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Role Of The Pkna And Pknb Kinases In Mycobacterium Tuberculosis

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ROLE OF THE PKNA AND PKNB KINASES IN *MYCOBACTERIUM TUBERCULOSIS*

by

TRIPTI ANANDAN

DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

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Approved by:

Advisor

Date

DEDICATION

To everyone who made this possible

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Chapter 1

Introduction

Tuberculosis

Tuberculosis (TB) is an infectious disease caused by the pathogen *Mycobacterium tuberculosis*. Scientists have been able to discover the DNA of *M. tuberculosis* in Egyptian mummies as early as 2000 B.C. (1). It is identified by various names in the historical texts, few of those terms being “consumption”, “wasting away”, and “the white plague”, etc. (2). As tubercle bacilli can be spread through air, TB is termed a contagious disease. The developing nations of the world are at utmost risk of being easily infected due to their high population size and poor living conditions. Although a lot of improvements have taken place in the therapeutic regimen for TB by improving diet, housing, education, and sanitation conditions there seems to be bigger challenges with increasing cost of drugs and the length of therapy (3).

Since the early 20th century Bacillus Calmette-Guérin (BCG) vaccine has been used to prevent TB infection. BCG vaccine consists of live attenuated tubercle bacilli that are aimed to act as a prophylactic measure to prevent TB. The duration of protection rendered by the vaccine seems to be around 15 years. However, BCG is ineffective to prevent adult pulmonary tuberculosis as well as reactivation of latent TB in individuals who carry the bacteria without displaying any symptoms of the disease (4). It is estimated that 2.2 billion people are infected worldwide annually and approximately 1.3 millions deaths occur in HIV-negative people and approximately half a million deaths in HIV-positive patients (5,6). Another pressing issue in the battle against TB is the increase in

the number of strains that are resistant to first line and second line TB drugs. These multi-drug resistant (MDR) and extensively drug resistant (XDR) strains of *M. tuberculosis* curtail the potency of chemotherapy associated with TB and thus defining a global emergency to overcome these roadblocks in TB treatment (7).

The infection begins through the inhalation of aerosols containing *M. tuberculosis* released from the lungs of an infected individual by coughing or intaking contaminated food. The disease typically affects the lungs but can also spread to other parts of the body. Upon inhalation of mycobacterium-laden aerosol, the microbe is engulfed by alveolar macrophage. During this stage the majority of the bacteria is destroyed, but some that resist the harsh intracellular condition are able to survive, multiply, and infect neighboring macrophages (8). In general, the disease has two patterns. In the first kind, known as the primary TB, the mycobacteria are engulfed by the host alveolar macrophage that is then carried to hilar lymph node in order to control the infection. During the primary infection, the initial focus of infection is identified as a tiny granuloma (tubercle) in the lung tissue also called Ghon's complex. The primary TB is seen mostly in children and majority of these cases resolve as the infant begins to develop immunity (80% of cases) whereas sometimes the bacteria enter into a state of dormancy and remains undetectable by the host immune system. A secondary TB is a pattern of the disease that arises as a result of reactivation of previous infection, exogenous reinfection, or gradual progression of primary TB into chronic form mainly due to deteriorating health status. The reactivation of latent TB infection can happen when the immune system is weakened due to another infection, drug abuse, or immune compromise that enable the dormant bacilli to break out of latency and enter an actively dividing state. In

the secondary TB the granulomatous inflammation is severe and widespread along with extensive cavitation and lung tissue damage. The symptoms of active disease include persistent fevers, night sweats, severe cough, sputum with blood, fatigue, and weight loss (9).

M. tuberculosis can remain latent inside the human host for decades and the immense reservoir of infected individuals created by this microorganism has a potential to develop active tuberculosis (TB) sometime in their life time (10). Approximately, 50% of latent infection can lead to reactivation of TB (Fig.1.1). According to the world health organization, it is estimated that about one-third of the world's population is infected with latent TB. Latency of *M. tuberculosis* is described as a state of the bacillus where it is metabolically minimally active or in a non-replicating stage for undefined periods of time. A person with latent TB is not yet ill with the disease and despite being a reservoir for the pathogen cannot transmit the disease to other people. Therefore, latent TB infection is marked by the presence of the tubercle bacilli in the body without displaying any symptoms, or radiographic, or bacteriologic evidence of TB disease (11). Fig. 1.1 describes different stages of TB infection in humans.

Diagnosis of latent TB and active TB can be difficult although the general guidelines observed for TB diagnosis entails Mantoux tuberculin skin test (TST) and interferon-gamma release assays (IGRAs). Although TST cannot distinguish between TB infection and TB disease, a more confirmatory screening is accomplished by chest X-ray studies that can provide details as to the location of an infiltrate, cavities and/or fluid in the lungs. Finally, a confirmatory laboratory test known as acid-fast bacilli smear (AFB smear) is performed in order to microscopically analyze the presence of mycobacteria in

sputum obtained from patients with active TB. Apart from these early diagnostic measures, an additional test that is made mandatory recently is the drug susceptibility test carried out with initial isolates in order to determine what should be the preferred anti-tuberculosis regimen.

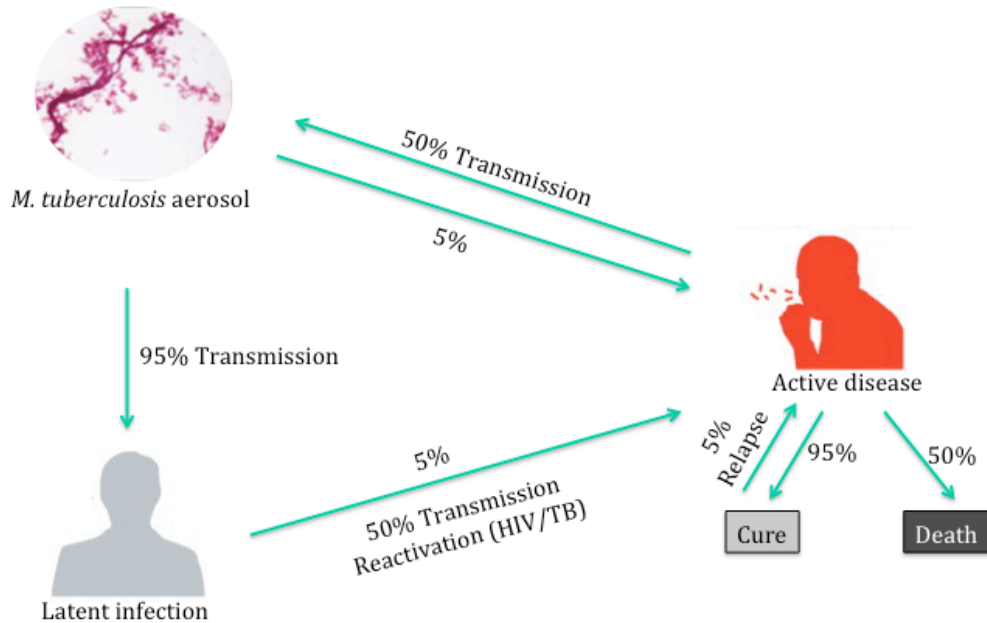


Fig. 1.1: Stages of *M. tuberculosis* infection. Inhalation of aerosol can either lead to active or inactive (latent) TB. Latent TB carriers have the potential to develop infectious, active TB anytime during their lifetime. Progression from latent to active TB can be triggered by immune suppression, especially during human immunodeficiency virus (HIV) infection. Treatment of drug-susceptible (DS)-TB (denoted by asterisk), is successful 95% of the cases, whereas 5% relapse. If untreated (denoted by two asterisks), chance of mortality is approximately 50%. Image adopted from (11).

Even though the bacterial burden in the latent TB infection is small compared to active TB disease, the treatment period for the latent TB is no different from the active TB therapy: both require around 6-9 months of treatment period. For latent TB infection, two drugs are administered; isoniazid and rifampicin. The treatment plan for active TB comes in two phases; an initial phase of 2 months, followed by a continuation phase of 4-

7 months (total of 6 to 9 months of treatment). It is very important that people under TB medication follow the treatment regimen without any lapses as it paves way for drug-resistance strains of mycobacteria. TB caused by drug-resistant strain is very hard and expensive to treat. Such long treatment plans also come with their own downsides of which the major threat is development of drug-resistance strains of *M. tuberculosis* (12,13). Therefore, better understanding of human host-*M. tuberculosis* interaction and *M. tuberculosis* physiology is believed to be crucial to develop more efficient drugs against TB.

Host-Pathogen interaction

As discussed earlier, tubercle bacilli are capable of both active and dormant survival inside host macrophage cells. To establish infection *M. tuberculosis* possesses a plethora of virulence factors whose expressions are regulated with progressing infection state concluding with latent infection and/or an eventual resuscitation from dormancy.

Invading *M. tuberculosis* comes face to face initially with macrophages and dendritic cells (DC). These two innate immune cells are able to recognize pathogen components through highly conserved pattern recognition receptors (PRRs), including Toll-like receptor (TLR) family players. This recognition initiates the activation of both DC and macrophage, leading to phagocytosis and internalization of the bacilli in the phagolysosome complex, where they undergo lysis. The DC and macrophages travel to the mediastinal lymph nodes, in order to present bacterial lipids and peptide antigenic determinants to CD4⁺ and CD8⁺ T cells via MHC-I and MHC-II, leading to T cell activation and clonal proliferation. These T cells return to the site of primary infection

along with the macrophages and secrete cytokines such as $IFN\gamma$, IL-12, and $TNF\alpha$ (14).

There are many defense mechanisms adopted by *M. tuberculosis* to resist host immune responses. One mechanism is dependent on the inhibition of phagosome-lysosome fusion inside infected macrophages. Without successful degradation by macrophage, the bacterium is able to adapt to intracellular conditions that can have two outcomes. One, where the infected macrophage is utilized as a home to replicate and releases more microbes that then infect other cells, and two, where the bacteria can shift to a metabolically inactive stage known as latency (15). It has been shown that mycobacterium prevents lysosomal maturation (lysosome-phagosome fusion) with the help of the eukaryotic type Ser/Thr kinase PknG (16,17).

Despite the importance of latency in the epidemiology and pathology of tuberculosis, it is yet not fully understood how *M. tuberculosis* alternates between a latent state and active replicating state inside the host. To achieve latency, however, mycobacterium must be able to respond to specific host signals and regulate its metabolism and growth. For this, the *M. tuberculosis* genome contains a wide variety of signaling molecules including eleven "eukaryotic-type" Ser/Thr protein kinases. Our lab has been studying two of these kinases, PknA and PknB, and their substrate proteins. The PknA/B signal transduction network is essential for the survival of *M. tuberculosis* (18). In addition, some of their known substrates (such as Wag31 and proteasome) are also known to be essential. Thus, understanding the roles of these kinases and their substrates will provide important information regarding the disease process in tuberculosis and thus can aid in the development of potent TB drugs.

Cell envelope of *Mycobacterium tuberculosis*

M. tuberculosis is a Gram-positive, aerobic, rod-shaped bacterium measuring 2 – 4 μm in length. Its cell envelope has a complex structure made up of peptidoglycan, mycolic acid, and arabinogalactan that are highly hydrophobic (19). The success of *M. tuberculosis* as one of the deadliest human pathogens can be easily attributed to its cell envelope make-up as it is this unique protective layer in *Mycobacterium* that guard the cell against the host's immune repertoire (20). Upon entry into the host system, the bacillus encounters several different cellular environments that it must adapt partly by changes in the cell envelope's physical and chemical composition. These physiological variations in cell envelope contribute to changes in the host-pathogen immunologic interplay that in turn plays a part in the success of the pathogen. A lot of focus has been given to understand the cell envelope composition of *M. tuberculosis* in hopes to determine the physiology and the changes that occur during host-pathogen interplay (21). Studies have been aimed at identifying different components of cell envelope and antigens present on the bacterial surface or secreted by the bacterium to determine virulence factors associated with cell envelope (22).

