different strains of *M. tuberculosis* had differential effects on the expression profile of TLR2 and TLR4 cytokines and MHC class II components[8]. Another study indicated that the ESAT-6 antigen of *M. tuberculosis* modulates T helper cell responses *via* TLR-2[51]. Further, in our studies, phosphorylation of NF- κB was evident in macrophages treated with recombinant Rv3131. Activation of NF-κB in the macrophages by the mycobacteria has been reported[52]. Given this, it is possible that Rv3131 might modulate the expression of TLR2

and may interact with it to influence the downstream signaling *via* NF-κB leading to the secretion of cytokines that favor the survival of *M. tuberculosis* in the granulomas. We used an *in vitro* system to study the immunogenic properties of Rv3131 protein at varying concentrations. The physiological concentration of Rv3131 in the granuloma during latency is very difficult to predict or estimate. However, it is an accepted practice to analyze the immunogenic properties of antigens using *in vitro* models. Hence, the results presented in our study strongly support that Rv3131 might induce pro-inflammatory cytokine expression *via* TLR2 signaling pathway and give an impetus to the understanding of the molecular mechanisms of regulation of DosR antigens.

In conclusion, we report that the DosR antigen, Rv3131 may possibly influence the innate immune responses to facilitate the survival of *M. tuberculosis* in the granulomatous microenvironment. This is brought about by induction of cytokine secretion mediated by its interaction with TLR2. Given paucity of knowledge on the mechanisms by which DosR antigens influence the *milieu* of the granuloma and support *M. tuberculosis* survival in a latent state, results of this study contribute suggestively to the understanding of the survival of *M. tuberculosis* executed *via* its latency specific antigens.

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Author Contributions

V.P. designed and performed all wet lab experiments and contributed to writing of the manuscript; S.N.D. helped in wet lab experiments and data analysis; I.A.Q. performed computational modeling of Rv3131 and its interactions and wrote the related portions of the manuscript; P.Y. helped in analyzing data and participated in discussions; N.A. conceptualized the study, provided reagents and materials, critically reviewed and refined the manuscript and provided overall supervision.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

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Genomes of Two Clinical Isolates of *Mycobacterium tuberculosis* from Odisha, India

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We report whole-genome sequences of two clinical isolates of *Mycobacterium tuberculosis* isolated from patients in Odisha, India. The sequence analysis revealed that these isolates are of an ancestral type and might represent some of the "pristine" isolates in India that have not admixed with other lineages.

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Tuberculosis caused by *Mycobacterium tuberculosis* is a chronic infectious disease that is often fatal if not effectively treated. Every year, 8 to 9 million new infections and a death toll of 1.5 million are recorded worldwide. It is estimated that about one-third of the human population is infected with *M. tuberculosis* (1). Comparative genomic studies have provided deeper insights into the genetic diversity and clonal architecture of *M. tuberculosis* (2). Recent studies conducted on isolates from India have shown that highly concentrated reservoirs of the ancestral *M. tuberculosis* lineages prevail in South and Central India (3–6). Only a limited number of *M. tuberculosis* genomes from India are sequenced. The whole-genome analysis of ancestral and modern lineages would facilitate deciphering of the genetic variability and evolutionary mechanisms of this obligate parasite. We describe the whole-genome sequences of two *M. tuberculosis* strains, NA-A0008 and NA-A0009, isolated in 2008 from patients in rural Odisha, India.

Genomic DNAs of both strains were isolated using the Qiagen kit method. Whole-genome sequencing was carried out on an Ion Torrent sequencing platform (Life Technologies). The process generated 3 million and 2.9 million reads amounting to 89× and 93× genome coverage for NA-A0008 and NA-A0009, respectively, with a mean read length of 250 bp. The reads after filtration were assembled into 280 and 310 contigs for NA-A0008 and NA-A0009, respectively, using the MIRA v.2 *de novo* assembler. These contigs were ordered and reoriented according to the *M. tuberculosis* CCDC 5180 genome using in-house written scripts. The resulting draft genomes were annotated using the RAST annotation server (7), and CDSs were validated by comparing outputs from EasyGene (8) and Glimmer (9), as done previously (10–13). The number of rRNA operons were predicted in both strains using RNAmmer (14), while tRNAscan-SE (15) was used to identify tRNA sequences. Artemis (16) was used to glean the genome statistics of both the strains. The genome sizes of NA-A0008 and NA-A0009 were 4,259,206 and 4,271,739 bp, with coding percentages of 89.4% and 89.3%, respectively. The G+C contents of both strains were high, as usually observed for the *M. tuberculosis* complex, 65.31% (NA-A0008) and 65.28% (NA-A0009). The two genomes, NA-A0008 and NA-A0009, were predicted to encode 4,400 and 4,453 CDSs with average lengths of 866 and 857 bp, respectively. Both of them contained a single rRNA operon and 45 tRNA genes.

