In vivo expression study of PrrA in *Mycobacterium tuberculosis* H37Ra

Dissertation submitted in partial fulfillment for the degree of

Master of Science in Applied Microbiology

Submitted By

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CERTIFICATE

This is to certify that the dissertation entitled **"In vivo expression study of PrrA in *Mycobacterium tuberculosis* H37Ra"** submitted to School of Biotechnology, Kalinga Institute of Industrial Technology-KIIT (Deemed to be University; Declared U/S 3 of UGC Act, 1956) Bhubaneswar, Odisha, India in partial fulfilment of the requirement for the **Master of Science in Applied Microbiology** is a record of work done by **Ms. Priyanka Roy** bearing **Roll No. 1662012** & **Registration No. 16530450269** during the period from **02/01/2018 to 04/05/2018** in the Department of Molecular Reproduction, Development & Genetics, Indian Institute of Science (IISC), Bangalore, India under my guidance and supervision. She has actively participated in studies carried out in the laboratory. Further, it is also to certify that the above said work has not been previously submitted for the award of any degree, diploma, or fellowship in any Indian or foreign University. She has good aptitude for research. I wish her success in her future endeavour.

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DECLARATION

I hereby declare that the dissertation entitled **"In vivo expression study of PrrA in *Mycobacterium tuberculosis* H37Ra"** submitted by me, for the degree of **Master of Science in Applied Microbiology** to School of Biotechnology, Kalinga Institute of Industrial Technology-KIIT (Deemed to be University; Declared U/S 3 of UGC Act, 1956) Bhubaneswar, Odisha, India is a record of bona fide work carried by me during the period from **02/01/2018 to 04/05/2018** under the supervision and guidance of **Dr. Deepak K, Saini**, Associate Professor, Department of Molecular Reproduction, Development, Indian Institute of Science (IISC), Bangalore, India.

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Abstract

Tuberculosis (TB) is always a matter of concern for the human society because of its high infectivity rate which almost covers 1/3 of the global population. It severely affects the social and economic status of infected person. Though this bacterium has been discovered a long ago but the effective therapeutic treatment and vaccines are not available till today. With the emergence of multidrug-resistant mycobacterial strains, better therapeutic strategies are required for the successful treatment of the infection. The present study has confirmed an *in vivo* model in *Mtb* with respect to one of its STPK PknK and its transcriptional regulator PrrA. PrrA was found to be phosphorylated in the presence of PknK. *in vivo* analysis of *Mtb* H37Ra cell lysate from different pH i.e. 4.4, 6.6 &7.2 and from starvation conditions showed that there were no significant changes in PrrA expression as compared to the control set. It was found that PrrA metabolism does not depend upon any varied pH and starvation conditions suggesting that it may constitute novel targets for antimycobacterial drugs.

Acknowledgement

With great pleasure and deep sense of gratitude, I take this opportunity to express my indebtedness to my supervisor **Dr. Deepak K Saini**, Associate Professor, Molecular Reproduction, Development and Genetics Department (MRDG), Indian Institute of Science (IISC), Bangalore, India for giving me the opportunity to work in his research a for my Masters Dissertation. His constant guidance, painstaking effort and encouragement at each and every step of my research work has led me to complete my work successfully. I got a chance to learn many new things. He is an inspiration to work and do something good in life. My motivation to continue in the field of research is all because of him. Working under him is an honor and a great learning experience.

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I owe; gratitude to my parents for their encouragement. They are the only people for whom I am working and want to achieve more just to make them proud. I can't quote in words the wholehearted sense of reverence to siblings. The inspiration behind successful completion of my project work is their endurance, everlasting strong support and endless prayers.

Last but not the least I would like to thank the almighty God for making me capable to understand a bit of this fascinating world of science and giving me the courage to work sincerely during my dissertation.

Priyanka Roy

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List of Abbreviations

TB	Tuberculosis
Mtb	*Mycobcaterium tuberculosis*
Amp	Ampicillin
Gen	Gentamycin
MTBC	Mycobacterium tuberculosis complex Species
MA	Mycolic Acid
XDR	Extreme Drug Resistance
MDR	Multi Drug Resistance
TDR	Total Drug Resistance
ROS	Reactive oxygen species
HNP	Human Neutrophil peptide-1
RNI	Reactive nitrogen intermediate
OADC	Oleic Albumin Complex
TCSS	Two-component signal transduction systems
RR	Response regulators
SK	sensor kinases
STPK	Serine/threonine protein kinases

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1. INTRODUCTION

1. 1. Tuberculosis: A Deadly Disease

Tuberculosis (TB) is a very widespread infectious disease triggered by an intracellular pathogen *Mycobacterium tuberculosis (Mtb)*, asymptomatically has infected 1/3 of the world's existing population over a death rate of ~2 million every year of which 10% of the population are susceptible for TB infection in their lifetime (Small, 1996; WHO, 2009, 2010 & 2011). TB has been a severe threat to human species for over 5000 years. In history of 5th century BCE Hippocrates, writing described a word '*phthisis*'-the Greek term for tuberculosis described as the utmost common disease of his time.

TB has been discovered almost 125 years back by Robert Koch. Since that time, we humans are fighting against this deadly pathogen and unable to find a complete cure against this disease and ultimately makes TB, the 2nd principal cause of death from an infectious agent worldwide (WHO, 2009). The ratio between the steady drops in number of cases with the new cases appears are almost equivalent. Moreover, an estimated 9 million freshly diagnosed cases appear per year. African countries have shown the highest occurrence rate, while 1/2 of the world's cases are found in Bangladesh, China, India, Indonesia, and Pakistan (Kumar *et al*., 2007; WHO, 2015). (Figure 1.1)

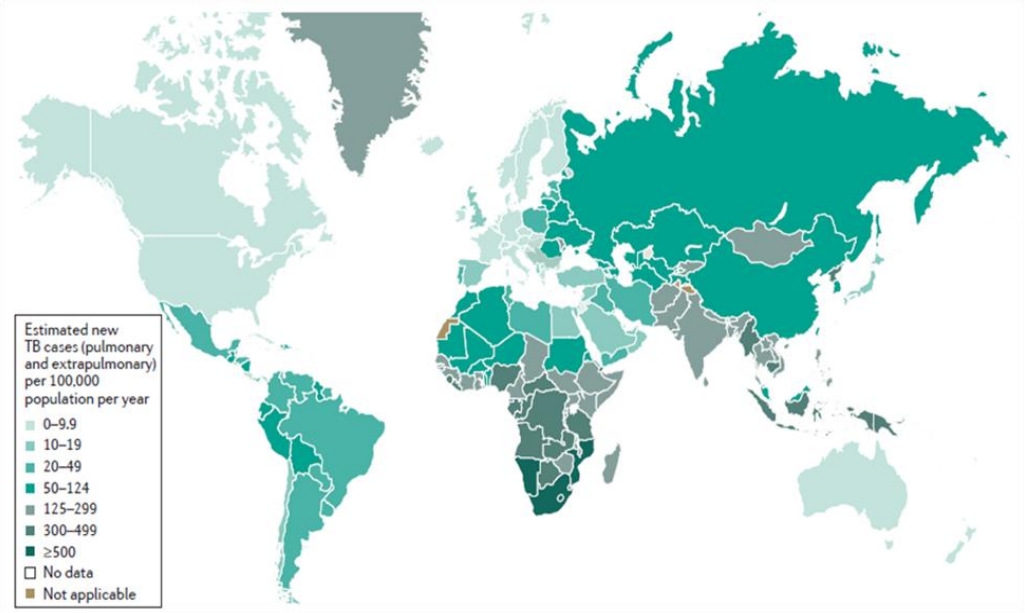


Figure 1.1 Global incidence of active Tuberculosis (pulmonary and extrapulmonary)
Tuberculosis Report: World Health Organization, 2015

As of now, more than 90% deaths occurred in developing countries and among them, the individuals aged 15-60 have accounted for 3/4 of TB morbidity and mortality. It was estimated that one out of every three people is infected with bacilli which could cause TB, but in case of most individuals, these bacilli stay dormant referred to as "Latent TB". These latent TB cases get infected once their immune system becomes compromised or in malnourished or old age people. The rising expense of TB infection along with the occurrence of bacterial resistant towards the first and second line of drugs have impacted the necessity to explore new drugs for treating this deadly disease. New treatment strategies are required for decrease the TB infection over the increasing drug resistance. Moreover, the boosting era of HIV has intensified the frequency of TB infections, as both these bugs work in parallel for killing a host. TB typically attacks the lungs referred as pulmonary TB which is regarded as the most common form of TB. Simultaneously TB can also affect other organs of the causing other forms of TB altogether known as extrapulmonary tuberculosis (EPTB) (Golden and Vikram, 2005). The infection sites for EPTB include the central nervous system (meningitis TB), pleura (pleurisy TB), genitourinary system (urogenital TB), and lymph nodes. TB may have accompanied with pulmonary TB (Golden and Vikram, 2005). There is another serious form of disseminated TB called military tuberculosis (Regnier *et al*., 2009). (Figure 1.2).

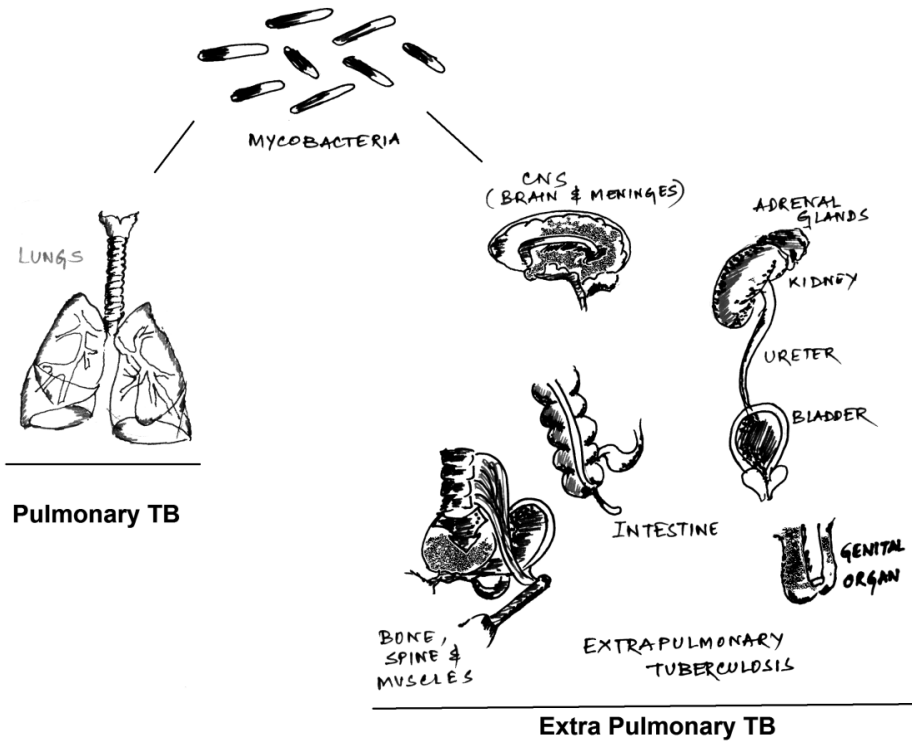


Figure 1.2 *Mycobacterium tuberculosis* infects lung (Pulmonary TB) and also in different human body parts (Extra Pulmonary TB). (Golden & Vikram, 2005)

1.2. *Mycobacterium tuberculosis*: The Driver of Tuberculosis

Mycobacterium tuberculosis (Mtb) is an aerobic, acid-fast bacillus, non-motile with a lipid rich cell wall, which makes this pathogen a unique one (Southwick, 2007). The generation time of this pathogenic bacteria is extremely slow (16-20 h growth rate) as compared to other bacteria which generally have a cell division rate of less than an hour (Cox, 2004). Another four TB causing mycobacteria; (a) *Mycobacterium bovis*, (b) *Mycobacterium africanum*, (c) *Mycobacterium canetti* and (d) *Mycobacterium microti* were also reported (van Soolingen *et al*., 1997). *M. bovis* is responsible for tuberculosis, but the concept of pasteurized milk was helpful in eradicating this public health issues in developing countries (Thoen *et al*., 2006). *M. africanum* is not prevalent; but some of the African regions have shown some substantial cases of TB (Niemann *et al*., 2002; Niobe-Eyangoh *et al*., 2003). *M. canetti* is an occasional pathogen limited to Africa for which very few cases were found in African emigrants (Pfyffer *et al*., 1998), whereas *M. microti* is mostly found in immunocompromised people (Niemann *et al*., 2000). Another known pathogenic mycobacterium includes *Mycobacterium lepra* & *Mycobacterium marinum*. There are also non-tuberculous mycobacteria exist, such as *Mycobacterium avium* & *Mycobacterium kansasii* cause neither TB nor leprosy, but cause resemblance to TB. Macrophages are considered as the 1st line of defence mechanism for its recognition, phagocytosis and killing of invaders in case of *Mtb* infection. These macrophages could kill non-pathogenic mycobacteria such as *M. smegmatis* (*Msm*), but pathogenic strains smartly avoid the host immune responses and cause the disease eventually.

Mycobacterium tuberculosis infected a projected 2.2 billion people worldwide with a death rate of 1.3 million in 2008-2009. New translational strategies in TB treatment are required to overcome the raising drug resistance features in this deadly bug. Eukaryotic STPKs have shown potential in fighting against cancer as it possesses specific targets (Huse & Kuriyan 2002). Similar to this, the homologous proteins from prokaryotic origin seem to be striking for development of possible drug targets. Mostly this kinase inhibitors role is to switch off/on themselves for tracking kinases activation. The systematic structural insights and biochemical characterization of the all protein kinases and their activation mechanism in *Mtb* might facilitate the development of analogs of molecular inhibitors for mycobacteria.