The chemical structure and composition of the mycobacterial cell wall and envelope have been extensively studied and reviewed (23,24). David Minnikin proposed the definitive model of the structural composition of the mycobacterial cell envelope in 1982 (25) (Fig.1.2). In this model, the cell envelope is similar to the lipid bilayer where mycolic acids are covalently linked to the cell wall associated arabinogalactan layer in order to form the inner leaflet. This inner leaflet is found to have the lowest permeability to organic chemicals in comparison to overall cell wall. The outer leaflet of cell wall is

believed to contain extractable phospholipids, glycolipids, peptidolipids, and mycosides (25,26). The mycolic acids (MA) are long chain (C60-C90) fatty acids and comprise a core structure covalently attached to an inner complex of arabinogalactan and peptidoglycan (mAGP) (26). Mycolic acids accounts for ~ 60% of the dry weight of the bacillus. The highly complex meshwork architecture of peptidoglycan (PG) is accomplished by having repeating units of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) or N-acylglycolic acid (MurNGlyc) that is also cross-linked to the arabinogalactan (AG) layer (27). The mycolic acids are also associated with other essential virulence factors, trehalose monomycolate (TMM) and trehalose dimycolate (TDM) (28). Apart from these elements, other lipid and glycolipid components form the cell wall framework also play a role in pathogenesis of the bacillus (29).

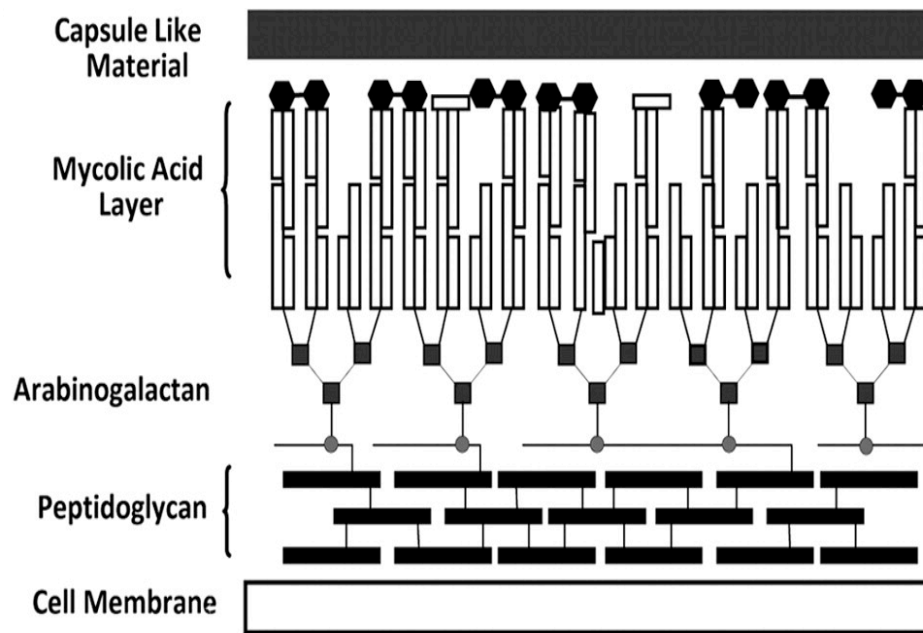


Fig. 1.2: The lipid cell wall composition of Mycobacteria and the structure of TDM. The arabinogalactan is associated with mycolic acid that forms a second membrane-like structure adjacent to the peptidoglycan-linked cell wall. Above arabinogalactan layer is a thick mycolic acid layer made of complex lipid profile containing trehalose monomycolate (TMM), trehalose and trehalose dimycolate (TDM). The outermost layer containing capsule-like material consists of lipopolysaccharide and protein lies on top of mycolic acid layer. This unique cell wall composition is a crucial aspect of mycobacterial resistance to several drugs used for treating TB infection. (Image taken from (30)).

The extremely hydrophobic waxy surface of mycobacterial cells is formed by the transfer of mycolate to the arabinogalactan chains by a group of mycolyl transferase enzymes called fibronectin binding proteins (Fbp) that are also known as antigen 85 complex (31). In *M. tuberculosis*, there exist four Fbp proteins (FbpA, FbpB, FbpC1, and FbpBC2), whereas *M. smegmatis* has five Fbp proteins (FbpA, FbpB, FbpC, FbpD, and FbpE) (32). Among these various types of Fbp proteins, FbpC2 contributes the most to the mycolic acid content of the cell wall in *M. tuberculosis*, and FbpA, apart from its role as trehalose dimycolate (TDM) synthesizing enzyme, has been suggested to be required for virulence (33,34). FbpB, which also has the mycolyl transferase activity, has been suggested to have a role in the pathogenesis of TB by disseminating within the host through fibronectin interactions on the mucosal surface and elevating expression of interferon-gamma leading to a reduced phagocytosis by monocyte-derived-macrophages (35).

Signal transduction in *M. tuberculosis*

The success of a pathogen depends on its capacity to quickly sense and adapt in the host in order to subvert the constantly varying hostile host environment. To support this aspect of an organism's adaptive nature, proteins that are capable of sensing and transducing external and internal cues are essential. Protein kinases and their cognate

phosphatases are enzymes that perform the important role in this kind of signal transduction pathways by catalyzing reversible protein phosphorylation (36,37). Many of those protein kinases respond to an external signal by autophosphorylation of themselves and then relay the signal to substrate protein(s) by trans-phosphorylation. Depending on the type of signal transduction system and the kinase involved, phospho-modification can occur on different amino acid residues such as serine (Ser), threonine (Thr), tyrosine (Tyr), histidine (His), or aspartate (Asp) (36,38).

In bacteria, two-component systems (TCS) and the 'eukaryotic-type' Ser/Thr protein kinases (STPKs) mediate signal transduction network. *M. tuberculosis* has eleven two-component systems and eleven STPKs (18). Bioinformatic analysis has revealed that TCS and STPKs are conserved to a varying degree across the genus *Mycobacterium* (39). In bacteria TCS typically comprises of a membrane-bound sensor protein kinase (histidine kinase) that senses a specific environmental stimulus and a response regulator protein that relays the cellular response. The sensor kinase undergoes autophosphorylation on histidine residue and subsequently modulates the function of response regulator by phosphorylating aspartic acid residues. TCS is commonly found in prokaryotes with some discoveries of their existence also in eukaryotes (40). In eukaryotes, however, STPKs and phosphatases constitute the canonical signal transduction systems. Until recently, it was believed that STPKs are specific to eukaryotes but sequence analyses of bacterial genomes have revealed that they are also present in prokaryotes including *M. tuberculosis* (41). The first STPK discovered in prokaryotes was in *Myxococcus xanthus* (42).

Protein phosphorylation by STPKs occurs on Ser, Thr, or Tyr residue. STPKs

have sequence homology in their kinase domain, which is used to classify STPKs into various STPKs superfamilies (43). The kinase domain contains twelve highly conserved subdomains that fold into a typical bi-lobed catalytic core structure that hides the catalytic active site in a deep cleft sandwiched by the two lobes (18). The N-terminal lobe is smaller compared to the C-terminal lobe and functions to bind and orient the phospho-donor ATP molecule. The bigger C-terminal lobe of kinase domain is involved in substrate binding and transfer of phosphate group (44).

Structure and function of Mycobacterial STPK

The *M. tuberculosis* STPKs contain nine kinases that have a transmembrane domains and remaining two soluble kinases (PknG and PknK) (45,46). These nine transmembrane kinases have an intracellular, N-terminal kinase domain that is linked to an extracellular, C-terminal sensor domain through a single transmembrane helix. The extracellular domain is predicted to interact with signaling ligands and is identified to be a folded structure in PknB, PknD, and PknE. For example, the extracellular sensory component of PknB has four PASTA (Penicillin-binding protein and serine/threonine kinase associated) domains. PASTA domains have low affinity toward beta-lactam antibiotics, possibly due to their similarity to their predicted natural ligand: stem peptides of peptidoglycans (47). Even though the sensor domain structure of PknB and PknD is solved, the various ligands that these kinases bind to are largely unknown (46,48). The kinase domain structures of PknB, PknD, PknE and PknG have been well characterized (45,49).

Hanks *et al.* (1988) described in their elaborate review that the kinase domain in

STPKs consists of approximately 250 amino acid residues spanning 12 conserved subdomains (43,45,50). The catalytic kinase domains can be organized into evolutionary clades based on sequence homology between STPKs of *M. tuberculosis* and other mycobacterial species. PknA, PknB, and PknL belong to the first clade/clade I and are conserved across the mycobacterial genus. Clade II contains PknH, PknE, and PknD, whereas clade III includes PknF, PknI, and PknJ. Clade IV and V are represented by the soluble kinases PknK and PknG (51). Apart from sharing high sequence homology, the three kinases in clade I are suggested to share a common function in cell division and morphology regulation (51,52). The operon containing *pknA* and *pknB* also contains the lone Ser/Thr phosphatase gene, *pstP*, whose product is a substrate of both PknA and PknB in *M. tuberculosis* (53). The start and stop codons in this open read frame (ORF) overlap, suggesting that transcription and translation could be coupled, and that they could have similar expression levels (52).

Like eukaryotic STPKs, *M. tuberculosis* STPKs including PknA and PknB are activated by autophosphorylation of the activation loop (49,54). Autophosphorylation has been identified to occur on T172 and T174 residues in PknA that has been suggested to be important mechanism for activation of the kinase (49,54). Studies indicate, kinase domains are dimeric, with dimerization probably occurring due to extracellular ligand binding to the sensor domain. Additionally, it is predicted that dimerization of kinase domains could be a regulatory mechanism to conduct *trans* or *cis* autophosphorylation of the activation loop leading to a conformational change for the activation of the kinase (55-57).

Phospho-proteome analysis in *M. tuberculosis* has led to the identification of a

large number of STPK substrates to be involved in cell division, morphology, and other important regulatory pathways. Kang *et al.* (2005) showed that *pknA* and *pknB* are expressed preferentially during exponential phase of growth and that overexpression of these two kinase genes results in growth and morphological defects in *M. tuberculosis*. They also showed that Wag31, a homolog of cell division protein DivIVA in other bacteria, is phosphorylated by PknA/B *in vivo* and *in vitro* and that its phosphorylation status determines cell shape in *M. tuberculosis* (52). Overexpression studies, especially with *pknB* led to reduced growth rate and viability of *M. smegmatis* that was not seen in the presence of kinase defective mutants. When *pknA* was overexpressed in *M. smegmatis* or *M. bovis* BCG, the cells appeared elongated with branched morphology. On the other hand, *pknB* overexpression in either mycobacterial species gave rise to bulging rods. Our lab has further provided evidence to suggest PknB phosphorylates Wag31, which then affects polar peptidoglycan synthesis and cell morphology (58). In another study conducted by Dasgupta *et al.* (2006) it was shown that PknA phosphorylated Penicillin-Binding-Protein A (PbpA) that is known to play a role in cell wall synthesis (59). Furthermore, *in vivo* studies based on *M. tuberculosis* survival inside macrophage also provided evidence of PknB as an important factor for growth during macrophage infection (60). These *in vitro* and *in vivo* data implicate that PknA and PknB are key regulators of cell shape, cell division, and growth.