The availability of these genome sequences would definitely complement the gene pool analysis of the Indian strains from different parts of the country. Besides this, comparative genomic analysis and phylogenetic study of these isolates with other *M. tuberculosis* strains might give us important insights into the biology and molecular epidemiology of this organism.

Nucleotide sequence accession numbers. The *M. tuberculosis* NA-A0008 and NA-A0009 whole-genome shotgun projects have been deposited in the GenBank database under the accession numbers ALYG00000000 and ALYH00000000, respectively. The BioProject designations for these projects are PRJNA168604 and PRJNA168605, respectively.

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Modern and Ancestral Genotypes of *Mycobacterium tuberculosis* from Andhra Pradesh, India

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Abstract

Traditionally, the distribution of the *Mycobacterium tuberculosis* genotypes in India has been characterized by widespread prevalence of ancestral lineages (TbD1+ strains and variants) in the south and the modern forms (TbD1$^-$ CAS and variants) predominating in the north of India. The pattern was, however, not clearly known in the south-central region such as Hyderabad and the rest of the state of Andhra Pradesh where the prevalence of both tuberculosis (TB) and human immunodeficiency virus (HIV) infection is one of the highest in the country; this area has been the hotspot of TB vaccine trials. Spoligotyping of 101 clinical isolates obtained from Hyderabad and rural Andhra Pradesh confirmed the occurrence of major genogroups such as the ancestral (or the TbD1+ type or the East African Indian (EAI) type), the Central Asian (CAS) or Delhi type and the Beijing lineage in Andhra Pradesh. Sixty five different spoligotype patterns were observed for the isolates included in this study; these were further analyzed based on specific genetic signatures/mutations. It was found that the major genogroups, CAS and ''ancestral,'' were almost equally prevalent in our collection but followed a north-south compartmentalization as was also reported previously. However, we observed a significant presence of MANU lineage in south Andhra Pradesh, which was earlier reported to be overwhelmingly present in Mumbai. This study portrays genotypic diversity of *M. tuberculosis* from the Indian state of Andhra Pradesh and provides a much needed snapshot of the strain diversity that will be helpful in devising effective TB control programs in this part of the world.

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Introduction

Tuberculosis (TB) today constitutes the second major cause of death due to infectious diseases. India being the hotspot region for TB witnesses one of the highest incidence rates although the mortality figures are on the decline due to effective implementation of the control programs. Nevertheless, the scientific challenge in TB control has become complicated with the emergence of new frightening forms of tuberculosis – the extensively drug resistant tuberculosis (XDR-TB) and the HIV-TB co-infection.

Correct identification of the underlying strains is of paramount importance in devising TB control strategies. Spoligotyping is one of the potentially powerful tools for simultaneous detection and differentiation of *M. tuberculosis* complex lineages [1]. Studies using multiple markers revealed predominance of ancestral (TbD1+) and modern (TbD1−) genogroups of *M. tuberculosis* strains in India [2]. The signature deletions RD239 and RD 750 [3] are specifically useful to define isolates belonging to the 'Indo-Oceanic' (TbD1+) [3] or 'East African/Indian' – EAI [2] and CAS lineages. Genotyping studies from India revealed a C→T specific silent mutation in 65th codon of the *pncA* gene which is specific for the CAS (Central Asian) lineage [4]. A new, ancient clade of strains, called as 'MANU' was identified in India which belongs to the ancestral family of principle genetic group (PGG) - 1 [5,6] and is heavily concentrated in Mumbai [7]. It is tentatively subdivided in to MANU1 (ST100; loss of spacer 34), MANU2 (ST 54; loss of spacers 33, 34) and MANU3 (ST1378; deletion of spacers 34–36) [5,7,8,9]. It has been suggested that spoligotypes evolve through the successive loss of spacer DNA sequences. In addition to the loss of spacer 34, MANU [this study] and 'ancestral' EAI or Indo-oceanic lineages [3] are characterized by the deletion of RD 239 and an intact TbD1 region; it thus appears that MANU and EAI lineages are closely related or were derived from a last common ancestor.