1.3. Pathogenesis and mode of transmission of Tuberculosis

The tubercle bacilli are transported through airborne particles (estimated 1-5 μm in size), or as droplet nuclei, which get further intensified upon exposure the surroundings with an infected person sneezing, coughing, speaking etc. The risk of infection in terms of few no. of TB bacilli is very low; but it gets intensified in people having immunosuppressed immunity such as during HIV/AIDS. Infection get intensified if a susceptible person inhales nuclei droplet carrying *Mtb* which further navigate through mouth or nasal passages reaching upper respiratory tract followed by bronchi and finally reach in lung alveoli (Behr *et al*., 1999; Nicas *et al*., 2005) (Figure 1.3). Alveolar macrophages engulf this organism and spread from the site of initial infection in the lung through lymphatic nodes and through blood vessels connected to other body parts. Within 2-10 weeks of *Mtb* infection, the immune response limits further proliferation of the tubercle bacilli; however, some of these bacilli persist towards dormant stage (called latent TB) and remain viable for several years. Microbes cannot transmit in latent TB stage onto others except in environments where HIV/AIDS is widespread. This risk is greatest during the first 2 years of post-infection. TB disease have symptoms include coughing blood, fatigue, fever, persistent cough, weight loss, and night sweats (WHO, 2009).

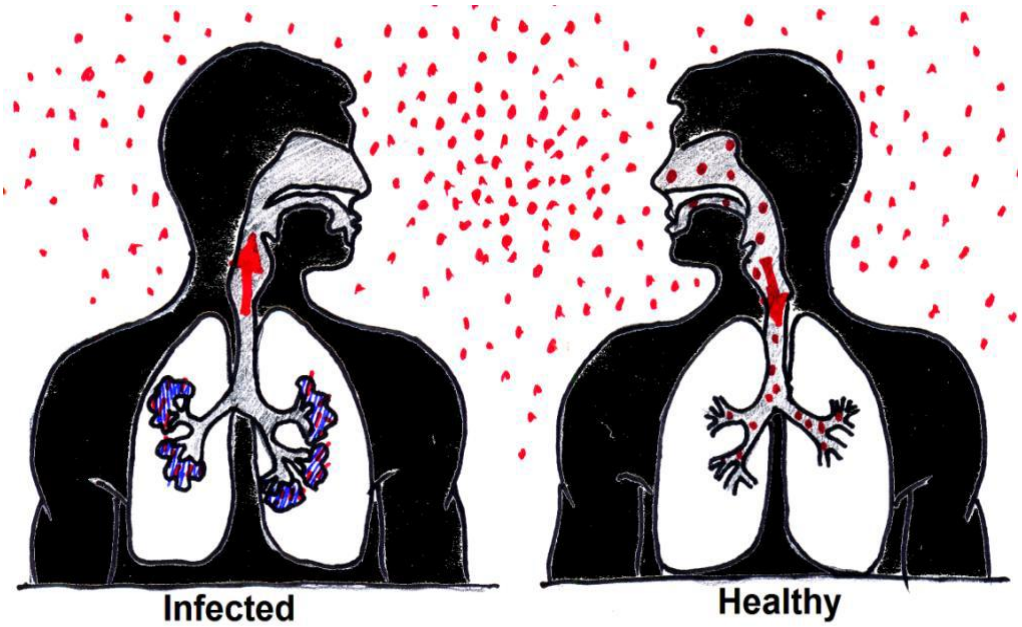


Figure 1.3 Transmission of *Mycobacterium tuberculosis* bacilli from infected to healthy person. (Behr *et al*., 1999; Nicas *et al*., 2005)

1.4. The Need of Signalling system in Mycobacteria

Various Signalling system are present in mycobacteria which play key role in their survival and persistence within the host system. There are five Signalling system i.e. TCS, STPK, Protein phosphatase 2C, Protein tyrosine phosphatase, Protein tyrosine kinase. Out of all these TCS and STPK are most important.

1.5. Two-component regulatory system (TCS)

Also, known as Two-component signal transduction systems, it serves as an important response towards any stimuli which is coupled with an activity that allow organisms to sense followed by respond and adapt in diverse environmental cues. These systems are generally involved a membrane-bound receptor histidine kinase which is responsible for sensing a specific signal and translates the same into a required action via phosphorylation of a cognate response regulator which facilitates the cellular response via target gene expression. Majority of the Gram-negative bacteria and cyanobacteria contain this two-component signalling systems. Both of these histidine kinases and their response regulators are amongst the major gene families found in bacteria, whereas these systems are much less conjoint in archaea and eukaryotes. The two-component signalling system acts as a strategy for coupling changes with varied environmental stress and modulates the cellular physiology accordingly. These signalling proteins have been positioned in the genomes of nearly all existing bacteria.

As depicted in the Figure 1.4, the TCS pathway involves (a) cytoplasmic membrane-bound sensor histidine kinase (HK) or (b) hybrid HK dimer and (c) a response regulator (RR) dimer. A signal (blue circles) generated in the periplasmic space is generally noticed by the HK (with additional phosphor transfer steps in between) or RR is being phosphorylated by hybrid HK. This attachment of the RR to promoter regions on the genome triggers the target gene expression.

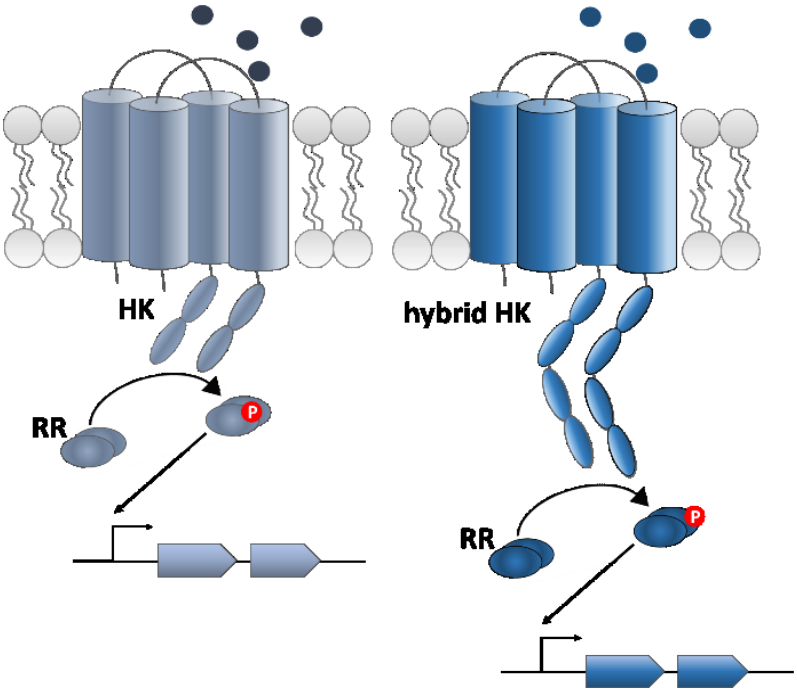


Figure 1.4 Schematic representations of two-component system (TCSs) Signalling pathways (Fischer *et al.,* 2016).

TCS is ubiquitous among bacteria. They modulate signalling events such as cell-cell communication, adaptation to environments, and pathogenesis in bacteria. Bacteria express its genetic system to adapt in altering environments. This TCSs are principal moderators of signal transduction that allows the bacteria to sense physical and/or chemical alterations and then transmit this signal through the cytoplasm to the bacterial nucleoid which act as the site of gene expressions. As TCSs are absent in humans and other mammals, these proteins are regarded as prospective targets for emergence of new antibiotics.

1.6. Cross-talk between Two Component Systems

Cross-talk generally takes place due to the cross interactions of two or more divergent Signalling pathways. TCSs acts paralleled to an enormous range of stimulus simultaneously. The domains and arrangement of TCSs is highly linked which give rise to cross-interactions for administering several cellular signals (Igo, *et al.,* 1989). It could take place on HKs level where a heterodimerization between non-cognate HKs can arise to form a higher-order Signalling complex (Figure 1.5A). Along with cross-phosphorylation between HKs (non-cognate) & RRs and the heterodimerization of RRs (non-cognate) are another signal notifications (Figure 1.5 B and C). Conclusively, crosstalk could take place on the promoter level where RRs control the gene expressions (Figure 1.5D)

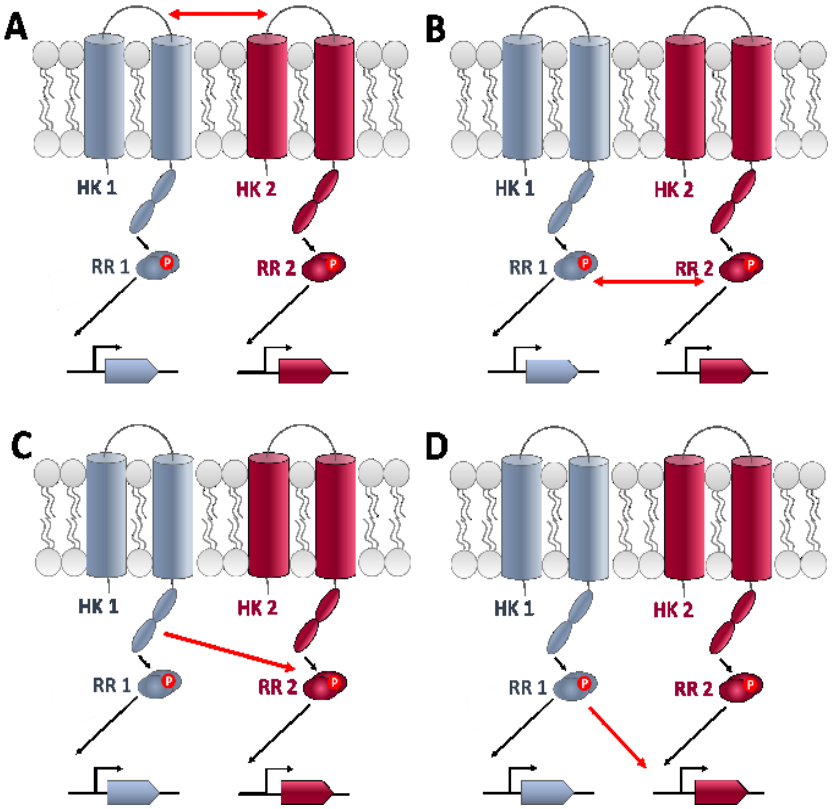


Figure 1.5 Levels of cross-talk between TCS pathways (Fischer *et al.*, 2016).

1.7. Ser/Thr protein kinases (STPK)

Protein kinases are present in eukaryotes in the most extensive and diverse gene families contribute to the system. Receptor protein kinases involves in regulation of cell physiology towards response to extracellular signals. His and Asp residues exchange phosphoryl groups with the help of TCSs, as a result cognate phosphatases reverse the stable phosphorylation which is again facilitated by spatial & temporal regulation of Ser/Thr/Tyr kinases (Cheek *et al.*, 2005). TCSs protein superfamily contain very well-derived class of enzymes which regulate a broad range of metabolic activities. Eukaryotic STPKs have already been proven for possible drug targets to fight against cancer (Huse & Kuriyan 2002). In bacterial system, many kinase inhibitors act to seize the kinase activation. This prokaryotic protein system are now fascinating targets in the area of drug development.

1.8. Ser/Thr protein kinases (STPK) of *Mycobacterium tuberculosis*

Mycobacterium tuberculosis (Mtb) genome contains 11 STPKs, 11 HKs with 11 cognate RRs, 1 Ser/Thr phosphatase followed by 2 Tyr phosphatases (Ortiz-Lombardia *et al.,* 2003) making this bug an attractive model system for studying intracellular Signalling pathways in prokaryotes. The STPKs belongs to the prokaryotic PKN2 kinases family which has phylogenetic resemblance as eukaryotic homologs (Kang *et al.* 2005). This machinery Ser/Thr phospho-Signalling in *Mtb* infection collectively with the cellular process control by the regulatory proteins provides robust basis for inspecting these mechanisms of this Signalling systems (Greenstein *et al.* 2005).

BACKGROUND

Antibody against PrrA~P was generated through rabbit immunization and purified through negative selection method.

OBJECTIVE

In vitro crosstalk has been established in lab PknK-PrrA (Personal Communication). This thesis aims to answer the questions by studying the *in vivo* expression of PrrA~P with respect to the various challenged environmental effects.

- *In vitro* crosstalk study PrrA and PknK
- Optimization of detection level of purified PrrA using anti-PrrA antibody.
- *In vivo* expression study of PrrA of H37Ra in different pH (4.4, 6.6 and 7.2) and starvation conditions.

2. REVIEW OF LITERATURE

Tuberculosis (TB) is a preventable and curable infection caused by *Mycobacterium tuberculosis.* However, it remains as a most important basis of mortality and morbidity worldwide as the effectiveness of standard treatment is often compromised by side effects, patient's failure for completion of the prolonged course of treatment. Moreover, the rise in multi-drug resistant (MDR) & extremely drug resistant (XDR) has emerged a hindrance in TB diagnosis and treatment. The current available treatment for MDR-TB not only requires a longer period of time, but is also less efficacious, more expensive and more toxic than the standard treatment presenting a double whammy for TB treatment and control. New anti-TB drug development has generally been a slower process, with bedaquiline being the first drug to be agreed in 40 years by US Food and Drug Administration (FDA).

Cell signalling plays an important role in all metabolically active cells. These signalling mechanism is the recognizing of a signal followed by its translation into an output which alters cell physiology. More precisely, signal transduction defines as a sensing of an extracellular signal that gets transduced throughout the cytoplasmic membrane and giving an intracellular response. Therefore, this mechanism of signal transduction is crucial for cellular adaptation to varying extracellular environment. In case of *Mtb*, these cellular adaptations allow growth and/or survival in the environments encountered by the pathogen during the course of infection in the human host.

The endurance of an organism depends on the capability towards respond followed by adaptation in a challenged environment. The origin of this adaptation is facilitated by the potential of cells to sense both external and internal signals and transduce them. With the help of respective cognate phosphatases, the protein kinases activate themselves for signal transduction followed by phosphorylation event to take place. Upon exposure to an external stimulus, these kinases get auto-phosphorylate followed by substrate protein transphosphorylation. Very precise amino acids like serine (Ser), threonine (Thr), tyrosine (Tyr), histidine (His), and aspartate (Asp), undergo phosphorylation for induction of the particular target protein directly by conformational changes or indirectly by regulating protein-protein interactions. These bacterial kinases have been considered for targeting His/Asp residues; but now a day, research on Ser/Thr kinases and their respective partner phosphatases is having equal demands.