Proteasome

Cellular proteins in general have a limited life span and their degradation is controlled by several types of protein degradation units. Intracellular degradation of protein in eukaryotes is dependent on two processes, namely the lysosomal pathway and

ubiquitin-dependent proteasomal pathway. Functionally, the lysosomal dependent proteolysis is believed to be less selective, compared to the proteolysis governed by proteasomes (61). This selectivity of proteasome is attributed to the protein called ubiquitin that are covalently linked to proteins marked for degradation (62). The proteasome degrades these ubiquitinated substrate proteins in the cytosol and nucleus of eukaryotes. These ubiquitin molecules are recycled and reused for proteolysis of other proteins (62). Proteasomes have a cylindrical multi-layered organization called 20S core particle (CP) that is sandwiched by cap-like structures called 19S regulatory particles (RP) (63). The active site of the proteasome is hidden in the catalytically active 20S CP and the substrate recognition and delivery into the 20S CP is carried out by the ATPase containing 19S RP (64).

Bacterial proteasomes were discovered only recently when the 20S proteasome was identified in *Thermoplasma acidophilum* (63,65). It was determined that proteasome is essential for this microbe's *in vitro* growth under heat shock conditions (65,66). Archaea and several eubacteria belonging to the order Actinomycetales, such as the genera *Streptomyces*, *Frankia*, *Rhodococcus*, and *Mycobacterium*, also possess 20S proteasomes, but they seem to lack both 26S proteasomes and ubiquitin (67).

Fig. 1.3 is a diagrammatic representation of the mycobacterial proteasome pathway. Unlike eukaryotic proteasome subunit organization, the bacterial 20S CP is made up of a single type of α -subunit and a single type of β -subunit (32,68). In *M. tuberculosis*, proteasome structural organization is predicted to be similar to that of *Rhodococcus species* (63). The β -subunits form two seven-membered ring structure stacked on top of each other, which then form the catalytic core of the proteasome; an N-

terminal Thr in the β -subunit is the catalytic nucleophile (65). The β -subunits is expressed as an unprocessed protein containing N-terminal pro-peptide that is cleaved when half-proteasome assemble into a holo-proteasome structure. On both sides of the double-stacked β -subunit are the seven membered α -subunit ring structures (Fig. 1.3). The proteasome assembly begins when an α -subunit ring structure interacts with a β -subunit ring it leads to the formation of a half-proteasome. Apposition of two half-proteasome leads to cleavage of β -subunit pro-peptide resulting in the formation of a catalytically active holo-proteasome complex (63). In addition to the 20S CP, *M. tuberculosis* also possesses proteasomal accessory factors. *Mycobacterium* proteasomal ATPase (Mpa) is similar in function to the 19S cap found in eukaryotes. Mpa functions to recognize and unfold proteasomal substrates via its N-terminal coiled coil domain. Recognition of proteasomal substrate occurs by tagging the target protein with prokaryotic ubiquitin-like protein (Pup) Proteasome accessory factor A (PafA) Pup is attached to lysine residue in proteasome target proteins with the help of the Pup ligase (69,70).

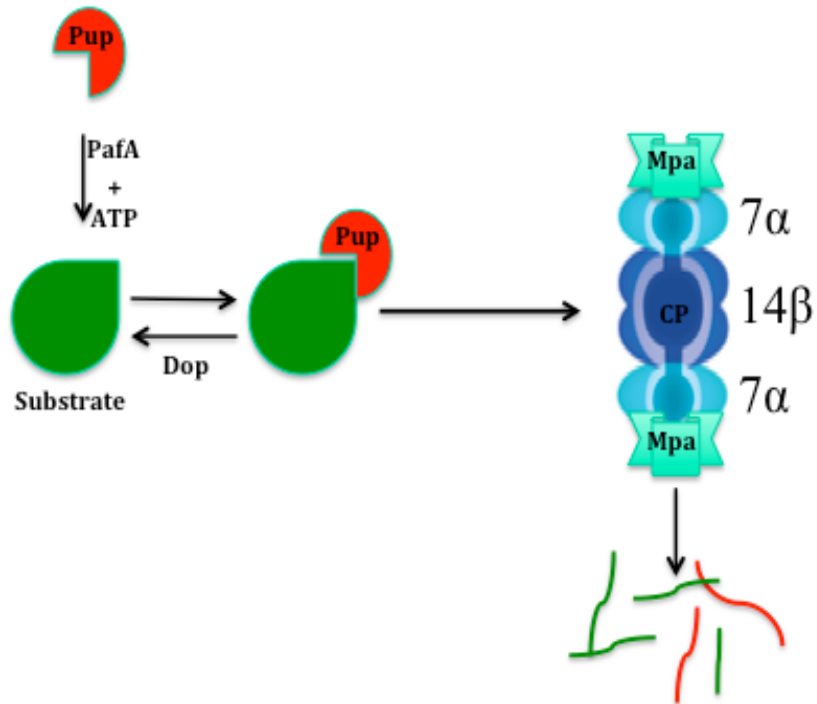


Fig. 1.3: Mycobacterial proteasomal pathway. In mycobacteria PafA is responsible for tagging the substrate protein with Pup for proteasomal degradation. Once pupylated, substrate protein is recognized by Mpa that also helps in its unfolding and entry into the catalytic core of 20S proteasome. Dop is another enzyme that can help in reversing the pupylation step so that the degradation Pup-tag is removed when deemed unnecessary.

In contrast to most bacteria that lack proteasome, *M. tuberculosis* requires proteasome for optimal growth and long-term survival *in vivo* (71). When the genes encoding the *M. tuberculosis* core proteasome, *prcB* and *prcA*, were depleted by conditional silencing, it affected their growth on solid media and *in vivo* survival in mice but not *in vitro* in liquid culture (71). Studies done using mutants depleting proteasome and proteasome associated genes such as *pafA* and *mpa* also suggest that *M. tuberculosis* proteasome plays an important role in nitric oxide resistance and in persistence during chronic mouse infections. During nitric oxide resistance, proteasome system may be responsible for targeted removal of nitrosylated proteins (72). On the other hand, similar

proteasome depletion in *M. tuberculosis* increased survival in response to hydrogen peroxide stress, suggesting that lack of proteasome is beneficial to oxidative stress response in *M. tuberculosis* (71). In Chapter 2, I describe my work on how phosphorylation of the proteasomal subunits by PknA may play a role in oxidative stress resistance by causing a proteasome depletion effect.

Fibronectin Binding Protein5 (Fbp)

M. tuberculosis cell wall has unique characteristics that attribute to the impermeability of most antibiotics. For example, *M. tuberculosis* contains a large amount of lipids, glycolipids like mycolic acid, and arabinogalactan-lipid complex, as described above. This robust and highly impermeable cell wall makeup is also known to play an important role in pathogenesis (73). Additionally, the genes that are responsible for their metabolism and transport are potential virulence factors that have been suggested as targets for drug design (74).

Fibronectin-binding proteins (Fbp) are such enzymes responsible for the production of some of those hydrophobic cell envelope molecules (75). Three independent genes (*fbpA*, *fbpB*, and *fbpC*) encode those Fbp proteins in *M. tuberculosis*, *M. leprae*, and *M. avium*. In *M. tuberculosis* FbpA, FbpB, FbpC2 and FbpC1 (FbpD) comprise the Fbp complex. Except FbpC1, these proteins have dual functions in *M. tuberculosis*; 1) to synthesize trehalose dimycolate and arabinogalactan-mycolate components of the mycobacterial cell wall, and 2) to modulate the immune system during infection by binding to fibronectin (75).

The Fbp proteins are also the major secreted proteins found in the culture filtrates of all pathogenic mycobacterial species examined to date (76). These proteins have molecular weights ranging from 30 kDa to 34 kDa with approximately 40 residues at N-terminal region processed when secreted as matured proteins (77). It has also been found that these proteins are not only secreted but also retained in the cell wall of *M. tuberculosis* (78,79). Their role appears to be redundant in that all of the Fbp complex members possess mycolyl transferase activity required for the biogenesis of trehalose dimycolate (TDM), a dominant structure necessary for maintaining cell wall integrity (Fig. 1.4). The significance of cell wall associated forms of the Fbp proteins is believed to be in fibronectin binding and in turn for the pathogenesis of the bacilli by promoting receptor mediated phagocytosis and adherence to mucosal surface (80). The role of completely secreted FbpB is not yet clear but it has been suggested to be involved in inducing cell-mediated and humoral immune responses in *M. tuberculosis*-infected patients at an early stage of the infectious process (81).

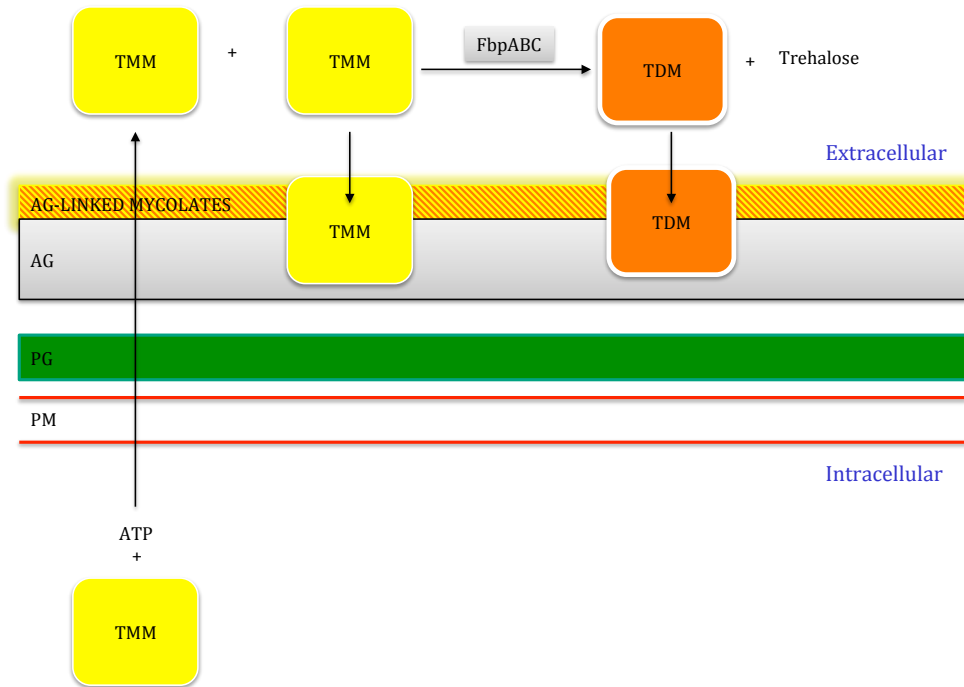


Fig 1.4: Trehalose Dimycolate synthesis pathway in *M. tuberculosis*. 1. Intracellular TMM is transported outside of cell with help of ATP and ABC transporter system. 2. Once outside, mycolic acid is transferred from the 6-OH of one molecule of TMM to the 6'-OH of another TMM molecule, forming trehalose and TDM. TMM and TDM are linked to arabinan in the arabinogalactan (AG) layer outside associated with peptidoglycan (PG) layer lying above plasma membrane (PM)(82).