Andhra Pradesh, with a population of about 80 million is one of India's states with high prevalence of HIV/AIDS. Patients with latent TB infection are at higher risk of progression if they are co-infected with HIV. A few TB vaccine trials have been initiated in

this region although the repertoire of circulating strains is largely unknown. Thus, the aim of this study was to characterize the predominant genotypes responsible for TB in urban and rural Andhra Pradesh and to generate a preliminary, baseline data for further epidemiological and infection control studies.

Materials and Methods

Ethics statement

All the mycobacterial strains analyzed in this study were available as a part of routine TB testing and surveillance programs being implemented at different centres and, therefore, human ethics committee (Institutional Review Board equivalent in India) approvals were not mandatory (also since no human biological samples were collected here). The study was part of a long term open ended project originally approved by the Institutional Biosafety Committee (IBSC) of the School of Life Sciences, University of Hyderabad.

Bacterial isolates

A total of 101 clinical isolates of *M. tuberculosis* representing TB patients from urban and rural Andhra Pradesh were analyzed in this study; 59 of these originated from a collection of MDR isolates cultured during 2000–2005 as a part of Hyderabad Urban DOTS program. The other isolates represented randomly selected collection at microscopy centres operating under the aegis of the revised national tuberculosis control program (RNTCP) (Table 1). Smear positive sputum specimens received for routine diagnosis were processed by modified Petroff's method and cultured on Lowenstein–Jensen slants at 37°C for 6–8 weeks. Drug susceptibility testing was performed by the absolute concentration (MIC) method for the anti-TB drugs, namely, isoniazid [H], ethambutol [E], rifampicin [R] and by resistance ratio method (RR method) for other drugs such as streptomycin [S], according to the protocol of Tuberculosis Research Centre, Chennai, India (TRC) [10]. Multi Drug Resistance (MDR) phenotype was defined as resistance to both isoniazid and rifampicin.

DNA isolation and spoligotyping

Isolation of DNA was carried out as previously described [11]. Spoligotyping was performed according to the standard method [1] and with the help of commercially available line probe arrays (Isogen Biosciences BV, Maarssen, the Netherlands). The hybridization pattern was visualized after incubation with streptavidin peroxidase using an enhanced chemiluminescence detection system (Amersham) followed by exposure to an X-ray film (Hyperfilm ECL, Amersham). Results were expressed as presence (n) or absence (0) of each of the 43 spacers and converted to an octal code format according to Dale *et al* [12]. The data were then compared to the SpolDB4 or SITVIT database and 'Spotclust' [12,13,14] to assign strain families based on standard definition/convention [13,14]. The isolates with spoligotype patterns present in the SITVIT database (SpolDB4) were automatically labeled with a 'shared type' number and unique profiles were mentioned as 'new'. The spoligotyping results were further analyzed with the help of Bionumerics® software program (Applied Maths, Belgium); a dendrogram was constructed (Figure 1) by un-weighted pair group method using arithmetic averages (UPGMA) [15].

TbD1 analysis

The presence (TbD1+) or absence (TbD1−) of the TbD1 region was analyzed by PCR using two primer sets complementary to the sequence of the deleted region or complementary to the internal sequence of the intact TbD1 region [16]. TbD1+ isolates generated a PCR product primed by the internal primers (2,153 bp), whereas the TbD1− isolates generated a PCR product based on the flanking primers (500 bp).

RD 239 and RD 750 deletion analysis

The RD 239 and RD 750 specific primers were used to amplify specific signature DNA and assign strains to either the EAI lineage or the CAS lineage [3,17]. RD239 deletions were denoted by an amplicon of size 888 bp and intact regions were scored based on the presence of an amplicon of 1730 bp. Similarly, an RD750 specific deletion resulted in a 734 bp PCR product and an intact region generated a product of 1533 bp.

PCR-RFLP and Sequencing

The *pncA* gene was analyzed for a silent mutation in 65th codon at the 195th bp [4]. Restriction digestion using BseLI enzyme (Fermentas) differentiated the mutated and wild type alleles. Gel electrophoresis was performed to resolve the digested products in 1.5% agarose gel (Figure 2).