2.1. *Mycobacterium tuberculosis* Serine/Threonine Protein Kinases (STPK)

Two component systems (TCSs) are the most extensively disseminated and intensively studied transmembrane Signalling systems in bacteria (West & Stock, 2001). These systems comprise of a sensor molecule and transducer which is referred as a response regulator, in which the sensor protein spans the cytoplasmic membrane and the response regulator is a cytoplasmic protein, usually a transcription factor that is activated in response to the phosphorylation event. A key mechanism involved in signal transduction across transmembrane in *Mtb* is via the serine/threonine protein kinases (STPKs). Unlike TCSs, STPKs are less widely distributed among different groups of bacteria. Two component systems were reported earlier along with the presence of less or no STPKs in bacterial pathogens and model organisms; but the genome of *Mtb* encodes 11 STPKs and equal no. of two-component systems (Cole *et al*., 1998; Fontan, 2004), indicating that these two mechanisms both perform noteworthy roles in signal transduction in *Mtb* which would further correlates with the pathogenicity of this smart bug.

Eukaryotic protein kinases display a broad diversity of regulatory mechanisms; but, prokaryotic STPKs and their activation mechanism is not well deciphered yet. The universal fact is, these kinases are usually maintained in a "switch off" state, only gets activated to "switch on" state upon response to a precise signal that reduce autoinhibition (Alber 2009). Several control mechanisms mediate these Signalling proteins activation since its binding to the allosteric effectors till alterations in subcellular localization. The "switch on" mode is defined by the binding of the Signalling molecule to a substrate followed by catalyzing phosphoryl-transfer and the "switch off" mode have other molecular events, which in turn allows the various classes of kinases to evolve by having a structurally diverse mechanism of autoinhibition (Huse & Kuriyan 2002; Alber, 2009).

For the very first time, this prokaryotic STPKs was found in *Yersinia pseudoturberculosis* and *Myxococous Xanthus* and afterwards the era of prokaryotic genome sequences have decoded the signs of several eukaryotic-like serine/threonine protein kinases (STPKs) along with histidine kinases as their response regulators. These Ser/Thr phosphoSignalling systems were also distributed in the archaeal genomes (Leonard *et al.*, 1998). The rising numbers of recognized eukaryotic-like STPKs have suggested their importance in signal regulation as two-component histidine kinases in bacteria (Kannan *et al.* 2007).

2.2. Structural insights into Serine/Threonine Protein Kinases (STPK)

STPKs demonstrate striking structural resemblance to the eukaryotic homologs, but the regulation mechanisms are different in *Mtb* kinases structures and eukaryotic kinases. Transphosphorylation is a signature metabolic event which activates all the 11 *Mtb* STPKs in "front-to-front" dimer form or in a "back-to-back" orientation allosterically (Greenstein *et al.* 2007; Mieczkowski *et al.* 2008) which in turn result in a phosphorylated active kinase. Autophosphorylation is a metabolic event where one kinase molecule phosphorylates another identical molecule. The mechanisms of STPKs trans-autophosphorylation via these two events are fairly established in *Mtb*. Alternate mechanisms for activation followed by conformational changes from phosphorylation event remain unknown. There are no reports available on transphosphorylation and phosphorylation of a kinase by other STPK in *Mtb* (Kang *et al.*, 2005). A major finding of kinase-kinase regulatory cascades in the form of cross-kinase phosphorylation in *Myxococcus xanthus* (Nariya & Inouye 2005), has added another step towards regulation control mechanism and possibly increased the complexity of *Mtb* Ser/Thr phospho-Signalling.

Activation of kinases is controlled by a predefined metabolic event leading towards structural modifications which further stabilizes the protein for achieving its active state. The mechanism between an inactive "off-state" and an active "on-state" is very important in terms of activating these kinases. Many reports were published on *Mtb* STPKs crystal structures which are active phosphorylated kinases. These structures of *Mtb* STPKs in different activation stages have provided key insights for activation machinery. Further to this, the thorough insights into biochemical & structural characterization mechanisms of all these kinase activations in *Mtb* will permit us to develop similar molecular inhibitors for mycobacteria.

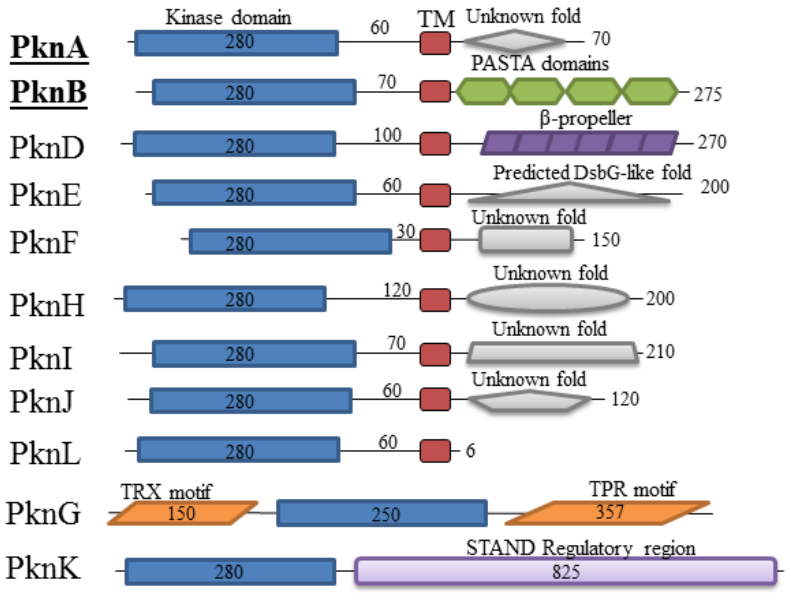


Figure 2.1 *Mtb* Ser/Thr complex contains 11 STPKS. The two essential kinases are PknA and PknB, PknG and PknK are the two soluble kinases. (Baer et al., 2010)

STPKs transfer the gamma-phosphate present in ATP to precise amino acids Ser, Thr or Tyr residues. Functionally these enzymes have diverse mechanisms: (a) binding the donor ATP phosphate to form a complex with Mg^{2+} or Mn^{2+}, (b) binding the substrate protein for positioning them, (c) transferring the gamma phosphate from ATP to the accepting hydroxyl group molecule (Hanks and Hunter 1995). As a result, the Ser, Thr, and Tyr phospho-esters are not likely to change for several weeks in a pH (7.0) which later required for STPKs regulation (Greenstein *et al.* 2005). This regulation events of STPKs enable them for an accurate cellular response towards external signals followed by allowing the bacteria to survive in a various environmental cue. The *Mtb* Ser/Thr complex comprises of nine kinases attached on the cell membrane with the support of a single trans-membrane (TM) helix along with two soluble solution kinases as shown in Figure 2.1 (Greenstein *et al.* 2005; Barthe *et al.* 2010).

2.3. Metabolic functions of *Mtb* Serine/Threonine Protein Kinases (STPK)

Amongst the 11 paired TCSs present in *Mtb* genome, the DosSR (DevSR) TCS is responsible for the mycobacteria adaptation in hypoxic environment (Kendall *et al.*, 2004; Lee *et al.*, 2008). All these eukaryotic like STPKs present in *Mtb* genome serve as central regulators of the metabolic process, growth & development, virulence, and host–pathogen interaction, (Cho *et al.*, 2009; Fernandez *et al.*, 2006; Fol *et al.*, 2006). For the first time, the junction of TCSs and STPKs which utilize phosphorylation/ dephosphorylation of proteins in mycobac-

teria were reported and showed the transcriptional activity of DosR which is controlled by DosS/DosT and PknH STPK in *Mtb* (Chao *et al.,* 2010). This PknB is membrane-bound in *Mtb*, conserved amongst mycobacteria and responsible for cellular events like for cell wall synthesis, cell division & elongation, and regulation of oxygen-dependent cell replication in *Mtb* and *M. smegmatis* (Chawla *et al.,* 2014; Ortega *et al.,* 2014). The adaptation ability of *Mtb* in hypoxic environments was newly studied and predicted to be impaired with *pknB* over expression (Ortega *et al.,* 2014).

STPKs presence in *Mtb* is very crucial once this bug invades and reaches in the surroundings of host macrophages. Endurance of *Mtb* and reproduce within host macrophages for over a long period of time because of its smartness in escaping from phagolysosome escape mechanism. This mechanism is driven by a STPK, PknG which is a necessary virulence factor of pathogenic mycobacteria (Walburger *et al.,* 2004) which down regulate macrophages PKC-α (Chaurasiya & Srivastava, 2008 & 2009) which is involved in phagocytosis, maturation of phagosome, immunity to infection, apoptosis, and the productions of cytokines/chemokines/immune effector molecules (Webb *et al.,* 2000; Holm *et al.,* 2003). PknG and PknK, do not have a transmembrane domain, are the only two cytosolic STPKs present in *Mtb* (Av-gay *et al.,* 2000). PknG is also reported for regulation of glutamate/glutamine metabolisms as *pknG* deletion from *Mtb* has resulted in reduction of cellular glutamate and glutamine levels confirming the PknG might act as a sensor for bacteria under starvation (Cowley *et al.,* 2004). Additionally, PknG and GarA are essential for virulence have been found to function as similar to *Mtb* and *M. smegmatis* (Ventura *et al.,* 2013). Gene expression in response to the extracellular environment are required for replication and survival of bacteria (Roxas *et al.,* 2009; Lund *et al.,* 2014; Li *et al.,* 2015; Ivy *et al.,* 2012; Deng *et al.,* 2014). Glutamate decarboxylation is a well understood acid resistance system in bacteria, proposed that PknG (as it regulates glutamate metabolism) might regulate acid tolerance system in mycobacteria (Su *et al.,* 2011; Damiano *et al.,* 2014). pH serve as a key signal to which mycobacterial pathogens respond (Rohde *et al.,* 2007) to acidic and oxidative stress as host defence mechanisms (Vandal *et al.,* 2009). Several studies have suggested the PknG is involved in not only regulation of structure &composition of cell envelope (Wolff *et al.,* 2009; Wu *et al.,* 2017); but also, play a role in increased antibiotic and stress resistance and deletion of *pknG* in *M. smegmatis* has been shown adverse effects towards antibiotic sensitivity (Wolff *et al.,* 2015).

2.4. Comparative genomics of mycobacterial Serine/Threonine Protein Kinases

The *Mtb* STPKs were primarily reported as "eukaryotic-like" protein kinases based on their sequence similarity to eukaryotic STPKs (Av-Gay *et al.*, 2000). Comparison of the *Mtb* STPKs to eukaryotic protein kinases has demonstrated that, *Mtb* proteins incorporate certain similar domains relatively limited sequence identity (Prisic & Husson, 2014). Sequence alignment of the *Mtb* STPKs had shown that all the 11 STPKs could be grouped into three clusters of three kinases and two kinase domains (KD) that are less similar to any of the nine clustered domains (Figure 2.2). The unclustered outliers, PknG and PknK, are also the two kinases that lack a transmembrane domain. This observation had suggested that the genes encoding the nine receptor-type kinases may have been derived from a single common ancestral gene via horizontal gene transfer, whereas PknG and PknK may have been acquired separately. In contrast to the intracellular KDs, the extracellular domains of the nine transmembrane STPKs had shown no sequence similarity, indicating that they might have bound and respond to distinct extracytoplasmic molecular signals (Prisic & Husson, 2014).

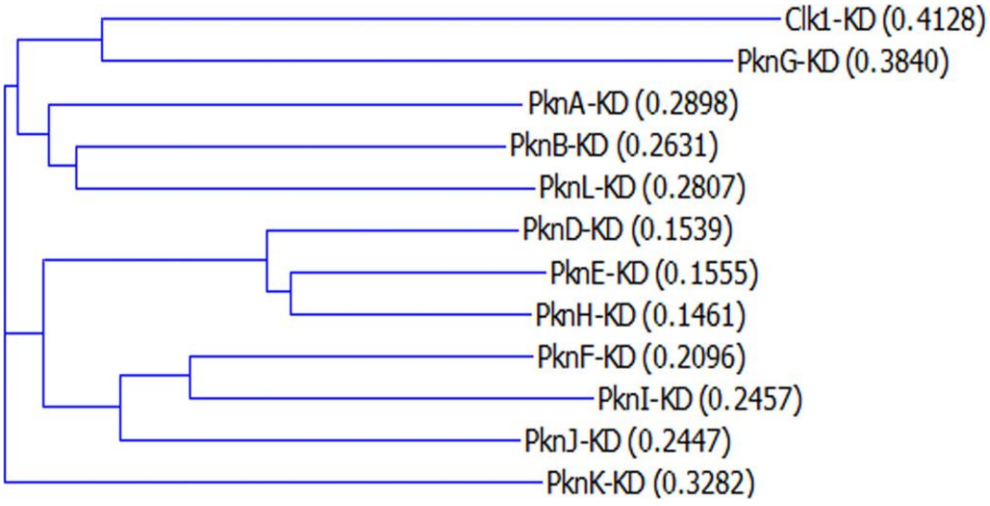


Figure 2.2 Dendrogram of KDs of *Mtb* STPKs. KDs identified by the SMART algorithm were aligned and grouped using the AlignX software (Prisic & Husson, 2014)

STPKs are not consistently dispersed among diverse bacterial phyla. Within the mycobacteria and closely related actinomycetes, their distribution is also uneven (Table 1., Prisic & Husson, 2014), which had compared the genomes of the pathogenic *Mycobacterium tuberculosis* and *Mycobacterium* leprae, the opportunistic pathogens *Mycobacterium avium* and *M. avium* subspecies paratuberculosis, the non-pathogen *Mycobacterium smegmatis*, and the more distantly related nonpathogenic actinomycete *Corynebacterium glutamicum*, showed these differences in the distribution of the STPKs. The presence of PknA, PknB, PknG, and PknL in all species have suggested that these kinases play important roles in regulating key

aspects of mycobacterial physiology, though only PknA and PknB are essential in *M. tuberculosis* (Sassetti *et al.*, 2003). The other STPKs likely have more specialized regulatory roles corresponding to the niches occupied by these different species (Prisic & Husson, 2014).