The three proteins of the Fbp family are expressed at a steady-state ratio of 3:2:1 (FbpB:FbpA:FbpC). 30 kDa FbpB is the most highly expressed of the three, and is also the most secreted protein, responsible for almost 41% of the total extracellular protein in liquid culture (83). In addition, during macrophage infection of *M. tuberculosis*, FbpB is one of the most secreted proteins observed in phagosomal space and on the bacterial cell wall (77). Tang *et al.* (2008) and others have suggested the use of the 30 kDa major secretory protein of *M. tuberculosis* (FbpB) as a vaccine candidate. In another study, it was shown that guinea pigs infected with *M. tuberculosis* aerosols could be protected against TB by vaccinating them with the purified FbpB (84). In Chapter, I will describe

that FbpB is phosphorylated and the phosphorylation of FbpB may play an important role in modulating its secretion and enzymatic activity.

Mycobacterial strains in my thesis work

M. tuberculosis belongs to the family Mycobacteriaceae and human TB is most often caused by *M. tuberculosis* strains, though cases have also been reported due to *M. africanum* and *M. bovis* infection (2). *M. tuberculosis* strains differ in phenotype and virulence, such as Beijing strain being highly virulent, mostly causing extra-pulmonary TB than other strains (85). In 1905, a H37 laboratory strain was isolated from the sputum of a 19-year old chronic pulmonary TB patient, that later dissociated into a virulent strain (H37Rv) and an avirulent strain (H37Ra) (86). It is only the virulent strain H37Rv that is capable of replication inside human macrophage although both the strains have been successfully cultured *in vitro* using appropriate media (87). There are genetic and phenotypic anomalies between H37Rv and H37Ra, and the major difference lies in a mutation in the *phoP* gene, which is important for survival and replication in the intracellular environment (88). In the last two decades H37Ra strain has been routinely used as a control in *M. tuberculosis* identification and investigation of its virulence and genetic properties (89).

Apart from H37-strains, BCG and different clinical isolates are commonly used to study the pathogenesis of mycobacteria. *M. marinum* is also commonly used, as it is genetically very similar to *M. tuberculosis* as well as safer to handle than *M. tuberculosis* (90). Another avirulent species of mycobacteria that is commonly used as a model system is *M. smegmatis*. The *M. smegmatis* genome has at least 14 counterparts of 19 virulence genes of *M. tuberculosis* (91). Additionally, *M. smegmatis* shares over 2000 homologs

with *M. tuberculosis*. In this study, I have used both H37Ra and *M. smegmatis* as model organisms although most of the preliminary work is based on *M. smegmatis* data. This species is preferred as a model organism since it is non-pathogenic, faster growing with a doubling time of 2.5 hours and only requires biosafety level 2 laboratory.

Scope and outline of dissertation

My thesis study is aimed at understanding the function(s) of the two Ser/Thr kinases, PknA and PknB, in *M. tuberculosis*. In the current scenario of TB where one in ten latently infected person has a potential to develop active TB disease, there is an urgent need for improving existing and discovering more effective anti-TB drugs. To achieve this, drugs that specifically target latent or persister cells are an attractive field of research especially due to their possibility of preventing post-exposure TB reactivation.

The prevalence of active TB is now at its peak in the world due to HIV-TB co-infection. The bacillus with its ability to resist a variety of host lesions and evade immune surveillance has many researchers puzzled. To overcome such harsh *in vivo* conditions, it is generally believed the bacterium must rely on a variety of cellular factors such as cell wall components, host-pathogen signaling mechanisms including proteins kinases and their cellular substrates. In order to understand these complex cellular processes, my research has been focused on determining the role of two key signaling molecules PknA and PknB. The overarching hypothesis is that identification of substrates of PknA and PknB will lead to understanding of function(s) of these two kinases and could serve as potential drug target(s). In the following chapters, I present data to describe; (i) An *in vivo* approach to identify substrates of PknA and PknB, (ii) the role of proteasome

phosphorylation in H₂O₂ resistance in mycobacteria, (iii) an *in vitro* technique to identify potential substrates of PknA and PknB, and (iv) the role of FbpB phosphorylation.

Chapter 2

In vivo* search for substrates of PknA/PknB and role of proteasome phosphorylation in *Mycobacterium tuberculosis

The work in this chapter is accepted in Journal of Microbiology.

Anandan T, Han J, Baun H, Nyayapathy S, Brown JT, Dial RL, Moltalvo JA, Kim MS, Yang SH, Ronning DR, Husson RN, Suh J, Kang CM (2014) Phosphorylation regulates Mycobacterial proteasome.

Abstract

Mycobacterium tuberculosis possesses eleven STPKs contributing to its complex and important network of signal transduction systems. Despite evidence suggesting their crucial role in several important biological pathways, the molecular mechanism by which they lead to the latency or the switch to actively replicating state is still unknown. In this chapter, I demonstrate an *in vivo* method to hunt for potential substrates of PknA/PknB. Previous studies based on whole proteome analysis using phospho-(S/T)Q antibody led to identification of two *in vivo* substrates (Rv1422 and Wag31) of these kinases. Here, I provide evidence for the *in vivo* phosphorylation of the proteasome by PknA and PknB using a proteomic approach, Western blot with a phospho-T antibody, and mass spectrometry analysis. Additionally, I show that PknA phosphorylation of unprocessed proteasome β -subunit (pre-PrcB) and α -subunit of the proteasome leads to assembly defect of the proteasome complex, which then contribute to mycobacterial resistance to H₂O₂. Finally, I show that H₂O₂ stress diminishes the formation of the proteasome complex in a PknA-dependent manner.

Introduction

Signal transduction pathways in many pathogens not only function as an adaptive response regulator, but also have implications in controlling virulence determinants (92). As the most ubiquitous post-translational modification, protein phosphorylation can modulate cellular activities such as protein function or subcellular localization, target proteins for proteolysis as well as modulate protein-protein interactions. Moreover, a single protein could be phosphorylated by more than one kinase depending on the desired cellular activity, thus paving the way for a range of combinatorial regulation at the post-translational level (51). Therefore, knowing which kinases are stimulated during a specific response and which proteins are their targets are integral to deciphering the mechanism underlying a wide range of biological processes. To accomplish this goal, our lab has been studying two STPKs, PknA and PknB, by identifying and studying the function(s) of their substrates in *M. tuberculosis* (52). We are interested in these PknA and PknB particularly because our previous studies had suggested that these two kinases have regulatory function in cell wall synthesis and cell shape, thus implicating their possible association in latency and/or active replication processes (52).

To determine the functions of PknA and PknB in *M. tuberculosis*, Kang *et al.* (2005) took a peptide-library screening approach to screen for phosphorylation-sequence preference, and found that PknA and PknB have a preference for phosphorylating a Thr (T) residue followed by Gln (Q) (52). In addition, by using a phospho-proteome analysis with anti-phospho-(S/T)Q antibody and mass spectrometry after over-expressing PknA and PknB, they identified two *in vivo* substrates: Rv1422 (a hypothetical protein) and Rv2145c (Wag31, a homolog of *B. subtilis* DivIVA). Purified PknA and PknB both

phosphorylated Rvl422 *in vitro*. On the other hand, PknA and PknB combined together maximally phosphorylated Wag31. PknA alone only weakly phosphorylated Wag31 and PknB could not phosphorylate Wag31 at all (52). Even though this initial search were successfully able to identify those two *in vivo* substrates, this approach was limited to only those proteins that had the phosphorylated TQ motif due to the phospho-(S/T)Q antibody being used.

In this chapter, I will describe another *in vivo* method to screen for potential substrate(s) of PknA and PknB. This technique is based on *in vivo* screening for substrates that required overexpression of PknA and PknB and whole proteome analysis using 2-D SDS-PAGE, western blot with a phospho-T antibody, and mass spectrometry. Furthermore, I report the phosphorylation of the *M. tuberculosis* proteasomal subunits by the PknA and PknB kinases. I determined that PknA phosphorylates unprocessed β -subunit (pre-PrCB) and α -subunit, which then affects proteasome assembly and renders mycobacterial cells more resistant to H₂O₂. My finding thus suggests that phosphorylation of the proteasome by PknA/B contributes to the survival of *M. tuberculosis* under oxidative stress conditions.

Results

1. *In vivo* approach to search for the substrates of PknA/PknB in *M. tuberculosis*

To further understand the role of PknA and PknB kinases, we attempted to identify their additional substrates by a similar *in vivo* proteomic approach, but with a

phospho-T antibody, instead of phospho-(S/T)Q antibody (Fig.2.1). To do this, *M. tuberculosis* H37Rv cells over-expressing *pknA*, *pknB* or vector alone were cultured in the presence of acetamide to induce kinase genes. Cells were harvested after 24 hr of induction and the whole proteome was purified. Whole proteome was separated using 2D-PAGE, followed by partial transfer on to PVDF membrane and immunoblot with phospho-T antibody. The residual proteins in 2D-PAGE gels after partial transfer were subjected to a flamingo staining. Approximately 15 protein spots were found to be stronger in *pknB*-overexpressing *M. tuberculosis* than the vector-only control (Fig. 2.1).

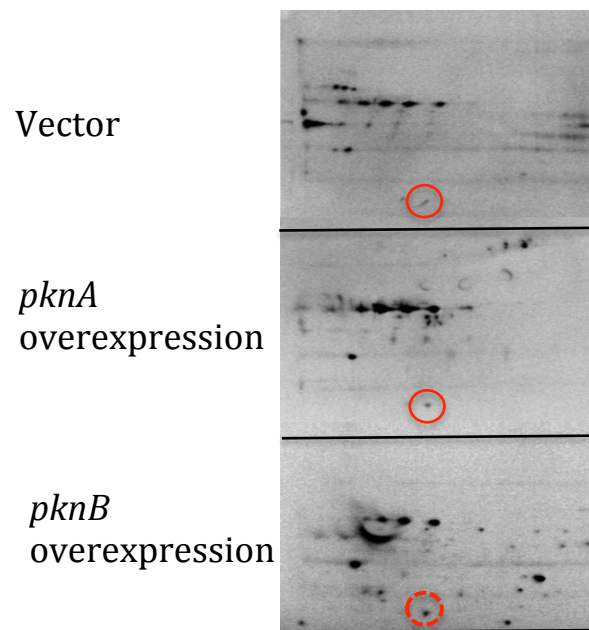


Fig. 2.1: *In vivo* approach to identify additional substrates of PknA and pknB. Whole proteome analysis of *M. tuberculosis* cells overexpressing *pknA* or *pknB* by 2D-Western Blot using phospho-T antibody. Upper panel: Vector only, middle panel: *pknA* over-expression, bottom panel: *pknB* over-expression. Mass spectrometry analysis of a spot (dotted red circle) in the *pknB* over-expression sample suggested PrcA to be phosphorylated at T84 and T202 residues. A weak spot of PrcA phosphorylation was also visible (red circle) in vector and *pknA* over-expression samples.

Mass spectrometry analysis of a spot from *pknB*-overexpression cells (dotted red circle, Fig. 2.1) revealed PrcA (α -subunit of the *M. tuberculosis* proteasome) as a potential substrate of PknB with two phosphorylation residues (T84 and T202). In this report, all the genes and proteins for kinases (PknA and PknB) and proteasome (PrcA and PrcB) are from *M. tuberculosis* unless otherwise indicated.

The presence of a weak but distinct immunoblot signal at the similar location in cells with vector alone or *pknA*-overexpression (red circle, Fig. 2.1) suggests that PrcA is a physiologic target of PknB *in vivo*. However, as overexpression of kinase genes can lead to non-specific targets being phosphorylated, I conducted a confirmatory experiment to test whether PrcA is phosphorylated even without *pknA*-overexpression. For this, wild-type *M. tuberculosis* was cultured in 7H9 liquid medium to mid-log phase where the kinase gene expression is known to be the maximum, and total lysate protein was subjected to 2D SDS-PAGE followed by western blot analysis, first with a phospho-T polyclonal antibody and subsequently with a PrcA polyclonal antibody after stripping off the phospho-T antibody (Fig. 2.2). Superimposition of the two images revealed that PrcA (pI=5.19, MW=26.9; bottom panel) from *M. tuberculosis* cells without *pknB*-overexpression appeared to be phosphorylated (upper panel).