Results and Discussion

Spoligotyping of 101 clinical *M. tuberculosis* isolates revealed 65 distinct spoligopatterns. Thirty nine out of 101 clinical isolates were represented by a unique pattern whereas sixty two isolates segregated into 26 different clusters or branches (Figure 1). Three clusters comprising of 13 isolates, 7 isolates and 7 isolates belonged to ST 26, ST126 and ST 100 respectively, followed by 6 isolates belonging to ST11 and 4 isolates belonging to ST591. Two clusters (ST1, ST288) were formed with only 2 isolates each. Two other clusters (ST127 and ST357) also comprised only 2 isolates each and seventeen spoligopatterns represented single isolates (Figure 1). Sixty three percent of all clusters/branches had only one isolate and ~39% of all the isolates were not previously reported in the SpolDB4, SITVIT or 'Spotclust' and were

Table 1. Details of the *M. tuberculosis* isolates studied herein.

Isolate/batch identity (Numbers)	Geographical origin	Selection criteria	Source centre type	Important genogroups
AP01 to AP59 (59)	Hyderabad and Ranga Reddy district (urban group)	Pulmonary TB MDR isolates	Hyderabad urban DOTS	CAS EAI Beijing
AP60 to AP101 (42)	Chittoor district (rural group)	Pulmonary TB Random selection/cross sectional (No resistotyping data)	RNTCP rural microscopy centres	EAI MANU CAS

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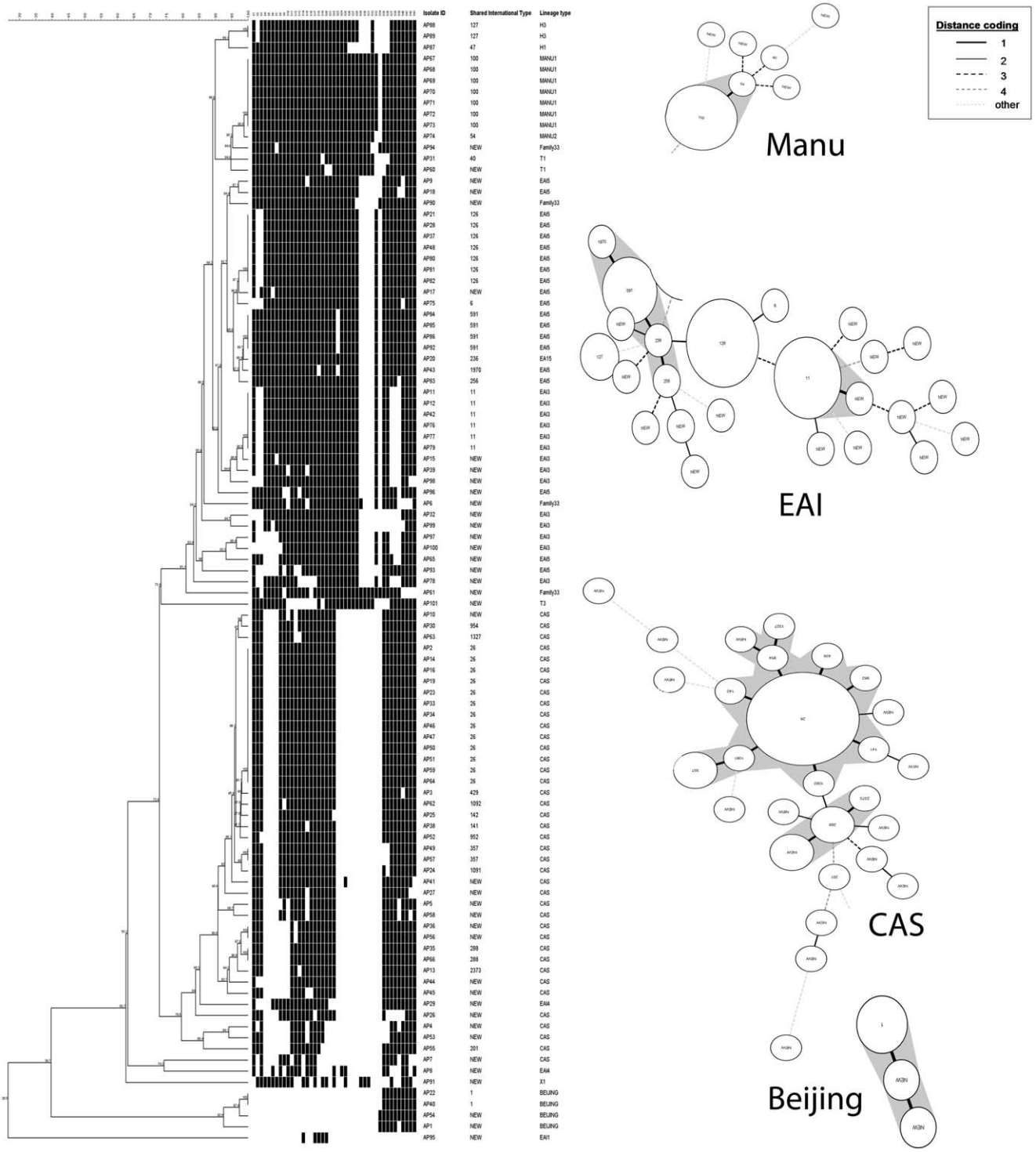