Species	PknA	PknB	Pkdd	PkdE	PknF	PknG	PknH	PknI	PknJ	PknK	PknL
Mtb	Rv0015c	Rv0014c	Rv0931c	Rvl743	Rvl746	Rv0410c	Rvl266c	Rv2914c	Rv2088	Rv3080c	Rv2176
Mmar	MMAR_0017	MMAR_0016	MMAR_4577	MMAR_2581	MMAR_2606	MMAR_0713	MMAR_1982	MMAR_1794	MMAR_1423	MMAR_2576	MMAR_32U
					MMAR_4174		MMAR_2444		MMAR_2408		
							MMAR_4156		MMAR_2941		
							MMAR_4171				
Mav	MAV_0019	MAV_0017	MAV_4238		MAV_3145	MAV_4751	MAV_1417				MAV_2318
							MAV_2158				
Map	MAP0018C	MAP0016C	MAP3387C		MAP1332	MAP3893C	MAP2026				MAP1914
							MAP2031C				
							MAP2504				
Msmeg	MSMEG_0030	MSMEG_0028			MSMEG_0886	MSMEG_0786	MSMEG_4366		MSMEG_5513	MSMEG_0529	MSMEG_4243
					MSMEG_3677						
Mle	ML0017	ML0016				ML0304					ML0897
Cgl	cg0059	cg0057				cg3046					cg2388

matis MC^2-155; *Mle, M. leprae* TN; *Cgl, C. glutamicum*

[a] Selected bacteria are shown: *Mtb, M. tuberculosis* H37Rv; *Mmar* ATCC 13032., *M. marinum;* Mav, *M. avium* 104; *Map, M. avium paratuberculosis* klO; *Msmeg, M. smeg*

Table 1. Closest orthologs of STPKs in various mycobacterial strains (Prisic & Husson, 2014)

2.5. Biological functions depicted in *Mtb* STPKs mapping

In an earlier report, these STPKs were being analyzed based on bioinformatics tools to systematically understand all 11 STPKs and their interacting proteins. It was found that, many of these metabolically active proteins were present in the inward provinces of the map depicted in the (Figure S7, Wu *et al.*, 2017) according to STRING analysis (Szklarczyk *et al.*, 2015), while the colors represented various biological processes. This study has proven that the STPKs not only played substantial functions in metabolic processes, but also help in regulation of many unknown functional proteins, which could be a suitable lead to establish the functions of these proteins. According to the recent analysis by latest version of TBDB, the biological functions of many proteins in *Mtb* are not well known (Lew *et al.*, 2011). Research were also conducted to decipher the role of the kinase protein interaction (KPI)s in a more explanatory way, the existing protein-protein interaction with well-defined functional annotations were further analysed. Bioinformatics tools like STRING and CYTOSCAPE were used for the network analysis shared by these proteins which has resulted a compact STPK-KPIs map (Figure 2.3). As per this metabolic network, many proteins might have shown to be involved in lipid biosynthesis, such as formation of peptidoglycan (PG) by RmlA, mycolic acids formation by LipR and FadB2, amino acids metabolism by SerS,

ArgH, HisH and ProA, nucleotide biosynthesis by AdoK, PryE, EpiA and Hpt. This metabolic network has given insights that STPKs might play key roles in cell cycle regulation (Parikh *et al.*, 2009; Griffin *et al.*, 2011).

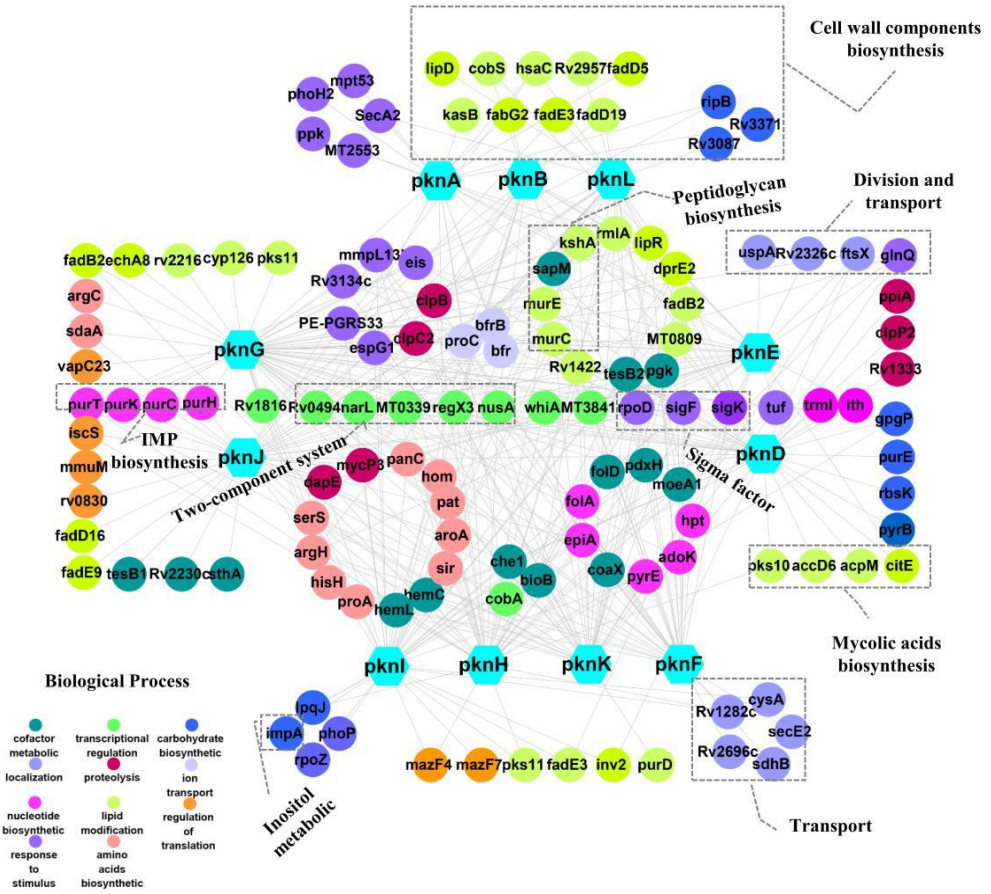


Figure 2.3 Variety of biological functions were enriched according to GO analysis based on the STPK-KPIs map (Wu *et al.*, 2017)

Other proteins like MmpL13b, EspG1 and PE-PGRS33 were found responsible for sensing chemicals. This network mapping has identified > 25 transcription-related proteins, including three sigma factors like SigK, SigF and SigA, and TCS proteins (NarL, RegX3, NusA and Rv1816). These proteins were shown their involvement in sensing various signals, and adaptation towards various environmental cues and maintenance of the cell envelope integrity during stress (Barik *et al.*, 2010; Hatzios *et al.*, 2013; Park *et al.*, 2013). STPKs might regulate the cell growth and respond by altering protein transcriptional processes. Surprisingly, ClpB and ClpP were shown to be involved in proteolysis processes. Previously Clp complexes family were reported for *Mtb* growth (Vaubourgeix *et al.*, 2015; Raju *et al.*, 2014). These results validated that the Clp protease complex activation and related biologi-

cal processes might have strongly regulated by STPKs. Another interesting fact was found that ATP-dependent Mur ligases were the crucial enzymes involved in peptidoglycan biosynthesis. It was shown that STPKs may regulate cell wall synthesis through the interaction with Mur ligases (namely MurC and MurE) (Wu *et al.*, 2017).

The major environmental factors which influence the *Mtb*'s signal transduction in relation to dormancy and reactivation are not well studied. Amongst these, oxygen stress was likely to be a key factor in mycobacterial survival both *in vitro* and *in vivo* (Rustad *et. al.,* 2009) and in granulomas, it was found low (Tsai *et. al.,* 2006; Via *et. al.,* 2008). Studies have also indicated that latency and reactivation, both were induced by hypoxia during infection process. Many studies have suggested that environmental cues in granuloma formation has promoted bacteriostatic followed by phenotypic drug resistance (Boshoff & Barry, 2005; Flynn & Chan, 2001; Gomez & McKinney, 2004). Reversible protein Ser/Thr phosphorylation is a crucial machinery towards external stimulus. As compared to other classical two-component systems present other bacteria, *Mtb* with similar genome size is expanded with 11 serine/threonine protein kinase (STPK) (Getahun *et. al.,* 2010; Alber, 2009). Recent reports implicated bacterial STPKs in stress response, development and host–pathogen interactions, *Mtb* latency and reactivation (Greenstein *et. al.,* 2005). PknA and PknB in *M. smegmatis* and *M. bovis* BCG have been reported for their function in cell wall generation and growth (Kang *et. al.,* 2005), and for PknB in *Mtb* (Gee *et. al.,* 2012). A recent study has provided an insight about the phosphoproteins present in *Mtb* (Prisic *et. al.,* 2010) which led to the identification of hundreds of Ser/Thr phosphorylation sites ultimately signifying mycobacterial physiology by Ser/Thr phosphorylation. Notably, most of this phosphorylation are specific to various growth conditions faced during infection such as acidic pH, exposure to nitric oxide and hypoxia and additionally supporting the idea of Ser/Thr phosphorylation mechanism in response to various environmental cues.

PknK, one of those *Mtb* STPKs that is deficient with a transmembrane segment, the other being PknG. Therefore, these two STPKs are predicted proteins located on cytoplasm (Prisic & Husson, 2014). As compared to the intracellular kinase domains, the extracellular domains other STPKs (nine transmembrane) does not share any sequence resemblance, indicating that PknK is predicted to bind and respond to distinct extra-cytoplasmic molecular signals. PknK is also reported to phosphorylate VirS (a transcription factor) in *in vitro* manner (Kumar *et al.,* 2009). Virulent *Mtb* H37Rv has shown greater *pknK* regulatory

expression as compared to avirulent strain. PknK protein was also found abundant in stationary versus log phase cells grown in broth culture *in vitro* (Malhotra *et al.,* 2010) whereas, the Δ*pknK* strain was found to grow to higher levels during stationary phase, and the cells of the mutant strain were shorter than wild type. These analyses have suggested the role of PknK in regulating translation in relation to growth and environmental conditions. Furthermore, the environmental signal for PknK activity is currently unknown. PknK is reported to regulate the gene expression involved in cell wall synthesis and lipid metabolism, clearly confirmed that the cellular abundance of PknK activity in *Mtb* reduces mycobacterial growth (Malhotra *et al.,* 2010).

TCSs comprise of the necessary skeleton of environmental sensing in bacteria and provide adaptation to variable environmental conditions. PrrA is a DNA-binding regulatory protein, considered as an essential TCS, required for early phase intracellular replication of *Mtb* (Mishra *et al.,* 2017). Though it is known to be important, the mechanism of PrrA mediated signalling is not well understood. The binding of PrrA on the promoter DNA and its consequent activation is cumulatively controlled via dual phosphorylation of the protein. *Mtb* PrrA/B has been identified essential for the viability. The role of PrrA phosphorylation was studied in an aspartate and a non-aspartate media which confirmed that slow growing mycobacteria might have employed an exceptional mechanism by engaging dual phosphorylation of the response regulator protein to confer its survival inside macrophages (Mishra *et al.,* 2017). The essential role of TCSs in next to STPKs might suggests the involvement of the genes in basic physiological processes other than the pathogenesis. That would further provide important novel information for understanding unique regulatory mechanism used by pathogenic mycobacteria to survive in the unfavorable environment of macrophage during its infection cycle.

3. MATERIALS & METHODS

3.1. Bacterial strains, Chemicals & Media

E. coli DH5-α and *Escherichia coli* artic were grown in (LB) broth supplemented with ampicillin (100 mg/ml) and gentamycin (50 mg/ml), used for clone maintenance and expression of protein respectively. Vector used for cloning was pPROEX-HTa (Lab stock) and pGEX-4T2 (Lab stock). For a range of experimental studies, chemicals were procured from Merck, HiMedia, India & Sigma Co., USA. The composition of various chemicals & buffers is mentioned in Appendices. *Mycobacterium tuberculosis* H37Ra was used for *in vivo* study.

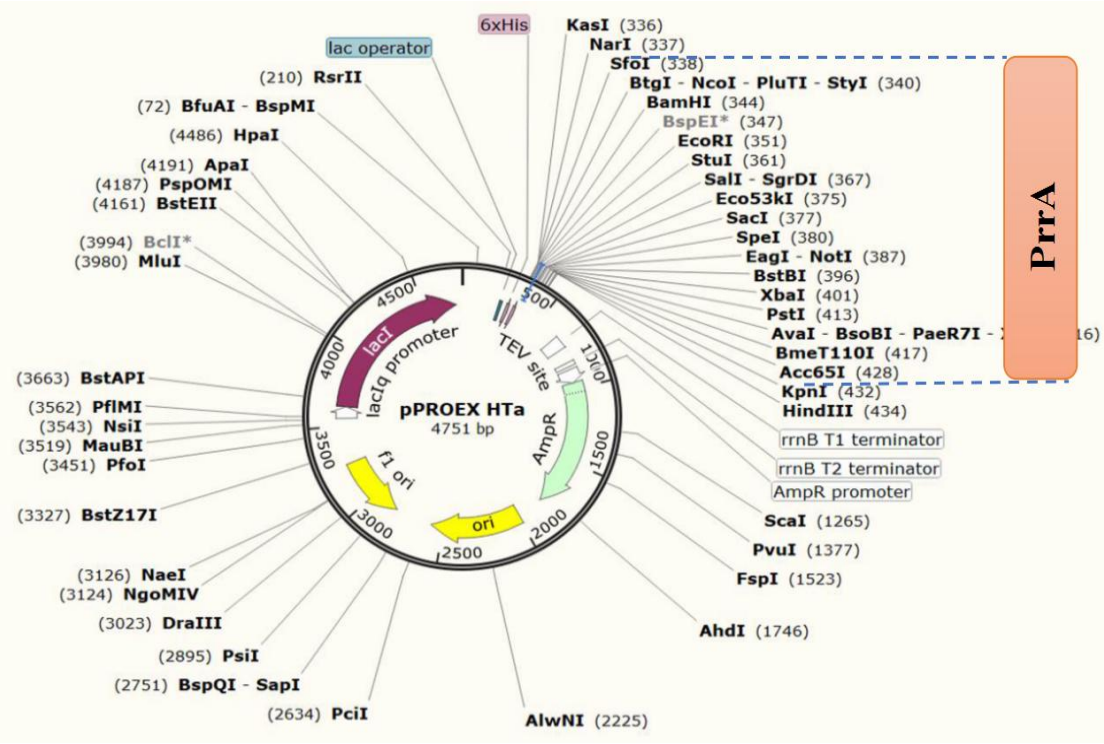


Figure 3.1 (a) pPROEX HTa where *prrA* was cloned.