Phospho-T Antibody

PrcA Antibody

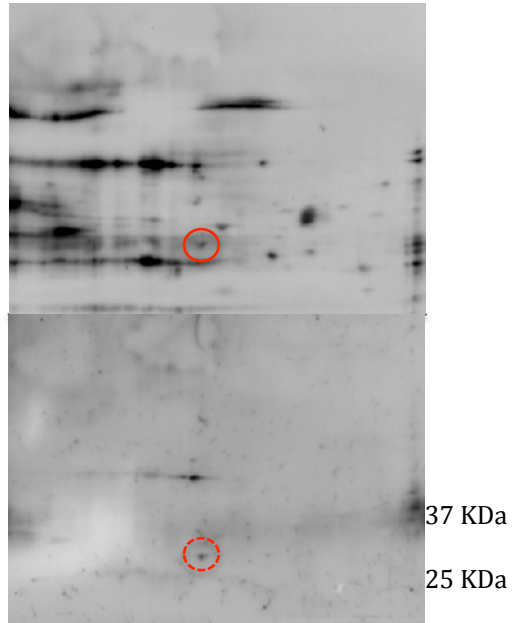


Fig.2.2: PrcA phosphorylation without *pknB*-overexpression in *M. tuberculosis*. Wild-type *M. tuberculosis* (attenuated mc²6230 strain) cells were cultured and whole cell lysate was prepared for 2D-Western Blot using phospho-T antibody followed by stripping and re-probing the same blot with a polyclonal PrcA antibody. Confirming that PrcA is still phosphorylated under PknB non-overexpression conditions *in vivo* in *M. tuberculosis*.

2. Phosphorylation of PrcA (α -subunit of the proteasome) by PknB *in vitro*

To study the role of phosphorylation of the proteasome subunit PrcA by PknB, Seeta Nyayapathi and Jae-il Han in our lab conducted further investigation. For this they transformed wild-type *M. smegmatis* with pMH94-*strep-prcA* (pCK206), and the expression of *strep-prcA* was induced by 0.2 % acetamide for 4 hr at mid-log phase. To examine the phosphorylation of PrcA *in vitro*, strep-tagged PrcA was affinity purified using Strep-Tactin Affinity Purification Kit (Novagen). Affinity purification of strep-PrcA afforded co-purification of PrcB_{MS}, the β -subunit of the *M. smegmatis* proteasome, in approximately equimolar amounts (bottom panel, Fig. 2.3). This complex of PrcA_{TB} and PrcB_{MS}, termed “chimeric proteasome” exhibited proteolytic activity against a

fluorogenic substrate Suc-LLVY-Amc (93), indicating that PrcA forms an active proteasome complex with PrcB_{Ms} in *M. smegmatis* (data not shown).

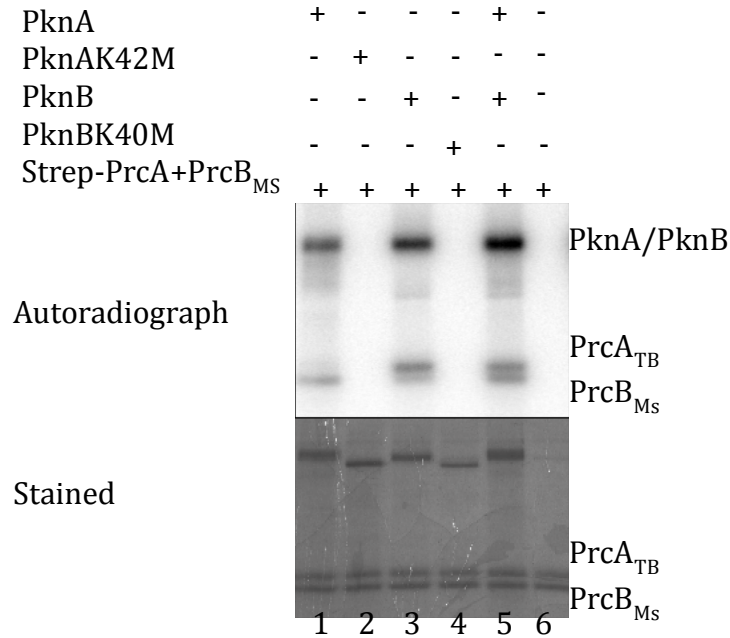


Fig. 2.3: *In vitro* phosphorylation of PrcA by PknB. Chimeric complex of strep-PrcA and PrcB_{Ms} was incubated with GST-PknA, GST-PknB, or kinase-inactive mutant protein (GST-PknAK42M or GST-PknBK40M) in the presence of [γ -³²P]ATP followed by SDS-PAGE, Coomassie blue staining (bottom row), and autoradiography (upper row).

When incubated with [γ -³²P]ATP, strep-PrcA was specifically phosphorylated by PknB but not by PknA (lanes 1 and 3, Fig. 2.3). Interestingly, PrcB_{Ms} from *M. smegmatis* was also phosphorylated by PknA and PknB (lanes 1 and 3, Fig. 2.3), suggesting that PrcB in *M. tuberculosis* may also be phosphorylated by one or both of these kinases. To validate the phosphorylation of PrcA *in vivo*, Jae-il examined the phosphorylation of strep-PrcA in the presence of *pknB*-overexpression in *M. smegmatis*. He purified strep-PrcA from cells with or without *pknB*-overexpression and analyzed by 2-D Western blots, first with a phospho-T antibody and subsequently with a strep antibody after

stripping off the phospho-T antibody. Mass spectrometry was performed on spots that were suspected to be phosphorylated and the results revealed PrcA to contain a third phosphorylation site at T178 in addition to the already identified T84 and T202. Jae-il also later demonstrated that PknB phosphorylated these three threonine residues in a sequential manner (T84, T202, 1178), and that PrcA phosphorylation is functionally important in regulating its proteolytic activity against a known substrate Ino1.

Note: The work described henceforth describes my results leading to the identification of phosphorylation of Pre-PrcB and PrcA by PknA and its implications in H₂O₂ resistance in *M. tuberculosis*.

3. PknA affects proteasome integrity in *M. smegmatis*

Since we found that PrcB_{Ms} from *M. smegmatis* was phosphorylated by PknA *in vitro* (Fig. 2.3), I wanted to investigate if PknA can also phosphorylate *M. tuberculosis* PrcB. To do this, I expressed *prcB-prcA-strep* in the $\Delta prcBA$ *M. smegmatis* strain overexpressing *pknA*, purified PrcA-strep to co-elute PrcB and examine its phosphorylation. To our surprise, PrcB was not co-eluted with PrcA-strep (lane 2, Fig. 2.4). In contrast, PrcA-strep purification from the $\Delta prcBA$ *M. smegmatis* strain overexpressing *pknB* (lane 1) or containing vector alone (lane 3) co-purified PrcB (lane 1 and 3) as shown in Fig 2.4. This result suggested that *pknA*-overexpression may affect the proteasome assembly or make the proteasome complex unstable (lane 2, Fig. 2.4).

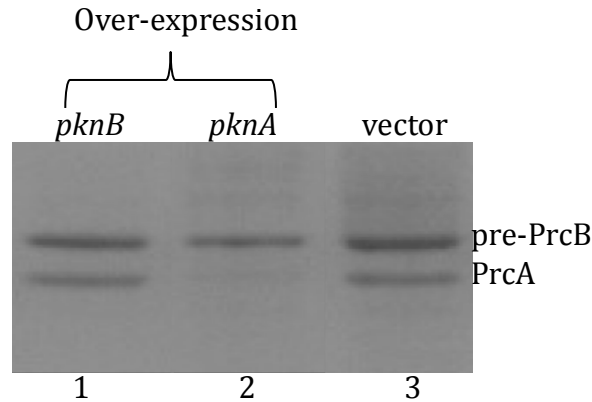


Fig. 2.4: PrcB is not co-eluted with PrcA under *pknA* overexpression. Purification of PrcA-strep and co-elution of PrcB was analyzed using a 1-D SDS-PAGE and Gel code blue staining. Approximately 5 μ g of protein was used for each lane. Lane 1: purified PrcA-strep and co-eluted PrcB from cells with overexpression of *pknB*, lane 2: purified PrcA-strep without PrcB co-elution from cells with overexpression of *pknA*, lane 3: purified PrcA-strep and co-eluted PrcB from cells with vector alone.

To further analyze the levels of processed and unprocessed PrcB in these samples, I used 2D-PAGE and loaded more of the protein sample (15 μ g) per gel in order to reassess the presence of PrcB in the purified proteasome samples. By loading more of the purified proteasome sample on 2D-PAGE and flamingo staining the gel, I was able to visualize processed and unprocessed PrcB along with PrcA (Fig. 2.5). Compared to the sample from the *pknB*-overexpressing cells, both pre-PrcB and processed PrcB levels were very low when *pknA* is over-expressed, indicating a possible role of PknA in processing of PrcB thereby affecting assembly or the stability of the proteasome complex (Fig. 2.5). In addition, the total amount of PrcA-strep plus PrcB purified from *M. smegmatis* expressing *pknA* was 5 to 10 fold lower than that of *pknB*-expressing or vector alone cells (data not shown) so that I had to load more volume of purification elution from the *pknB*-overexpressing cells to achieve the gel picture presented here. This result

suggests that, once again, PknA may affect the proteasome assembly or the stability of the proteasome complex.

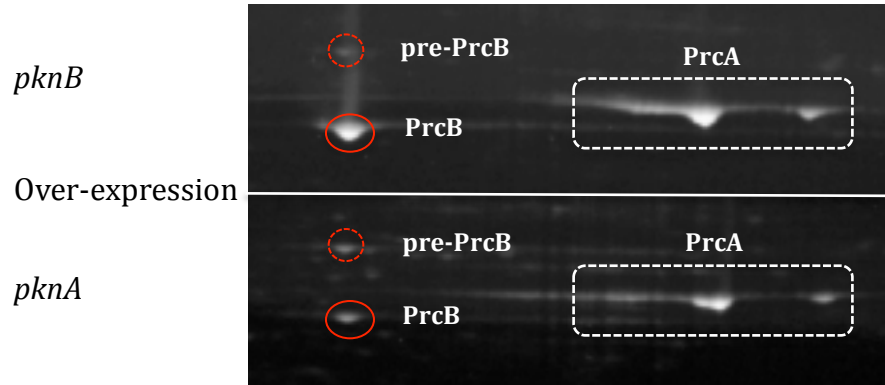


Fig. 2.5: PknA affects the integrity of the proteasome core complex in mycobacteria. Proteasome purified from *M. smegmatis* containing *pknA*- or *pknB*-overexpressing constructs was analyzed using 2D-PAGE and flamingo staining. Top panel: purification from cells with *pknB*, bottom panel: purification from cells with *pknA*. PrcA-strep (dotted box), pre-PrcB (dotted red circle), and processed PrcB (red circle) are shown.