Figure 1. Genetic affinities within the *M. tuberculosis* isolates based on spoligotyping. Different clades corresponding to prevalent genotypes are prominently highlighted. In the inset is the distance coding convention relevant to the genetic relatedness of different isolates within a clade.
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identified as 'new'. These unique patterns were variously affiliated to CAS, EAI1, EAI3, EAI4, EAI5, family33, X1, T1, T3 and Beijing genogroups.

The predominant spoligo families corresponded to CAS (~40%), EAI (~38%) and MANU (~8%). The CAS and EAI groups together comprised 77.22% of the total isolates. Other families represented were Beijing (~4%), family33 (4%), Haarlem (H) [~3%], X (1%) and Tuscany (T) [~3%]. The EAI family comprised of 55.26% EAI5, 36.84% EAI3, 5.26% EAI4, and ~2.63% EAI1. While the ST26 belonged to CAS1_Delhi lineage,

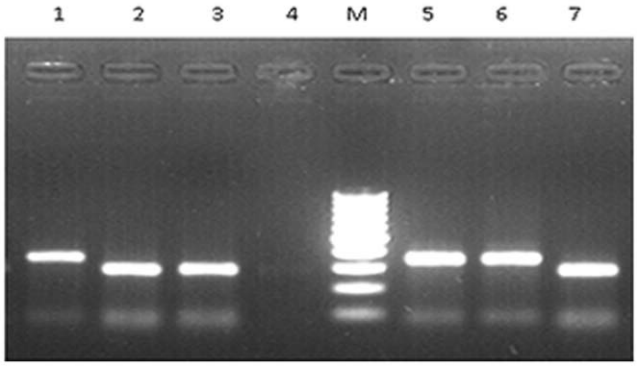

Figure 2. PCR-RFLP analysis of the *pncA* gene after restriction digestion with *Bse*LI. The *pncA* gene was analyzed for a silent mutation in the 65th codon at the 195th bp. Mutated alleles correspond to lanes 1, 5 and 6 (344 bp product and one minor fragment of 81 bp). Lanes 2, 3 and 7 denote wild type pattern (280 bp product and two minor fragments of 81 and 64 bp sizes) while lane 4 represents a negative control. Lane M corresponds to the profiling of a 100 bp DNA molecular weight marker.
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ST11 was grouped under EAI3_IND lineage. ST100 belonged to MANU clade and ST126 was grouped under EAI5 (Figure 1)

The drug susceptibility testing of the 32 representative isolates obtained by random selection out of the urban MDR group (Table 1) showed 25 isolates (78.12%) of the families CAS, Beijing, EAI5, EAI3 and EAI4, were MDR. Although a single isolate from EAI4 was found to be resistant to streptomycin and isoniazid (but sensitive to ethambutol and rifampicin), 6 other isolates belonging to different genotypes were found sensitive.

Random samples representing the EAI/Indo-oceanic lineage were reconfirmed based on 1) the presence of RD239, 2) an intact RD750 region, 3) an intact TbD1 region together with 4) non-mutated codon 65 of the *pncA* gene. Similarly, representative isolates from the CAS lineage were reconfirmed by 1) an intact RD239 region, 2) a deleted RD750 region, 3) the TbD1deletion and 4) mutations in the codon 65 of the *pncA* gene. The seven isolates belonging to MANU1 lineage were analyzed for mutations in the *pncA* gene. PCR-RFLP for the *pncA* gene (Figure 2) was developed and standardized in house. Six of the MANU1 isolates had a wild type gene and a single isolate was found to be mutated at codon 65. Sequencing of the amplicons from four representative MANU1 isolates confirmed the PCR-RFLP data (Gen bank Accessions for the *pnc*A: GU817406, GU817407, GU817408, GU817409).