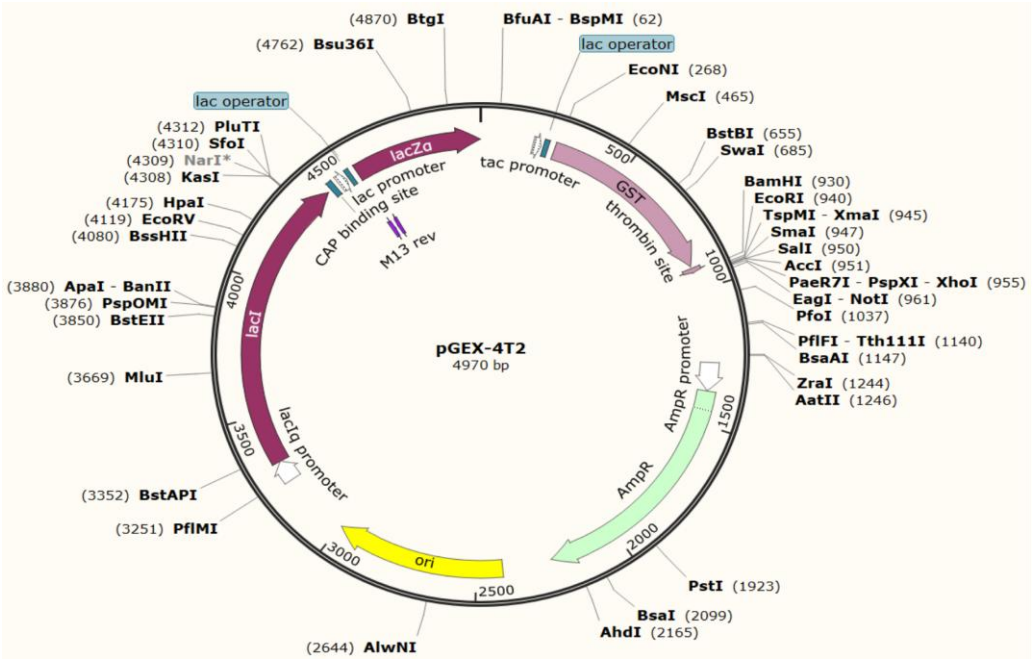


Figure 3.1 (b) pGEX-4T2 where *pknK* was cloned.

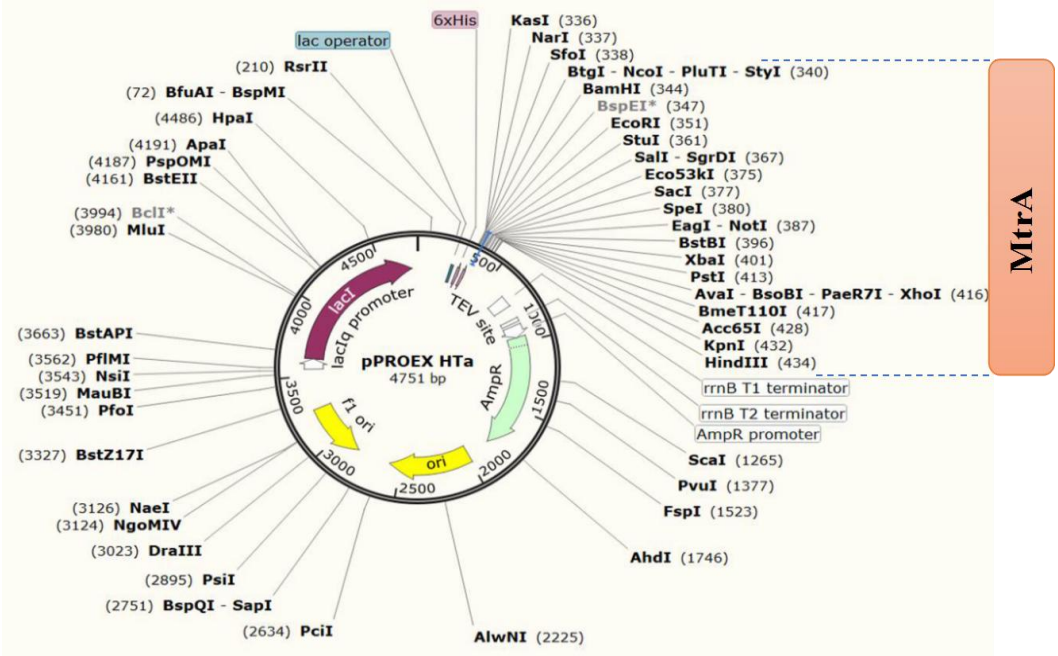


Figure 3.1 (c) pPROEX HTa where *mtrA* was cloned.

3.2. Plasmid isolation

The pure bacteria strains were inoculated in sterile LB (3-5 ml) supplemented with specific antibiotic and incubated at 37^{o}C for overnight. The grown cells were transferred into a micro centrifuge tube and harvested @ 5000 rpm for 5 min at RT. The resulted supernatant was discarded and pellet was further used for plasmid isolation. To the pellet, 200µl of Solution-I

(See Appendix) was added, mixed thoroughly by tapping the tube or pipetting for several times and kept for incubation on ice for 3-5 min. Followed by this, 300µl of Lysis Buffer (See Appendix) was added and contents were mixed by gently in inverting the tube 4-5 times and kept again for incubation on ice for 3-5 min. 300µl of Solution-III (See Appendix) was added and the contents were mixed by inverting the tube for 4-5 times. The tube was incubated on ice for 3-5 min. 450µl of chloroform ($CHCl_3$) was added and centrifuged @ 12000 rpm for 15 min at RT. The resulted supernatant was transferred to a fresh sterile labeled 1.5 ml micro centrifuge tube. 2µl of RNaseA (10mg/ml) was added to the supernatant and gently mixed by inverting the tube 4-5 times and incubated at 37 ^oC for 2-3 hrs. Later, 450µl of $CHCl_3$ was added and centrifuged @12000 rpm for 3 min at RT. The resulted aqueous layer was transferred into a clean sterile tube and $CHCl_3$ step was repeated. 650µl of isopropanol was added to the aqueous layer. The tube was gently mixed by inverting the tube for 4-5 times and centrifuged @ 12000 rpm for 20 min at RT. The supernatant was discarded. To the pellet, 500µl of ice-chilled 70% ethanol was added and centrifuged @12000 rpm for 5 min at 4^oC. The supernatant was carefully removed and the tubes were left open on the bench to allow all residual ethanol to evaporate. The final pellet was resuspened in 20µnuclease free water (NFW). This DNA was stored in -20^oC for further analysis. Quality of the isolated DNA was checked in agarose gel electrophoresis (discussed in 2.4) and quantification was done using appropriate method.

3.3. Agarose Gel Electrophoresis

To visualize the isolated plasmid, agarose gel electrophoresis was conducted. Agarose gels are normally used in a concentration of 0.7% to 1.5 % based on the expected size of DNA fragment need to be separated. Gently 0.8% of agarose solution was made in electrophoresis buffer (SB) (See Appendix). The mixture was heated in a microwave oven until the agarose got dissolved. After cooling of the gel, ethidium bromide (EtBr) solution was added to a final concentration of 0.5 µg/ml. The gel solution was mixed thoroughly by gentle swirling and poured into the electrophoresis casting unit. An appropriate comb was placed (0.5-1.0 mm above the casting unit) for making wells for sample loading and the gel was allowed to polymerize completely (20-45 min at room temperature). A small amount of electrophoresis buffer was poured on the top of the gel, and then the comb was carefully removed without disturbing the wells. The gel was placed in the electrophoresis tank in a submerged condition and the tank was filled with 1X SB electrophoresis running buffer

(See Appendix). To the DNA samples, a loading dye (See Appendix) was added to a final concentration of 1:5. Very carefully the DNA samples were loaded into the wells using a disposable micropipette. This entire system was connected with a power source with a voltage of 1-5 V/cm was applied and bubbles formed at the anode and cathode end resulted due to the same. Within few minutes, the bromophenol blue (a constituent of loading dye) migrated between the wells from cathode towards anode end which acted as a tracking component. The DNA sample was allowed to migrate simultaneously from the wells into the body of the gel till 3/4 of the gel. After sufficient migration, the voltage source was turned off; the gel was removed from the tank and placed onto a Gel Doc^{TM} (Biorad, USA). EtBr present in the gel stained the DNA molecular and it was visualized under ~300-nm.

3.4. Preparation of Competent cell by $CaCl_2$ heat shock method

Single colony of appropriate bacterial strain *E. coli* (freshly revived glycerol stock on medium plate) was inoculated in a 5ml sterile LB and incubated at 37°C, 150 rpm overnight. 1% started culture was inoculated in 100ml sterile LB and kept for incubation as per above mentioned condition. Once the optical density (OD_{600}) reached to 0.4-0.6, the culture was placed on ice for 30 min. The culture was then harvested aseptically with a precooled rotor at 3500 rpm for 10 min at 4^{o}C. Supernatant was discarded and the pellet was resuspended in ice-chilled sterile 0.1 M $CaCl_2$ and kept again in ice for 30 min. Harvesting the culture and resuspension step was repeated twice. The culture was finally harvested at 3500 rpm for 10 min at 4^{o}C. Supernatant was discarded and the pellet was resuspended in 2 ml chilled 0.1 M $CaCl_2$ containing 15% glycerol. Aliquots of 100µl was made in sterile micro centrifuge tubes and stored in -80^{o}C.

3.5. Transformation of the recombinant DNA into a suitable host

The competent cells stored in -80°C were allowed to thaw on ice for 10-15 mins. 2-3µl of plasmid pPROEX-HTa carrying *prrA* and pGEX-4T2 carrying *pknK* was added to 100 µl of competent cells and incubated on ice for 30 mins. Another negative control competent cell was used which was devoid of any plasmid. The tube was then given a heat shock at 42^{o}C for 90 seconds and immediately placed in the ice afterwards for 5 mins. Then 800 µl of prewarmed sterile LB was added to the tube and incubated for 1 hour at 37^{o}C. The tube was then harvested at 5000 rpm for 5 mins. Followed by this, ~700 µl of supernatant was discarded and ~100 µl of supernatant was used to resuspend the cells. The cells were then

spread plated on LB agar plate containing appropriate antibiotic (Ampicillin 100 mg/ml) and incubated at 37^{o}C for overnight.

3.6. Protein Purification

3.6.1. Over-expression of recombinant protein PknK & PrrA

Recombinant over-expression plasmids were transformed in *E. coli* artic strain and incubated at 37^{o}C. The starter culture was prepared using a single positive transformed colony in a 10-ml sterile LB [with 10µl Ampicillin (100 mg/ml) and 5µl Gentamicin (50 mg/ml)] and incubated at 37^{o}C. 1% of the active grown culture was inoculated further into 400 ml 2XYT media (See Appendix) containing 400µl of Amp (100 mg/ml) & 200µl Gen (50 mg/ml) and incubated at 37^{o}C for 4 h. Once OD_{600nm} ~ 0.5, 0.2-1 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) was added to the culture for inducing expression and allowed this induction at 16^{o}C for overnight. The induced culture was then harvested @ 3500 rpm for 10 min at 4^{o}C. The supernatant was discarded and the pellet was resuspended in lysis buffer (See Appendix). 10µl of 100mM PMSF (phenylmethane sulfonyl fluoride) was also added and sonicated for 15 min with an amp 25%, pulse 3 sec ON, 2 sec OFF. The cell lysate was harvested at 12000 rpm for 30 min at 4 ^{o}C. Supernatant was used for purification.

3.6.2. GST-fusion protein purification

The resulted supernatant from over expression step (3.6.1) was transferred to a new sterile microcentrifuge tube and kept in ice until use. The ethanol was allowed to flow out from GST-fusion column (usually ethanol is kept inside column when it is not in use). The column was washed 3 times with sterile MQ. To pre-equilibrate the column, 2 ml of lysis buffer (See Appendix) was added on top of bead and settled for 45 min. Column was centrifuged at 2000 rpm for 5 min at 4^{o}C. After complete washing, the supernatant (previously kept in ice) was added to the column; column was kept in rotor and incubated at4^{o}C for overnight. Then the column was centrifuged @ 2000 rpm for 10 min at 4^{o}C, discarded the supernatant followed by washing. The elution buffer was added to the column and incubated in ice for 60 min. Then it was centrifuged @ 2000 rpm for 5 min 4^{o}C and the supernatant was collected in the microcentrifuge tube. Same process was repeated thrice to collect the elution.

3.6.3. His-tag protein purification

The resulted supernatant from over expression step (3.6.1) was transferred to a new sterile microcentrifuge tube and kept in ice until use. The ethanol was allowed to flow out from Ni^{+2}-NTA column (usually ethanol is kept inside column when it is not in use). The column was washed 5 times with sterile MQ. 2 ml of His lysis buffer (See Appendix) was added on top of bead and settled for 30 min and allowed to flow out. The supernatant containing the protein was loaded into the Ni^{+2}-NTA column pre-equilibrated with native lysis buffer at 4°C. After 45 minutes of incubation with intermittent shaking, the unbound proteins were discarded as flow through followed by washing the column with wash buffer.

The nozzle of the column was opened for flow through and washed it with wash buffer (See Appendix) for 7 times. After that 500µl of elution buffer was added on top and keep the column remain undisturbed for 15 min. The elution was collected and the step was repeated until 5 elutions.

3.7. Dialysis

3.7.1. Dialysis Bag preparation

The dialysis bag was cut as per appropriate size and washed with sterile Milli-Q for five times. The bags were incubated with 0.3% sodium sulfide (Na_2S) for 1 min at 50°C. Then it was washed twice with warm water (60°C) for 2 min, followed by another washing with 0.2% v/v sulfuric acid (H_2SO_4). It was rinsed with sterile water and stored in 50% Ethanol.

3.7.2. Protein dialysis

The forceps & scissors (dipped in ethanol) were used to take out and cut the dialysis bags. The bag was put in sterile MQ water and was clamped from outside. The elution fraction was filled into the membrane and the other end was sealed with a clamp. The dialysis bag was kept in hanging position in a sterile beaker. The beaker was filled with dialysis buffer (See Appendix) and a sterile magnetic bead was put inside the beaker. The entire beaker was covered with an aluminum foil was kept stirred for overnight at 4 °C. The protein was taken out and stored in a sterile micro-centrifuge tube at -20°C.