In order to investigate whether the lack of the co-eluted PrcB in the *pknA*-overexpressing strain is due to direct effect of PknA on proteasome, I conducted a second round of analysis in *E. coli*. *E. coli* was chosen for this experiment because it lacks the proteasomal system as well as PknA/PknB kinases, and therefore can provide an environment for a direct measure of PknA effect on proteasome integrity. As before, recombinant PrcA-strep was purified from *E. coli* expressing *pknA* or *pknB*, and co-elution of PrcB was examined in 1-D PAGE. Fig 2.6 shows that while the formation of holo-proteasome was unaffected by *pknB*-expression, *pknA*-expression resulted in a mixture of half-proteasome (pre-PrcB + PrcA) and holo-proteasome (PrcB + PrcA), suggesting that PknA affects the processing of PrcB during the assembly of holo-

proteasome. Taken together with the results from *M. smegmatis*, *E. coli* experiments suggest that this effect of PknA is probably via directly phosphorylating PrcB or PrcA because *E. coli* lacks both a PknA homolog and the proteasomal system.

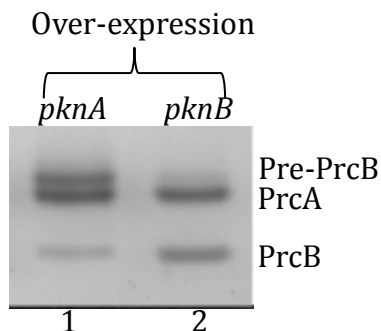


Fig. 2.6: PknA affects proteasome integrity in *E. coli*. *E. coli* cells expressing *prcBA*-strep in the presence of *pknA*, *pknB* was used and PrcA-strep was purified using strep affinity column, and co-purification of PrcB was tested. As in *M. smegmatis*, *pknA*-overexpression led to decreased level of PrcA-PrcB and therefore 5-10 times more of elution from PknA-expressing cells was loaded than those of PknB-overexpression to achieve similar intensities of PrcA.

4. PknA phosphorylates both PrcA and pre-PrcB

As the *E. coli* experiment in Fig. 2.6 suggests a probable direct effect of PknA on proteasome integrity, I hypothesized that PknA exerts this effect on the proteasome via directly phosphorylating proteasome subunit(s). To test which proteasomal subunit(s) is/are phosphorylated by PknA, the mixture of PrcA-strep and PrcB purified from *E. coli* expressing *pknA* was analyzed using immunoblot with phospho-T and strep antibodies (Fig. 2.7). Strong phospho-signals were found from pre-PrcB and PrcA but not from processed PrcB (lane 1, Fig. 2.7), suggesting that PknA phosphorylates the half-proteasome and thereby may regulate its assembly into the holo-proteasome.

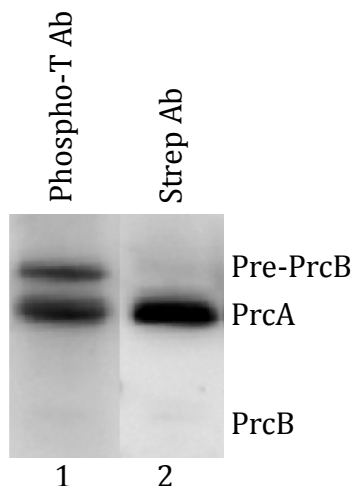


Fig. 2.7: Pre-PrcB and PrcA are phosphorylated by PknA. Purified PrcA-strep and co-purified PrcB in the presence of *pknA*-overexpression in *E. coli* BL21 (DE3) was analyzed using western blot with a phospho-T antibody (lane 1) or a strep antibody (lane 2).

To identify residues phosphorylated in pre-PrcB and PrcA by PknA, I conducted the following experiment in *M. smegmatis* expressing *pknA*. The $\Delta prcBA$ *M. smegmatis* strain that constitutively expresses *prcBA*-Strep and *pknA* was cultured with 0.2% acetamide for 7 hours. The cells were harvested in order to purify recombinant PrcA-strep and PrcB using Strep column. Purified sample was dialyzed and subjected to 2D-PAGE analysis, visualized using Flamingo stain and PrcB spots were cut out and analyzed by mass spectrometry. Mass spectrometry revealed that PrcA is phosphorylated at T195 and PrcB at T239. To test if these phospho-sites are correct, I made PrcAT195A and PrcBT239A mutants by site-directed mutagenesis, and tested their phosphorylation by using western blot with a phospho-T and a strep antibody (lane 2 and 3, Fig. 2.8). Briefly, the $\Delta prcBA$ *M. smegmatis* strain that constitutively expresses *prcBA* or *prcB-prcAT195A* or *prcBT239A-prcA* and contains *pknA* was cultured with 0.2% acetamide for 7 hours. Cells were harvested, proteasome was purified using Strep-column, and

phosphorylation of each phospho-mutant form was analyzed by immunoblot with phospho-T antibody.

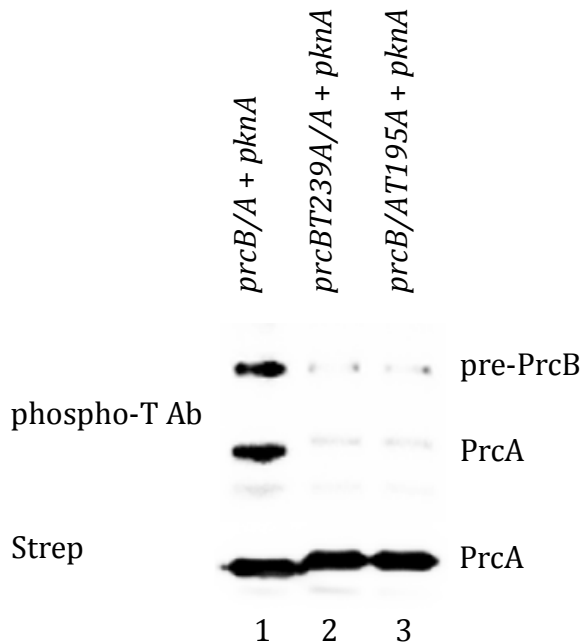


Fig. 2.8. Confirming phosphorylation site in Pre-PrcB and PrcA. Confirmation of proteasome phosphorylation sites. *M. smegmatis* cells overexpressing *pknA* was used to purify proteasome mutant proteins PrcB/AT195A or PrcBT239A/A and analyzed by westerns with phospho-T and strep antibodies.

Fig. 2.8 depicts the result of the phospho-mutant analysis, where both the phospho-ablative (T to A) mutants have lost most of phosphorylation (lanes 2 and 3, Fig. 2.8) while wild-type proteasome showed phosphorylation of both pre-PrcB and PrcA. Protein loading was comparable in each sample as shown from the bottom western with a strep antibody. While I still have to perform another control experiment that show the levels of pre-PrcB in each mutant purification, it appears that mutation of one phospho-T residue results in the abolition of phosphorylation in another residue, suggesting that

phosphorylation of these residues by PknA may be dependent on each other's phosphorylation.

5. PknA does not affect the proteasome stability

Since the total proteasome level was decreased by *pknA*-expression in both *M. smegmatis* and *E. coli* (Fig. 2.4 and 2.5), it was also possible that PknA destabilized the half-proteasome and/or the holo-proteasome complex after the assembly. To test this possibility, 3 μ M proteasome complex was incubated alone (lane 3, Fig. 2.9), with 1 μ M GST-PknA (lane 4), or GST-PknB (lane 5) in the presence of 1 mM ATP for 2 hr at 37 °C. Kinase alone controls (lanes 1 and 2) were also included.

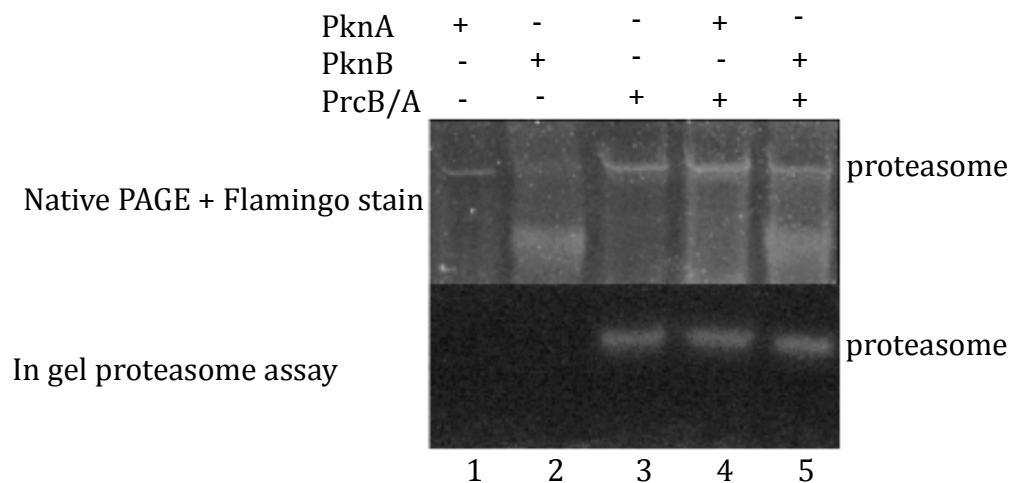


Fig. 2.9: PknA does not affect proteasome stability. To determine if PknA affect the stability of the holo-proteasome, proteasome purified from *E. coli* cells was incubated with PknA, PknB, or without any kinase in the presence of ATP followed by an in-gel proteasome assay. Duplicate of the same reaction was also used in a native PAGE and stained with Flamingo staining.

Proteins in each reaction were then separated on two native PAGE gels; one was stained with Flamingo Fluorescent stain to test the integrity of the proteasome complex and the other was used for enzymatic assay in an in-gel proteasome assay by incubating

the gel with 100 μ M Suc-LLVY-Amc for 30 min at 30 °C. Fluorescence from Amc released was analyzed by the ChemiDoc XRS system and Quantity one 1-D analysis software (Bio-Rad). Proteasome integrity or activity for the peptide substrate was not affected by the incubation with PknA or PknB (Fig. 2.9), suggesting that PknA affects the integrity of the proteasome before the assembly step into the holo-proteasome.

As a control experiment, the activities of kinases used in Fig. 2.9 were determined by an *in vitro* kinase assay with purified PknA or PknB and Rv1422, a known substrate of these kinases. Kinase assay reactions were resolved on SDS-PAGE followed by immunoblot with phospho-T antibody (Fig. 2.10). Fig. 2.10 confirmed that PknA and PknB were active as the substrate Rv1422 was phosphorylated only in the presence of PknA or PknB (lanes 1 and 2, Fig. 2.10).

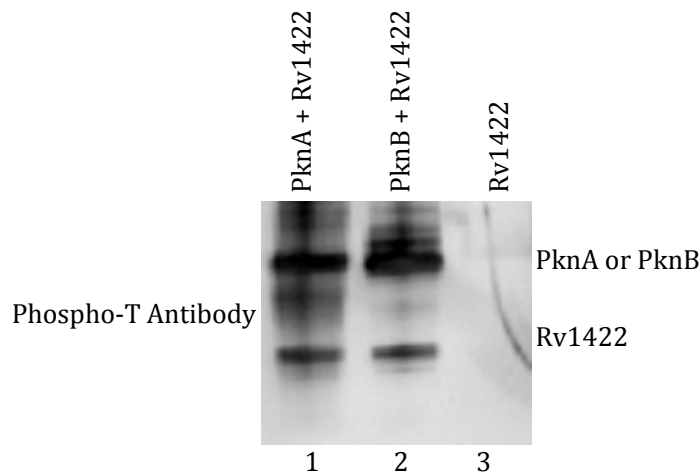


Fig. 2.10: Confirming the activity of PknA and PknB. To determine PknA and PknB are active, a kinase assay was performed using purified PknA and PknB along with a known *in vivo* substrate of these kinases called Rv1422. Kinase assay reactions run on SDS-PAGE followed by immunoblot with a phospho-T antibody.