Spoligotype data from this study indicate that the known lineages ST26, ST100, ST126 and ST11 predominate in Andhra Pradesh; this trend was also reported from elsewhere [2,6], although the most significant finding from our data was the occurrence of orphan spoligotypes (new) not identified in such high proportion in any previous studies from India.

According to other studies from India, ST26, ST11, ST1 and ST126 together accounted for a major proportion of all the isolates studied from this country [2,4,5,6,7,8]. Although, traditionally, ST100 was found to be a minor clade in different studies from India, except its high occurrence in Mumbai [7], we in our study found that the ancestral isolates of ST126 and ST100 type (MANU) could be much more concentrated in southern Andhra Pradesh (Chittoor district). Moreover, the most predominant spoliogotype, ST26 (CAS1_Delhi), was found in 12.87% of all the clustered isolates and was highly prevalent in Hyderabad region. Shared type ST11 which belongs to EIA3_IND clade accounted for only 6% of all the clustered isolates.

For this collection, the CAS (TbD1−) and EAI (TbD1+) lineages were found almost in an equal ratio (~40% CAS and ~38% EAI). However, if we take in to account the MANU types, then the incidence of ancestral type isolates (TbD1+) becomes clearly dominant. It has previously been shown that the CAS lineage predominates in north and EAI in south India [2,4,5,6]. In our earlier study carried out on a nation wide sample, EAI genotypes were clearly less predominant in north India (32%), followed by 52% in central India and 80% in south India [2]. The present data are in agreement with such findings and also with respect to the prevalence of Beijing strains [18] that accounted to be only about 3–5% [2]. We did not find any Beijing isolates in our rural group and it is possible that southern Andhra Pradesh may not have any prevalence of this exotic genotype.

Both mutated and wild type forms of the *pncA* gene were present in the MANU types. Thus, the CAS - lineage specific silent (C→T) mutation [4] was also noticed in the MANU isolates characterized in this study. Since both the EAI and MANU lineages have RD239 deleted along with an intact TbD1, it is interesting to know whether EAI or Indo-oceanic lineage is indeed evolved from MANU1 through successive loss of spacer DNA sequences. Since our data are based on limited isolates, there is a need for an elaborate study to understand this possibility as well as the significance of MANU strains with mutated *pncA* gene. As described above, the codon 65 mutation seems to be common among ST26 and a single ST100 genotype, however, it is difficult to relate them based solely on single gene mutations. On the other hand, TbD1 positivity and deletion of RD239 constitute much stronger evidence to portray them as ancestrally very closely related.

Both the modern and ancestral *M. tuberculosis* strains are prevailing in this region with a north-south compartmentalization, respectively, and the isolates show a high degree of spoligotype signature diversity. The present data once again remind of the possibility that the ancestral strains are somehow more adapted to southern peninsula. Although we did not analyze our samples based on demographic data of patients, it is possible that the cosmopolitan nature of Hyderabad population could have lead to more diverse spoligotype patterns and representation of different lineages without clear dominance of a single lineage. Although 'modern' (TbD1−) *M. tuberculosis* strains are far more prevalent worldwide, the ancestral clones of *M. tuberculosis* are responsible for a majority of TB cases in India with the exception of major metropolitan cities. Our results therefore corroborate with the findings of Gutierrez *et al.* [2] who reported similar trend on the basis of genotyping of a 'national collection' of isolates.

To sum up, this study has proven useful in identifying the predominant spoligotypes responsible for disease transmission in Andhra Pradesh. Significant presence of the ancestral type bacteria in the TB patients from Andhra Pradesh as shown here assumes importance in the light of our earlier espousal [19,20,21] and one recent finding that *M. tuberculosis* belonging to the ancestral lineage (EAI) could show reduced transmission as compared to other lineages [22]. This perhaps explains why the Indian population has never suffered with institutionalized TB outbreaks as seen in some other parts of the world where ancestral type bacteria are not so prevalent. Having said this, we sincerely wish our observations may form path forward to more rigorous future studies, based on whole genome sequencing of the underlying isolates, to obtain better picture of the existing diversity, transmission patterns and the preponderance of drug resistant strains in this high-incidence region.

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Author Contributions

Conceived and designed the experiments: NA VV SEH. Performed the experiments: SKT CCI BHM BVA. Analyzed the data: AK MM PSR YP. Contributed reagents/materials/analysis tools: SEH VV. Wrote the paper: NA. Obtained permission from biosafety committee: NA.

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