3.8. Estimation of protein (Bradford assay)

Protein estimation was carried out by a standard protocol using Bovine Serum Albumin (BSA) as a standard (Bradford, 1976). This assay is used to determine the total protein content (concentration) of a sample. This method is based on the proportional binding of the dye Coomassie to proteins which is captured in a calorimetric calculation. The absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when it binds to the protein. 100 µl of 1X Bradford reagent (See Appendix) was added to the sample and mixed. This mixture was kept in dark for 5 min and the OD_{595nm} was calculated afterwards. Different concentrations of BSA was prepared and used for standard curve.

3.9. Protein analysis by SDS-PAGE

SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis) is a standard technique for qualitative analysis of protein nature. The strongly anionic detergent SDS is used in combination with a reducing agent and heat to dissociate the proteins before they are located onto the polyacrylamide gel. The denatured polypeptides bind SDS and become negatively charged and migrates through the well. Different proteins carried different molecular mass. Lighter polypeptides migrate faster than the heavy polypeptides. 12% resolving gel pH 8.8 (See Appendix) and 5% stacking gel pH 6.8 (See Appendix) were used for this PAGE analysis. Purified proteins were verified through 12% SDS-PAGE.

3.10 *In vitro* kinase assays of STPKs

The purified STPKs were incubated in the PIPES buffer (100 mM PIPES, pH 7.0, 80 mM NaCl and 20 mM $MgCl_2$) containing 1-2µCi of [γ-^{32}P] ATP for 10 minutes at 25°C. Autophosphorylated STPks were transferred onto mutated SK and WT RR for *in vitro* substrate phosphorylation. The reaction mixture was incubated at 25°C for 30 minutes, followed by adding of 5X SDS sample loading buffer. Then the reaction mixture was heated at 95°C for 10mins and proteins were separated by SDS-PAGE. After electrophoresis, the gel was wrapped in cellophane sheet and exposed to a phosphor imaging plate for 2-12 hrs followed by scanning with Typhoon 9210 imager. The gel was then stained with Commassie R_{250} to visualize the protein bands.

3.11 Western blotting

Western blotting (also known as protein blotting or immunoblotting) is a rapid and sensitive assay for detective and characterization of proteins. Western blotting technique exploits the inherent specificity by polyclonal or monoclonal antibodies. It is an analytical method wherein a protein sample is electrophoresed on an SDS-PAGE and electrotransferred onto nitrocellulose membrane.

The nitrocellulose membrane was cut in advance according to the size of the gel and the blot paper was soaked in transfer buffer (See Appendix). The nitrocellulose membrane was soaked in methanol for 10 min and in transfer buffer for 5 min. The pre-soaked blot paper was placed onto the platinum anode of the casting unit. Then pre-wetted nitrocellulose membrane was placed on top of the blot paper. Carefully the gel was placed on top of the nitrocellulose membrane, aligning the stack as perfect as possible. Another piece of soaked blot-paper was placed on top of the gel. Make sure that no air bubbles are present in between this setup. Then the cathode plate was placed onto the stack and the latches were pressed with the guide posts without disturbing the nitrocellulose membrane. The complete unit was kept at 20 V for 45 min in RT. Afterwards, the membrane was kept in blocking solution (See Appendix) for 1 h at room temperature in slow agitation (agitation was done using rocker). The membrane was then incubated with primary antibody (See Appendix) for overnight at 4^0C and then washed with PBST (See Appendix) for 3 times in fast agitation for 5 mins each. The membrane was then incubated with secondary antibody for 1 hour at room temperature in slow agitation. The membrane was again washed with PBST for 3 times in fast rocker for 5 mins each. Then the reaction mixture containing enhanced luminol and Peroxide solution (1:1) was prepared in a vial under dark condition. To the nitrocellulose membrane, this reaction mixture was added and placed in a gel doc system to capture the specific protein bands as an image using appropriate software.

3.11.a Specificity detection of anti-PrrA antibody

The protein PknK, cold ATP, PIPES buffer and MQ was added in a sterile microcentrifuge tube and incubated for 1 hour at 25 ^{o}C for auto-phosphorylation. Then Prr A was added and incubated for 2 hour at 25 ^{o}C for phosphorylation. Then the sample (PrrA phosphorylated,

and MtrA) was loaded into the designated wells of the polyacrylamide gel. The gel was allowed to run in an electric pulse at 120 V, followed by western blot.

3.11.b Sensitivity study of anti-PrrA antibody

Different concentration of PrrA was loaded onto 12% SDS-PAGE to detect the sensitivty of anti-PrrA antibody. The gel was then transferred onto nitrocellulose membrane followed by blocking and developing.

3.12. *In vivo* study via western-blot

0.047 gm of Middlebrook 7H9 media (BD Difco^{TM}) in 10 ml distilled water supplemented with 20 µl glycerol was prepared and autoclaved. Post autoclaving, 1 ml of sterile OADC Growth Supplement (See appendix), 25µl of sterile Tween-80 was added to the media. 1 ml of *M. tuberculosis* H37Ra seed stock was inoculated and kept it under incubation for 7 days at 37^{o}C in 150 rpm. Meanwhile Middlebrook 7H9 media supplemented with 0.2% (v/v) glycerol, 10% (v/v) albumin–dextrose–catalase (ADC) and 0.025% (v/v) Tween-80 with different pH (4.4,6.6 & 7.2) and starvation condition (Middlebrook 7H9 media supplemented with 0.025% (v/v) Tween-80 with normal, pH 6.6) were made. The pH 7.2 was adjusted using MOPS buffer and the final volume of all the media was 100 ml. All these media were sterilized as per instruction. 1 ml of primary culture was inoculated and kept at 37 ^{o}C for 10 d in 150 rpm. Post incubation, the log phase culture was harvested at 4000 rpm for 10 min at 4 ^{o}C. The pellet was resuspened and washed with 1X PBS thrice at 4000 rpm for 15 min at 4 ^{o}C. The resulting pellet was resuspened in H37Ra lysis buffer (See appendix) and was bead beating for 5 times at 400 rpm for 30 s each. The lysate was harvested at 12000 rpm for 40 min at 4 ^{o}C. The resulting supernatant was transferred into a sterilized micro-centrifuge tube for further protein estimation by Bradford Assay (as per section 3.8), SDS PAGE (as per section 3.9) and western blotting (as per section 3.10).

4. RESULTS

Mtb is major intracellular pathogen of tuberculosis. Extensive studies have been dedicatedly undergone to determine the signaling network, cellular dynamics, formation of granulomatous and molecular metabolism of this host-pathogen interaction. As of now, few researches have been targeted towards the illustration of bacterial signaling network in infected host. A lot of information were illustrated from the mycobacterial proteome during infection through *in vitro* studies in infected cell lines (Fisher *et al.*, 2002; Mattow *et al.*, 2006) and under hypoxic stress (Rosenkrands *et al.*, 2002; Sherman *et al.*, 2001; Voskuil *et al.*, 2004; Wayne & Sohaskey, 2001). Moreover, further studies have also been shown on nutrient starvation (Betts *et al.*, 2002) and non-replicative persistence (NRP) (Cho *et al.*, 2006) have also advantage *Mtb*'s life style. The *in vitro* studies on TCSs and STPKs of *Mycobacterium tuberculosis* is being established in Dr. Saini's Lab at IISC, Bangalore, India (Personal Communication). This present study aimed to analyze the environmental effects like pH variation and starvation condition on PrrA in *in-vivo* model.

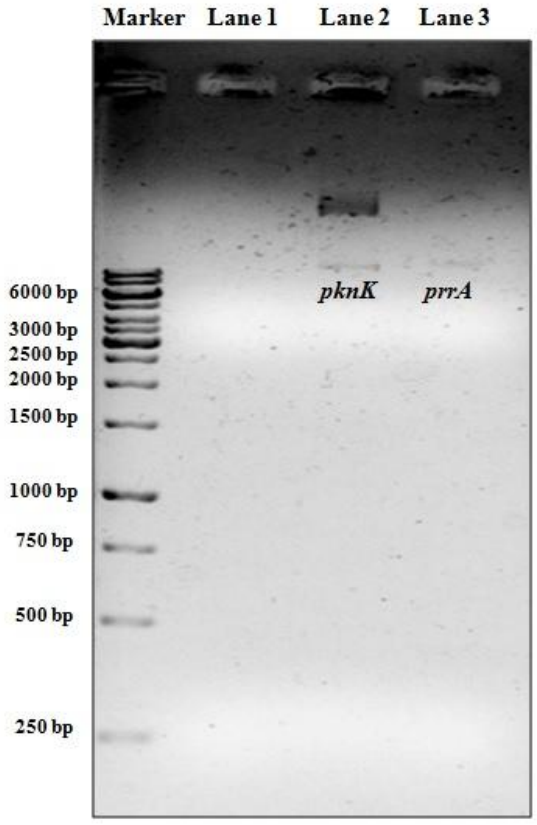


Figure 4.1 Plasmid isolation: pPROEX-HTa carrying (*prrA*) and pGEX-4T2 carrying (*pknK*)

prrA & *pknK* genes from *Mtb* were cloned in pPROEX-HTa & pGEX-4T2 respectively and transformed into *E. coli* DH5 alpha (cloning maintenance) and arctic (expression) strains. Both of these plasmids containing these genes were isolated successfully for quality check in agarose gel electrophoresis (Figure 4.1). As depicted in this picture, pPROEX-HTa + *prrA* was 5.5kb and pGEX-4T2 + *pknK* was 5.7kb in size. These transformed cells were used for protein expression followed by protein purification.

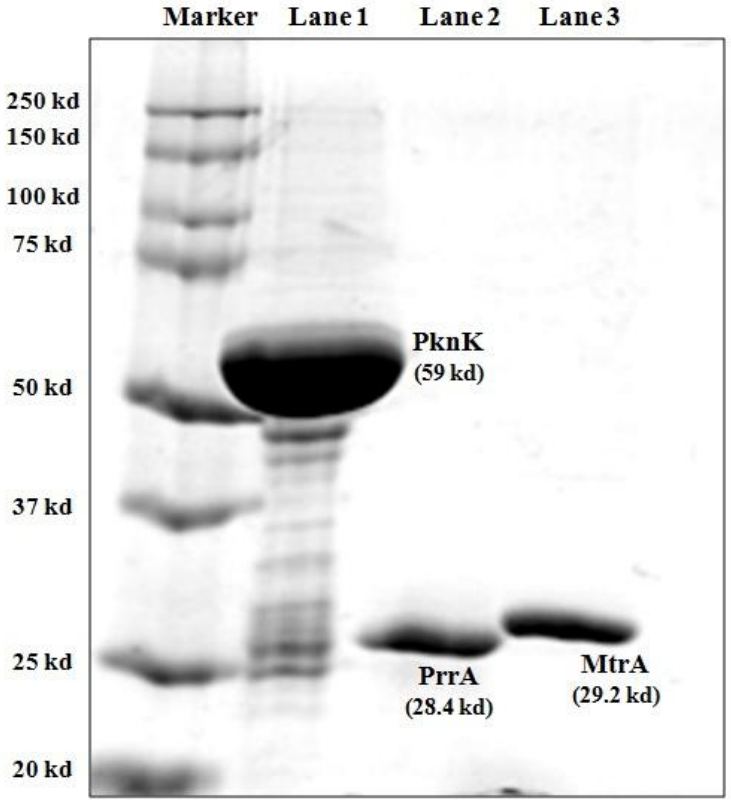


Figure 4.2 Specificity analysis of purified protein PknK, PrrA and MtrA

The transformed cells were grown in 2XYT and protein purification was performed using GST-tag fusion (for PknK) and His-tag purification system (PrrA and MtrA) (as mentioned in the section 3.6.2 & 3.6.3). The protein analysis was checked on a 12% SDS-PAGE electrophoresis at (120V, 20m Amp). It was confirmed that PrrA and MtrA were specific response regulators. The size of these proteins PknK, PrrA and MtrA was found to be 59 kDa, 28.4 kDa and 29.2 kDa respectively (Figure 4.2).

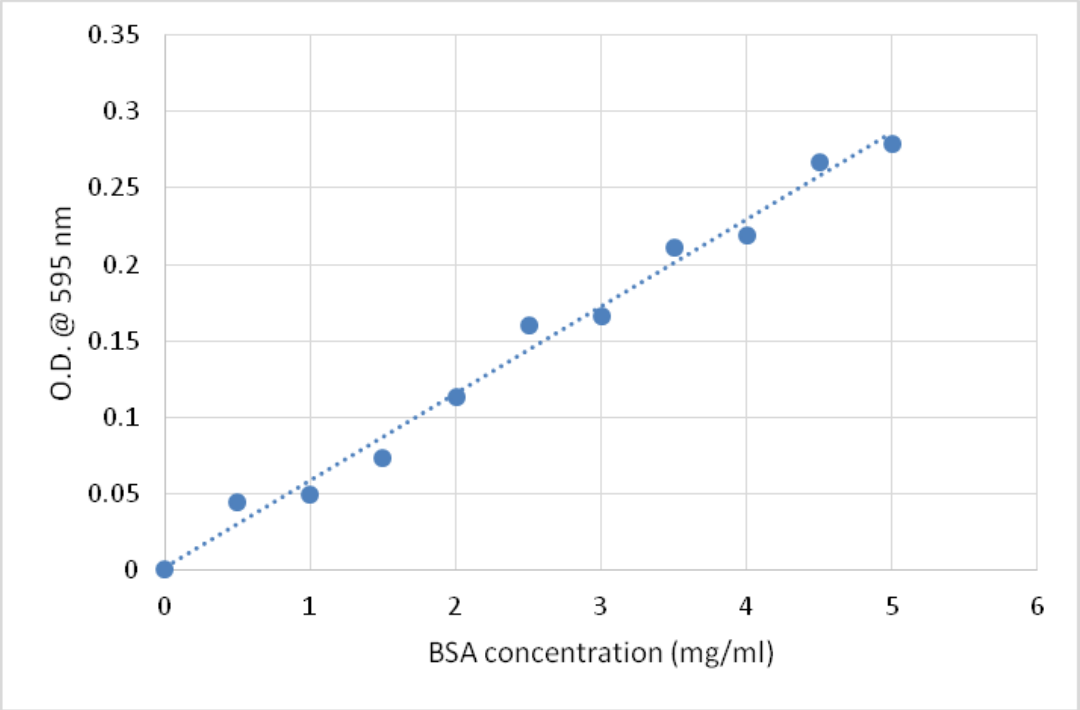


Figure 4.3 BSA standard curve using Bradford Assay method to determine the concentration of purified protein.