6. Effect of the proteasome phosphorylation on oxidative stress resistance

Previous studies demonstrated that depletion of the proteasome and the proteasome-associated proteins (Mpa and PafA) rendered *M. tuberculosis* more susceptible to nitric oxide but increased its resistance to H₂O₂ by about 2 - 3 fold (71,94). Since I found that *pknA*-expression diminished the holo-proteasome level (Fig. 2.4), I sought to determine whether *pknA*-expression also enhances mycobacterial resistance to H₂O₂. To test this possibility, I first examined if depletion of the proteasome causes a similar phenotype in *M. smegmatis*. To do this, the $\Delta prcBA$ *M. smegmatis* strain constitutively expressing *prcBA* or containing pUAB100 vector alone was constructed along with wild-type *M. smegmatis* containing vector. These cells were treated with 0 mM or 4 mM H₂O₂ for 45 min and CFUs were determined.

Introduction of *M. tuberculosis prcBA* into the $\Delta prcBA$ *M. smegmatis* strain decreased the resistance to H₂O₂ as in *M. tuberculosis* (survival rate of $\Delta prcBA$ *M. smegmatis* was 75% higher than that of the mutant carrying *prcBA-strep*; $p < 0.038$) (compare bar 1 and 2 in Fig. 2.11). In contrast to the results in *M. tuberculosis*, however, the survival of $\Delta prcBA$ *M. smegmatis* following exposure to H₂O₂ was almost identical to that of wild-type *M. smegmatis*, suggesting that the proteasome in *M. smegmatis* may have different physiological functions than that of *M. tuberculosis*, possibly reflecting their different habitats. (compare bar 2 and 3 in Fig. 2.11).

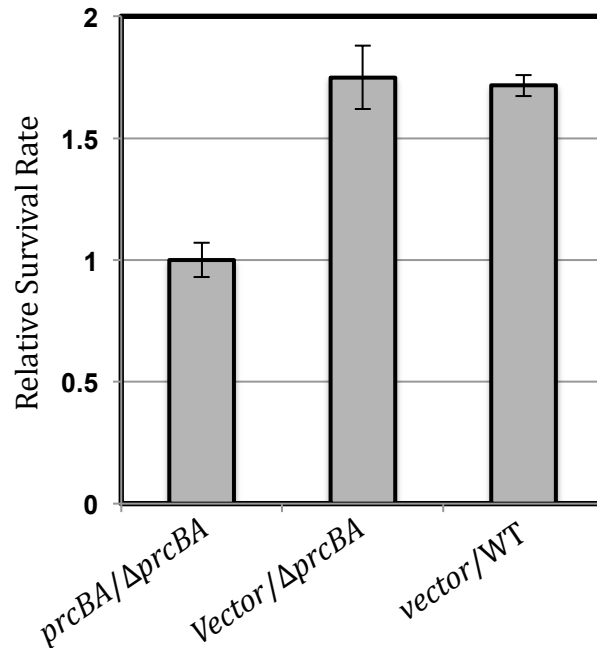


Fig. 2.11: Mycobacterial resistance to H₂O₂ is increased by absence of proteasome. Δ*prcBA* or wild-type *M. smegmatis* cells with constitutive *prcBA* expression or vector alone was treated with 0 or 4 mM H₂O₂ for 45 min and CFUs were determined. Relative survival rate was calculated by dividing CFU from 4mM H₂O₂ by CFU from 0 mM H₂O₂ sample after 45 min.

Even though the phenotype of proteasome depletion in *M. smegmatis* was different from the one in *M. tuberculosis*, this result provided us a tool to test whether *pknA*-expression can enhance resistance to H₂O₂ of Δ*prcBA* *M. smegmatis* carrying *prcBA*. To do this, I conducted another survival rate experiment where the Δ*prcBA* *M. smegmatis* cells expressing *prcBA* in the presence of *pknA*-, *pknB*-overexpression, or vector alone was treated with 0 mM or 4 mM H₂O₂ for 45 min and the relative survival rate was determined as described above. As a control, Δ*prcBA* *M. smegmatis* that contains only *pknA*-overexpression cassette without *prcBA* was also included.

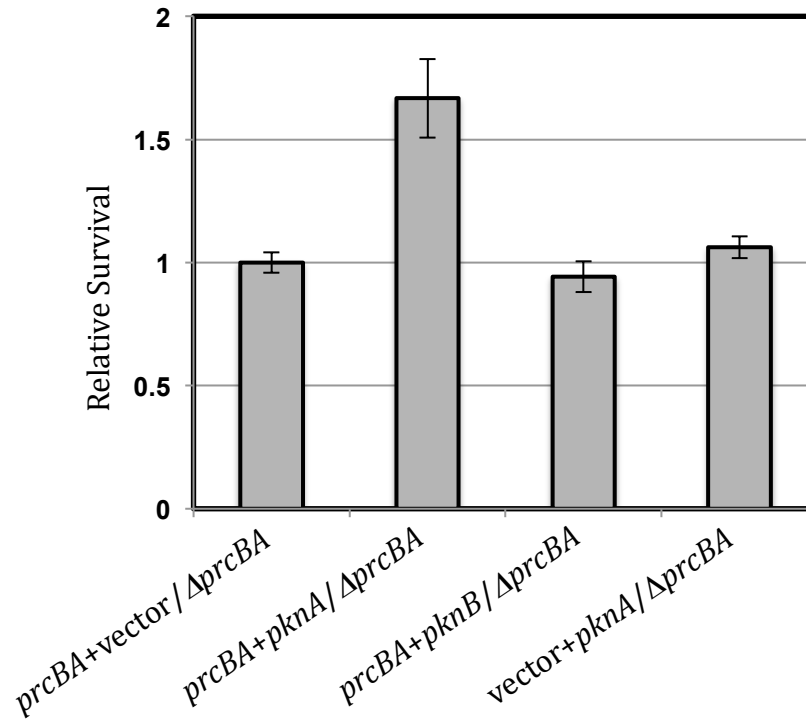


Fig. 2.12: Mycobacterial resistance to H₂O₂ is increased by *pknA*-expression. Δ *prcBA* *M. smegmatis* cells expressing *prcBA* in the presence of *pknA*-, *pknB*-overexpression or pMH94 vector alone was treated with 0 or 4 mM H₂O₂ for 45 min and the relative survival rate was determined as described in Fig. 2.11. As a control, Δ *prcBA* *M. smegmatis* that contains only *pknA*-overexpression cassette without *prcBA* was included. This result is a representative experiment of three replicates where each performed with triplicate plating.

Fig. 2.12 shows that *pknA*-expression in the presence of *prcBA* confers 67% higher resistance to H₂O₂ than cells containing vector only ($p < 0.012$) (compare bar 2 and 1). Though the percent change seems modest, the increased resistance to H₂O₂ by *pknA*-expression was statistically significant and was obtained consistently over three independent experiments. In contrast, co-expression of *pknB* and *prcBA* did not change the resistance to H₂O₂ (compare 3 and 1, Fig. 2.12), consistent with the intact proteasome complex formation under *pknB*-overexpressing condition shown earlier in Fig. 2.4. In addition, *pknA*-overexpression alone (bar 4) did not change the bacterial survival rate in

the presence of H₂O₂, indicating that PknA contributes to H₂O₂ resistance mainly through the proteasome.

7. H₂O₂ impedes the formation of holo-proteasome in a PknA-dependent manner

If PknA increases the resistance to H₂O₂ via the proteasome, I predicted that H₂O₂ stress would inhibit the formation of holo-proteasome in a PknA-dependent manner. To test this prediction, I wanted to examine the formation of the holo-proteasome under H₂O₂ treatment in $\Delta prcBA$ *M. smegmatis* that constitutively expresses *prcBA* in the presence of *pknA*- or *pknB*-expression (Fig. 2.13). For this, levels of PrcB processing were measured to examine the integrity of the holo-proteasome, by performing an immunoblot with a PrcB antibody.

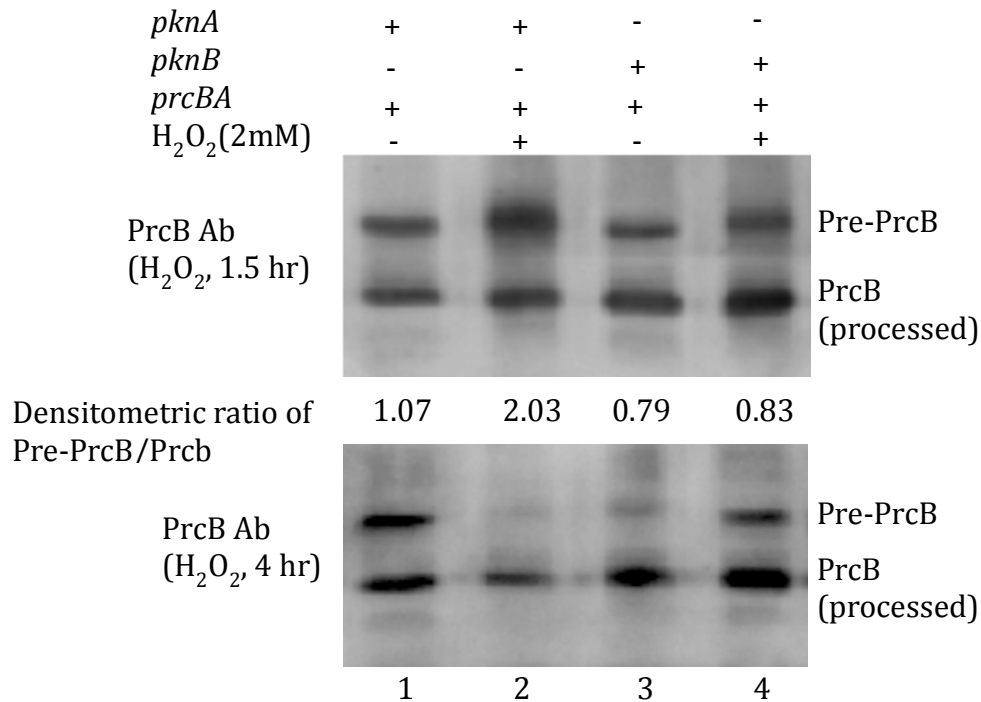


Fig. 2.13: H₂O₂ affects Pre-PrcB processing. The $\Delta prcBA$ *M. smegmatis* strain that constitutively expresses *prcBA* (pCK322) and contains *pknA*- (pCK5) or *pknB*-expression (pCK7) were treated with 2 mM H₂O₂ for 1.5 and 4 hr. 20 μ g total lysate protein from each culture was analyzed in immunoblot with a polyclonal PrcB antibody to test the processing of PrcB.

Two-fold more pre-PrcB was accumulated in cells expressing *pknA* after 1.5 hr H₂O₂ treatment (compare lanes 1 and 2, Fig. 2.13, upper panel), but not in cells with *pknB*-expression (lanes 3 and 4). Longer treatment of H₂O₂ dramatically diminished the level of both pre-PrcB and processed PrcB in *pknA*-expressing cells while processing of PrcB was near complete in *pknB*-expressing cells (Fig. 2.13, lower panel). Taken together, these data support that PknA plays an important role in oxidative stress response by impeding the formation of holo-proteasome.