The protein concentration was estimated through Bradford method. Standard curve was done using variable concentration of BSA (0-5mg/ml) (Figure 4.3). Protein concentration was determined using y = mx+c, where y = O.D of definite protein at 595 nm and m = 0.056. Concentration of purified proteins were as followed: PrrA 0.92 mg/ml, MtrA 0.77 mg/ml and PknK 1.056mg/ml.

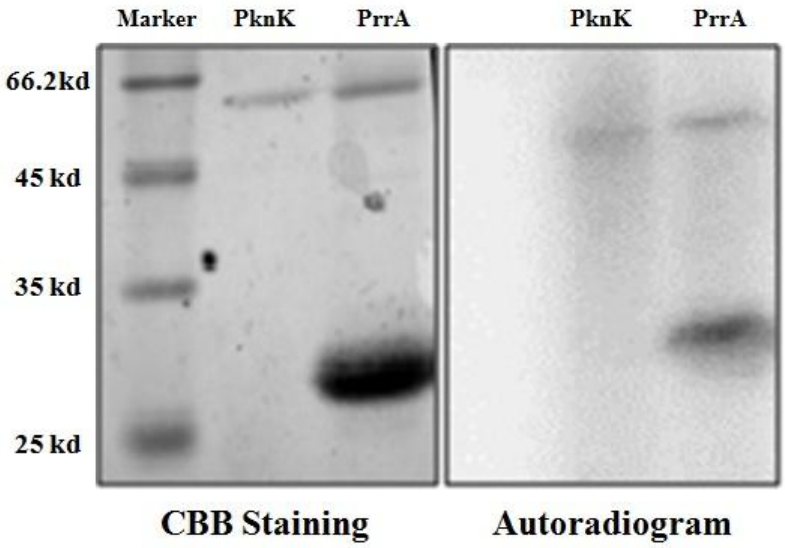
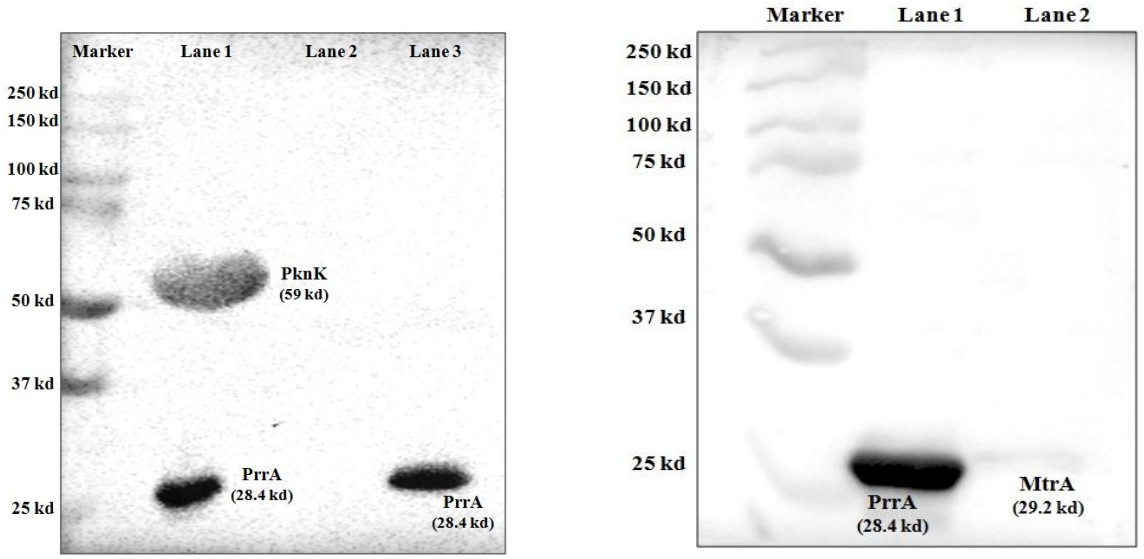


Figure 4.4 *In vitro* cross talk study between PknK and PrrA



(a) (b)

Figure 4.5 Specificity detection of anti-PrrA~P antibody. (a) Western blot is showing image of protein against anti-PrrA antibody Marker, lane 1 contains Phosphorylated PrrA and lane 3 contains unphosphorylated PrrA. (b) Marker, lane 1 unphosphorylated PrrA and lane 2 unphosphorylated MtrA.

The phosphorylation event was analyzed for PrrA and PknK proteins. In *in vitro* reaction, the PknK was autophosphorylated at 25^{o}C within an hour; and phosphorylated PrrA (RR of TCS) at 25^{o}C within 4 hours (Fig. 4.4).

In vitro study showed that there was cross talk between two signaling pathway, antibody was generated against PrrA~P through GST pull down method followed by immunization of Rabbit. The specificity of antibody was checked (Fig.4.5). The same phosphorylation reaction of PknK followed by substrate phosphorylation of PrrA~P was done using cold ATP. The reaction was stopped using 1X-sample loading buffer. This antibody can detect both of the phosphorylated and unphosphorylated PrrA (Fig. 4.5a). To check the specificity of antibody against other RR, MtrA (RR of TCS) also ran on 12% SDS-PAGE along with PrrA. Antibody detected specifically PrrA (Fig.4.5b).

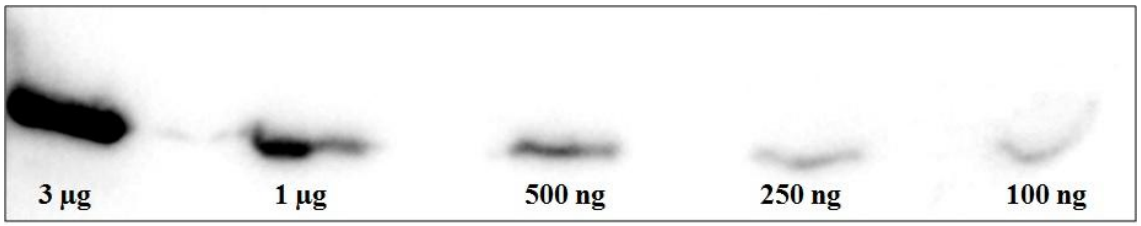


Figure 4.6 Optimization of unphosphorylated PrrA concentration

To optimize the ideal concentration of PrrA for further experiments, the same was standardized using various concentrations (3µg, 1µg, 500ng, 250ng, 100ng) of PrrA. It was found that 500 ng was an ideal concentration of PrrA for further *in vivo* work as depicted in figure 4.6. Higher and lower concentration were of PrrA was not significant as it could have given nonspecific signals in further experiments.

Earlier, the antibody was tested against PrrA and MtrA was performed. It was found that the generate antibody was specific to PrrA as it binds to only PrrA, not to MtrA (Figure 4.5b). This analysis had helped us to work further *in vivo* model.

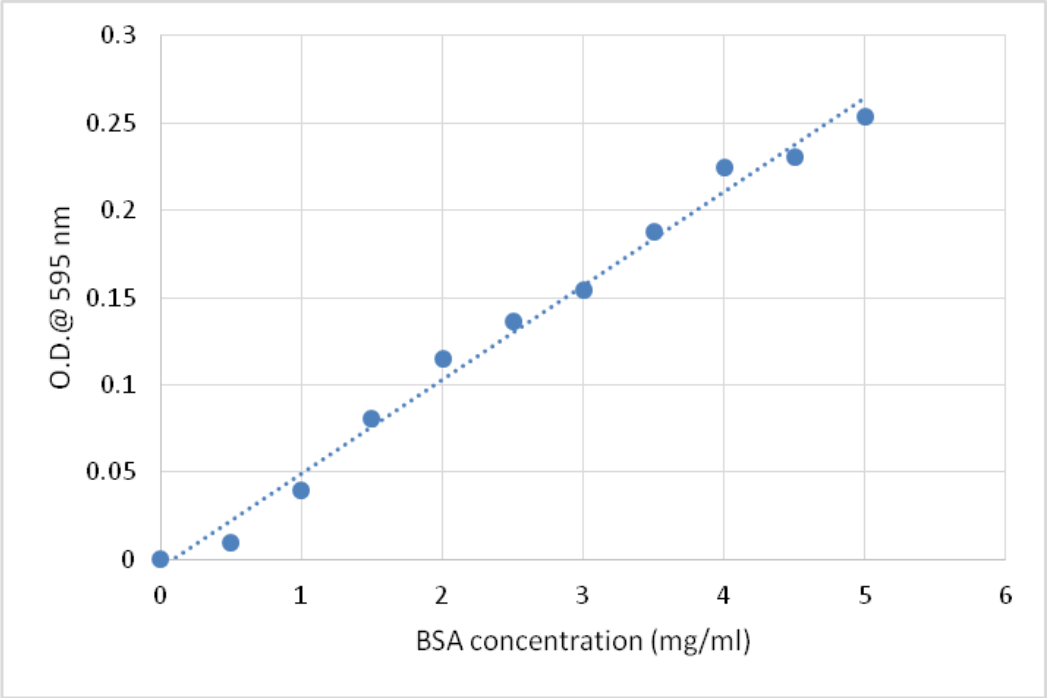


Figure 4.7 BSA standard curve using Bradford Assay method to determine the concentration of H37Ra cell lysate

The protein concentration was estimated through Bradford method. Standard curve was done using variable concentration of BSA (0-5mg/ml) (Figure 4.7). Protein concentration was determined using y = mx+c, where y = O.D of definite protein at 595 nm and m = 0.052. Concentration of H37Ra cell lysate: 0.801 mg/ml

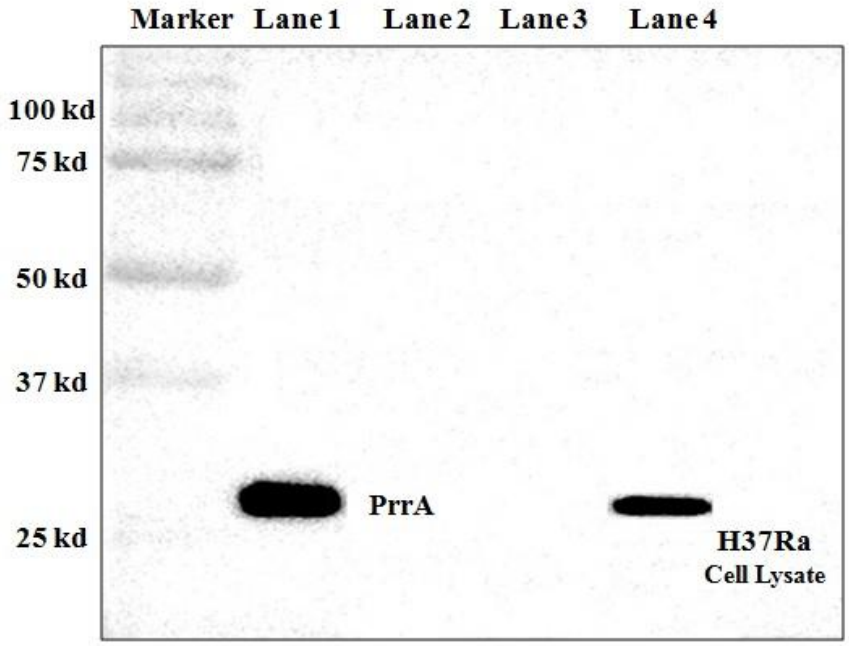


Figure 4.8 *in vivo* detection of pure PrrA and H37Ra lysate

Pure PrrA and *Mtb* H37Ra cell lysate, both were subjected to check the structural integration. It was found that there were no such structural changes (in terms of quality) in both the form (Figure 4.8) as these two protein were found intact and specific in both the conditions.

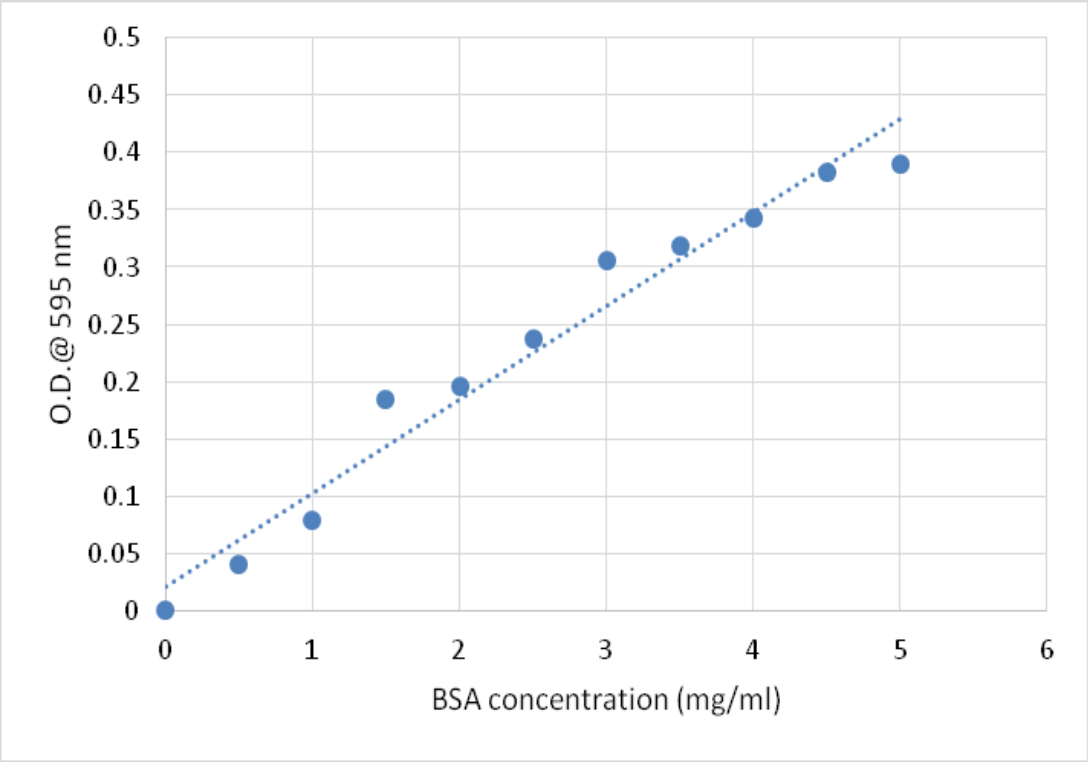


Figure 4.9 BSA standard curve using Bradford Assay method to determine the concentration of H37Ra cell lysate at different environmental conditions.

The protein concentration was estimated through Bradford method. Standard curve was done using variable concentration of BSA (0-5mg/ml) (Figure 4.9). Protein concentration was determined using y = mx+c, where y = O.D of definite protein at 595 nm and m = 0.088. Concentration of H37Ra cell lysate at different environmental conditions are pH 4.4: 0.397 mg/ml, pH 6.6: 0.622 mg/ml, pH 7.2: 0.359 mg/ml and starvation: 0.161 mg/ml .

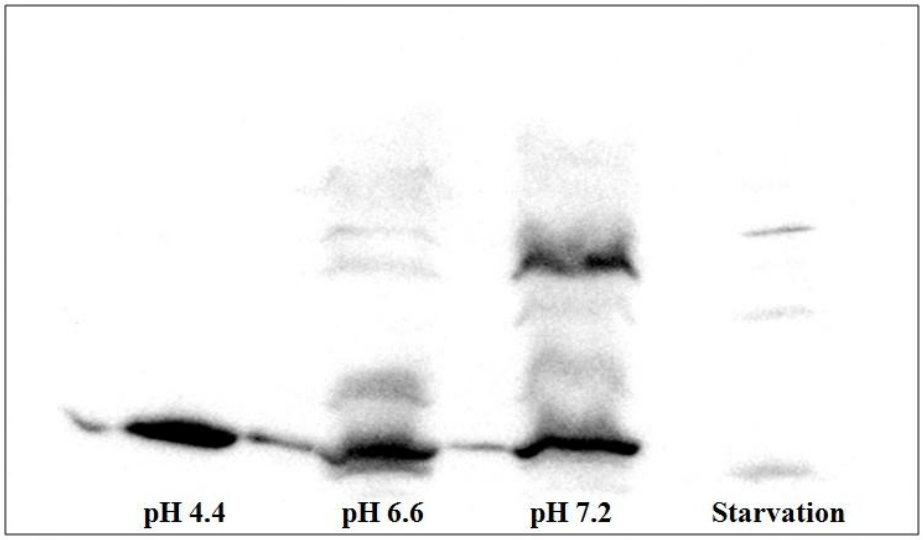


Figure 4.10 Effect of pH 4.4, 6.6 &7.2 and Starvation condition on PrrA

The PrrA protein was analyzed in various pH (4.4, 6.6 and 7.2) and starvation condition (nutrient limiting). It was found that, PrrA was not altered w.r.t to its structure in response to varied pH (Figure 4.10). A very faint band of PrrA signifying a less nutrient condition was observed in case of starvation condition.

5. DISCUSSION

Mtb has the ability to infect and survives within the macrophages and could persist within the granulomatous lesions in host for decades. Adapting in a challenged environment is a peculiar character in pathogens which is mostly mediated by activation of transcriptional regulators, STPKs, extra-cytoplasmic function (ECF) sigma factors, and TCSSs. In this environment, *Mtb* is exposed to a varied environmental stress like nutrient limitations, hypoxia, reactive oxygen species (ROS), reactive nitrogen intermediates (RNI), altered pH etc. Being a very smartest pathogen, *Mtb* possess ~190 regulatory proteins which include 11 TCSSs, 5 RRs, and 2 SKs (Tekaia et al., 1999; Cole et al., 1998). *Mtb* is considered to be in a condensed state of replication and metabolic activities as part of the chronic infection. Several *in vitro* studies have been explored the environmental conditions post infection and have decoded the information on the bug's response to intracellular pH, starvation and hypoxia. These studies have undoubtedly given insights to many untold stories of the pathogen; but the overall outlook seems to be incomplete. While these experiments have afforded great insight, the picture remains incomplete. Therefore, an approach was made to study the combined effects of varied environmental conditions with response to *Mtb* in *in vivo* conditions.

The laboratory adapted strains of *Mycobacterium tuberculosis* are usually used for both *in vitro* and *in vivo* studies. However, it is unknown, whether the heterogeneity of *Mtb* stocks used by various laboratories can result in different outcomes or not. This project aims to detect the endogenous overexpression of PrrA proteins and to understand the expression levels within the host during its survival. It was observed anti-PrrA~P showed response in presence of PrrA~P and PrrA, as well as in presence of PknK (purified protein); this might have happened due to presence of mixed antibody in antiserum collected from rabbit. The possible reason behind generating mixed antibody was, while performing GST Pulldown method to separate PrrA~P from PrrA certain amount of these proteins could have remained back. To check whether the antibody generated is nonspecific it was probed against non-specific antigen like MtrA, no response of anti-PrrA~P as found against MtrA. In head to head studies, we tried to decipher the *in vivo* expression analysis of PrrA with the laboratory adapted strain H37Ra could have any effect on environmental factors like pH, starvation etc. The *in vitro* phosphorylation of PrrA was already established in the lab. For *in vivo* analysis, *Mtb* H37Ra cell lysate was prepared from different pH i.e. 4.4, 6.6 &7.2 and from starvation conditions. It was found that, there was no significant changes in PrrA from cell lysate as

compared to the pure PrrA in both pH and starvation condition. Starvation condition had shown a less expression of PrrA due to the nutrient limiting factors like absence of ADC and glycerol for exponential growth. Therefore, it was concluded that, PrrA metabolism does not depend upon any varied pH and starvation conditions.

6. CONCLUSION

Tuberculosis (TB) is always a matter of concern for the human society because of its high infectivity rate which almost covers one third of the global population. This is the second most common infectious disease caused from a single infectious agent after HIV. It severely affects the social and economic status of infected person. Though this bacterium has been discovered a long ago but the effective therapeutic treatment and vaccines are not available till today. Science has developed in due course to combat TB, but the complexity of *Mycobacterium tuberculosis* always created a major problem in drug discovery. The rise in MDR and XDR strains of *Mtb* has made this bug an extremely clever pathogen. Moreover, co-infection of *Mtb* with HIV has threatened our capability to control tuberculosis. The progress in making new drugs with enhanced efficacy against *Mtb* is the core area to work on for governing control over this deadly disease. The TCSSs of *Mtb* have been suggested as new targets for making possible anti-TB drugs. Along with these, the attenuated mutants have been recommended for making possible.

Our study has validated an *in vivo* model in *Mtb*, where a STPK PknK is necessary for its transcriptional regulator PrrA. Along with that it was also proved that PrrA gets phosphorylated in the presence of PknK. This study also validated that varied pH and starvation does not have any implications of *Mtb* survival within host. Recent development in *Mtb* TCSS research and the utilization of present state-of-the-art techniques like transcriptomics, DNA microarrays etc. have proven the fact that TCSSs expression is being a noteworthy role in accelerating the effective adaptation of *Mtb* in various environmental cues like nutritional starvation, hypoxia, altered pH, NO and CO, exposure etc. within host. Significantly, continuous evaluation of this TCSSs of *Mtb* would be giving insights to novel therapeutic approach or suitable vaccine candidates against this deadly disease.

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8. APPENDIX

1. Luria Bertani (LB) Broth

Composition	g/L
Casein Enzymic Hydrolysate	10.00
Yeast Extract	5.00
Sodium Chloride	10.00
Final pH	7.5± 0.2

*Autoclave the media at 15 psi for 30 minutes at 121°C.

2. Luria Bertani (LB) Agar

Composition	g/L
Casein Enzymic Hydrolysate	10.00
Yeast Extract	5.00
Sodium Chloride	10.00
Final pH	7.5± 0.2
Agar	15.00

*Autoclave the media at 15 psi for 30 minutes at 121°C.

3. Antibiotic Solutions

(a) Ampicillin: Stock concentration was made 100 mg/ml in nuclease free water (NFW) and filter sterilized. Stored in -20^{o}C until use.

(b) Gentamicin: Stock concentration was made 50 mg/ml in nuclease free water (NFW) and filter sterilized. Stored in -20^{o}C until use.

4. SDS-PAGE Loading Dye 5X

Composition	Conc
Tris-Cl (pH 6.8)	0.225 M
Glycerol	50%
SDS	5%
Bromophenol Blue	0.05%
Dithiothreitol (DTT)	0.25 mM

5. SDS-PAGE Gel Running Buffer

Composition	for 1000 ml
Glycine	14.4 g
Tris	3.03 g
SDS	1 g

*Add these above components and make up the volume up to 1000 ml.

6. Staining solution for SDS-PAGE

Composition	**for 1000 ml**
Coomassie Brilliant Blue	0.25%
Distilled water	50%
Methanol	40%
Glacial Acetic Acid	10%

*Add these above components and mix it well. Filter it using a whatman filter paper.

7. Staining solution for SDS-PAGE

Composition	**for 1000 ml**
Distilled water	50%
Methanol	40%
Glacial Acetic Acid	10%

8. 30% Acrylamide Stock Solution

Composition	**for 100 ml**
Acrylamide	29 gm
N, N'-methylenebisacrylamide	1 gm

* Add these two components and mix it well. Make up the volume to 100 ml.

9. 2XYT Buffer

Composition	**for 400 ml**
Yeast extract	4 gm
Tryptone	8 gm
NaCl	4 gm

10. Solution I for Plasmid Isolation

Composition	**Conc for 100 ml**
Glucose	50 mM
Tris-Cl (pH 8.0)	25 mM
EDTA (pH 8.0)	10 mM
deionized water	For volume make up

* Glucose solution is filter sterilized using 0.22µm

11. Solution II for Plasmid Isolation

Composition	**Conc for 100 ml**
NaOH	0.2 N
SDS	1% (w/v)
deionized water	For volume make up

12. Solution III for Plasmid Isolation

Composition	Conc for 100 ml
Potassium acetate	5 M
Glacial acetic acid	11.5 ml
deionized water	For volume make up

13. Lysis Buffer for His-Tag Protein purification

Composition	Conc for 100 ml
Tris	50 mM
NaCl	500 mM
Imidazole	10 mM
Glycerol	10%
MQ water	For volume make up

14. Wash Buffer I for His-Tag Protein purification

Composition	Conc for 100 ml
Tris	50 mM
NaCl	500 mM
Imidazole	20 mM
Glycerol	10%
MQ water	For volume make up

15. Wash Buffer II for His-Tag Protein purification

Composition	Conc for 100 ml
Tris	50 mM
NaCl	500 mM
Imidazole	20 mM
Glycerol	10%
MQ water	For volume make up

16. Elution buffer for His-Tag Protein purification

Composition	Conc for 100 ml
Tris	50 mM
NaCl	500 mM
Imidazole	250 mM
Glycerol	10%
MQ water	For volume make up

17. 10X TG Buffer

Composition	Conc for 100 ml
Tris	250 mM
Glycine	20 mM
pH	8.3
MQ water	For volume make up

18. Dialysis Buffer I (for 4 hours)

Composition	Conc for 100 ml
Tris	50 mM
NaCl	50 mM
Glycerol	10%
DTT	0.1 mM
MQ water	For volume make up

19. Dialysis Buffer II (for overnight)

Composition	Conc for 100 ml
Tris	50 mM
NaCl	50 mM
Glycerol	50%
DTT	0.1 mM
MQ water	For volume make up

20. Lysis Buffer for GST-Tag Protein purification

Composition	Conc for 100 ml
Tris-HCL (pH 7.4)	50 mM
NaCl	150 mM
EDTA	1 mM
Glycerol	10% v/v
PMSF	1 mM
MQ water	For volume make up

21. Wash Buffer for GST-Tag Protein purification

Composition	Conc for 1000 ml
Na_2HPO_4	1.44 gm
KH_2PO_4	0.24 gm
KCl	0.2 gm
NaCl	8.0 gm
pH	7.2

***** It was made in 1X PBS

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22. Elution Buffer for GST-Tag Protein purification

Composition	Conc for 100 ml
Tris-HCL	50 mM
DTT	1 mM
$MgCl_2$	5 mM
Glutathione	15 mM

23. Dialysis Buffer (in case of GST-Tag Protein purification)

Composition	**Conc for 100 ml**
Tris HCL (pH-7.4)	50 mM
NaCl	50 mM
Glycerol (100%)	100 ml
DTT	20 µl
MQ water	For volume make up

24. Transfer Buffer

Composition	**Conc for 100 ml**
10X TG	10 ml
Methanol	20 ml
MQ water	For volume make up

25. 10X PBS Buffer

Composition	**for 1000 ml**
NaCl	80 gm
KCl	2 gm
Na_2HPO_4	14.4 gm
KH_2PO_4	2.4 gm
pH	7.4
deionized water	For volume make up

26. PBST Buffer (500ml)

Composition	**Conc for 500 ml**
PBS buffer	495.5 ml
Tween-20	0.5 ml

27. Blocking buffer

1X PBST with 5% w/v BSA

28. H37Ra Strain Lysis Buffer

Composition	**for 100 ml**
Tris-HCL (pH-7.4)	50 mM
NaCl	150 mM

29. OADC Growth Supplement (per vial)

Composition	**per vial**
BSA	2.50 g
Dextrose	1.00 g
Catalase	0.002 g
Oleic acid	0.025 g (only for plate)
Sodium chloride	0.425 g
Distilled water	50 mL

30. 20X SB Buffer

Composition	for 1000 ml
Boric acid	61.83 gm
NaOH	40 gm
Distilled water	for make up the volume

31. PIPES buffer (10X)

Composition	for 100 ml
NaCl	0.58 gm
PIPES (piperazine-N,N′bis[2-ethanesulfonic acid])	3 g
NaOH	1M
$MgCl_2 \cdot 6H_2O$	0.2 g
Distilled water	for make up the volume

* filter sterilized using 0.22µm

32. MOPS buffer (10 X)

Composition	for 10 ml
MOPS free acid, Ultrapure	0.209 gm
pH	7.0 (adjusted with 10N NaOH)
Distilled water	for make up the volume

* filter sterilized using 0.22µm

33. Resolving Gel for SDS-PAGE (12%)

Composition	for 10 ml
Distilled water (dH_2O)	3.3 ml
30% Acrylamide Mix	4.0 ml
1.5 M Tris (pH 8.8)	2.5 ml
10% SDS	0.1 ml
10% APS (ammonium persulfate)	0.1 ml
TEMED	0.004 ml

34. Stacking Gel for SDS-PAGE (5%)

Composition	for 3 ml
Distilled water (dH_2O)	2.1 ml
30% Acrylamide Mix	0.5 ml
1.0 M Tris (pH 6.8)	0.38 ml
10% SDS	0.03 ml
10% APS (ammonium persulfate)	0.03 ml
TEMED	0.003 ml