8. H₂O₂ affects holo-proteasome activity in *M. tuberculosis*

To further confirm the effect of H₂O₂ on the PrcB processing, I wanted to investigate whether in wild-type *M. tuberculosis* holo-proteasome formation and its activity is affected in the presence of H₂O₂. Wild-type *M. tuberculosis* cells were treated with 0 mM or 2 mM H₂O₂ for 24 hr. Cells were harvested and total lysate protein from each culture was analyzed by immunoblot with a polyclonal PrcB antibody. As shown in Fig. 2.14, exposure to H₂O₂ affects the processing of pre-PrcB and leads to defect in holo-proteasome formation in *M. tuberculosis*.

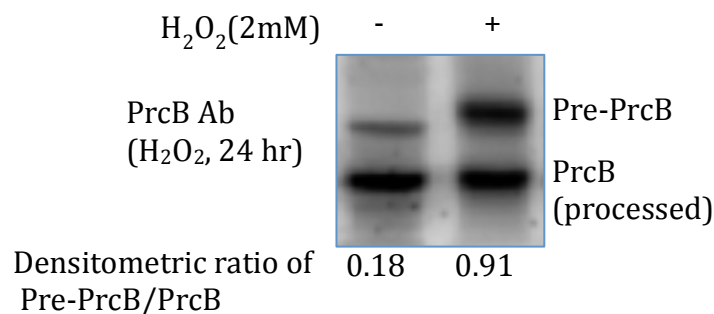


Fig. 2.14: H₂O₂ impedes the formation of holo-proteasome in *M. tuberculosis*. *M. tuberculosis* mc²6230 was cultured to log phase and treated with or without 2 mM H₂O₂ for 24 hr. 20 µg total lysate protein from each culture was analyzed in immunoblot with a polyclonal PrcB antibody. The ratio of pre-PrcB to PrcB was determined as in Fig. 2.11.

T

To analyze if this defect in holo-proteasome formation is reflected in its proteolytic activity as well, I conducted a proteasomal activity assay using whole cell lysate of wild-type *M. tuberculosis* that were treated with 0 mM H₂O₂ or 2 mM H₂O₂. Proteolytic activity was tested against a peptide substrate (Suc-LLVY-AMC). Fluorescence emitted by cleaved Amc was measured in a fluorometer at $\lambda_{\text{ex}} = 340$ nm, $\lambda_{\text{em}} = 460$ nm (Fig. 2.15) as describe earlier. Proteolytic activity was absent in the cell lysate prepared from cells incubated with H₂O₂ but seen in control without H₂O₂ treatment (Fig. 2.15). These experiments provided clues that processing of PrcB in *M. tuberculosis* was also greatly retarded by H₂O₂ treatment thereby causing defects in holo-proteasome formation and proteolytic activity in *M. tuberculosis* (Fig. 2.14 and Fig. 2.15). Fig. 2.14 suggests that there may be some holo-proteasome formed even in the presence of H₂O₂ although the proteolytic activity tested of that lysate in Fig. 2.15 does not indicate that the holo-proteasome is proteolytically active. One possibility could be that oxidative stress leads to phosphorylation of the proteasome by PknA that not only leads to defects in integrity but also on the proteolytic activity of the holo-proteasome. oxidative stress conditions.

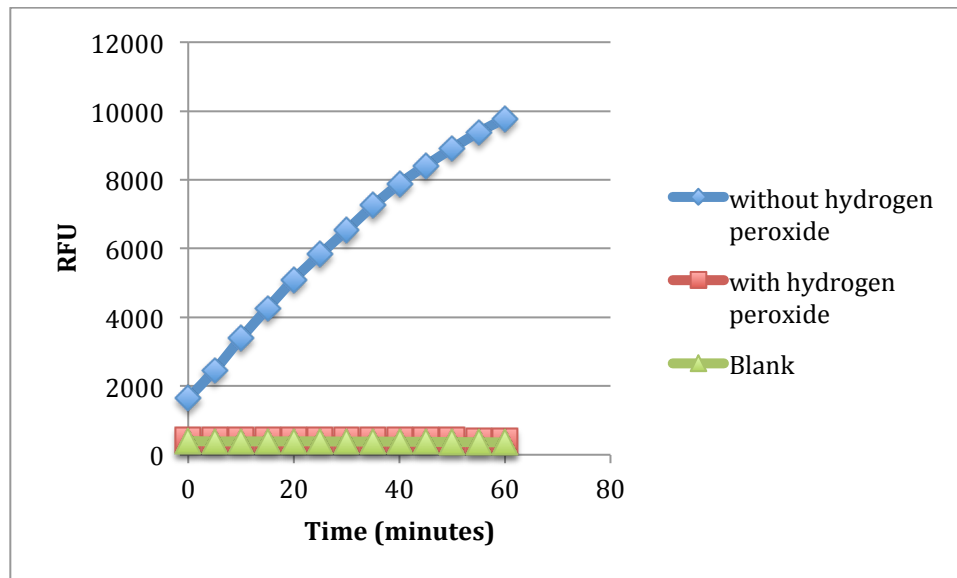


Fig. 2.15: Presence of H₂O₂ affects proteasomal activity in *M. tuberculosis*. *M. tuberculosis* cells were harvested and lysate was used for a proteasomal activity assay. Lysates were treated with 2mM H₂O₂ and as controls lysate without H₂O₂ or blank containing no lysate were used. X-axis: Time (minutes), Y-axis: Relative Fluorescence Units (RFU).

9. H₂O₂ activates PknA

So far, my data suggest an important role of PknA in H₂O₂ resistance via proteasome but the mechanism underlying this process was not answered. To pursue this goal, I wanted to know whether H₂O₂ could activate PknA that then could lead to defects in proteasome integrity and H₂O₂ resistance. For this, *E. coli* BL21 (DE3) containing a pGEX-4T-3-*pknA* plasmid was cultured in the presence of 0.5 mM IPTG for 6 hr, and then exposed to 2 mM H₂O₂ for 0, 60, and 120 min. The levels of the PknA phosphorylation and the GST-PknA protein were determined by immunoblot with phospho-T (row 1, Fig. 2.16) and GST (row 2, Fig. 2.16) antibodies, respectively. Fig 2.16 shows an increase in autophosphorylation levels of PknA in cells incubated with H₂O₂ with increasing incubation time, suggesting that PknA can be activated by H₂O₂.

This result is similar to that seen in the I κ B kinase (inhibitor of NF- κ B kinases) of some eukaryotes, where H₂O₂ has been shown to promote autophosphorylation of I κ B kinase by phosphorylating two serine residues on activation loop thereby activating the kinase (95). Taken together, these data suggest that PknA plays an important role in the oxidative stress response by impeding the formation of holo-proteasome in *M. tuberculosis*.

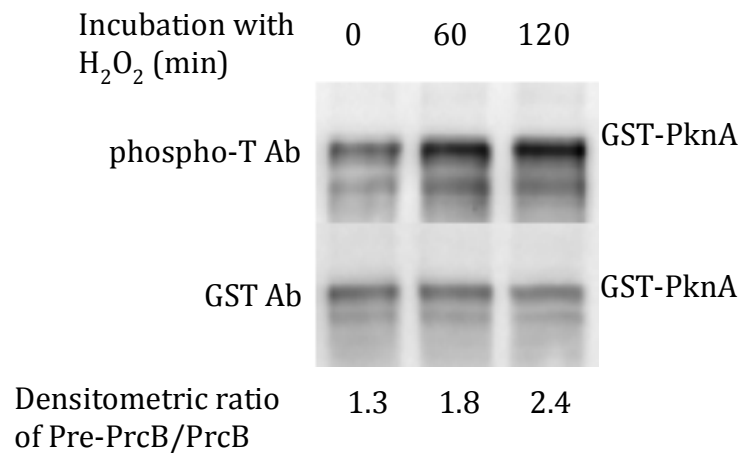


Fig. 2.16: Autophosphorylation of PknA increases in response to H₂O₂. *E. coli* BL21 (DE3) containing a pGEX-4T-3-*pknA* plasmid (pCK3) was cultured in the presence of 0.5 mM IPTG for 6 hr, and then exposed to 2 mM H₂O₂ for 0, 60, and 120 min. The levels of the PknA phosphorylation and the GST-PknA were determined by immunoblot with phospho-T (row 1) and GST (row 2) antibodies, respectively. Densitometric ratio of the phosphorylation level and the GST-PknA level was determined as in Fig. 2.11.

Discussion

In this work, I determined that proteasome subunits, pre-PrcB and PrcA, are phosphorylated by PknA and that their phosphorylation enhances the resistance against H₂O₂ stress in mycobacteria. Together with the holo-proteasome assembly defects, H₂O₂ resistance, and activation of PknA data presented here, these results indicate that PknA plays an important role in regulating proteasome integrity, oxidative stress response, and

possibly pathogenesis in mycobacteria. Fig. 2.17 describes a model speculated based on my findings of role of proteasome phosphorylation in increasing resistance to H_2O_2 .

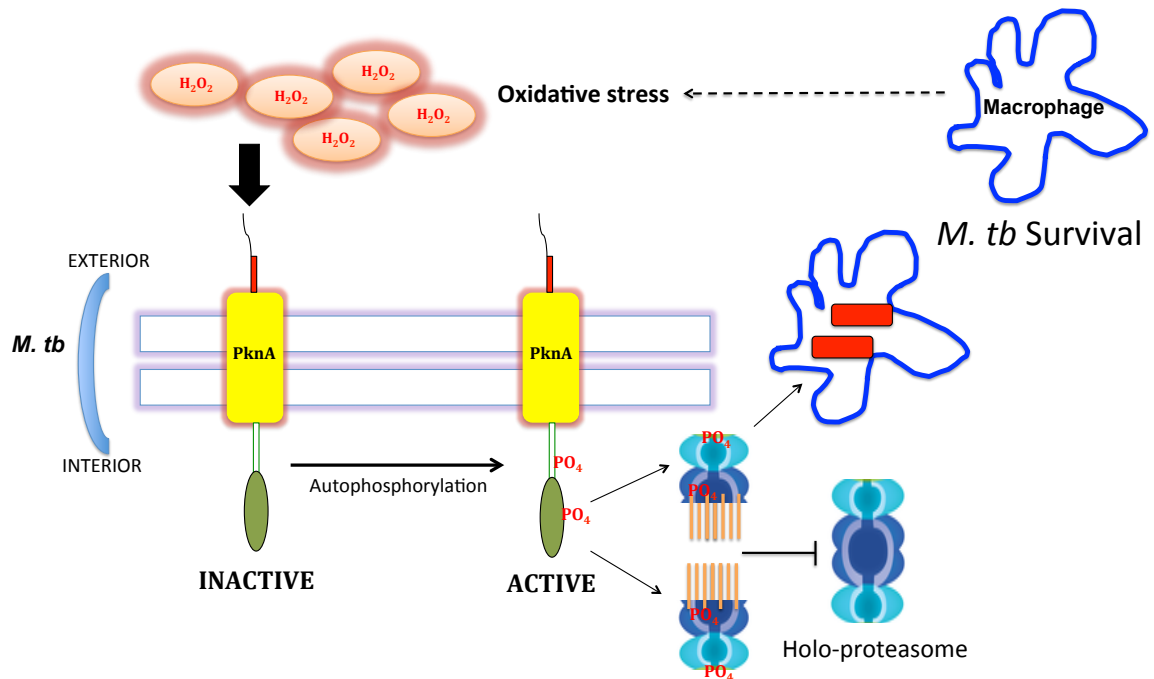


Fig. 2.17: Model for role of proteasome phosphorylation in *M. tuberculosis* (*M. tb*). Host induced defense factors such as H_2O_2 are made against an invading pathogen such as *M. tb* cells that are then available for them to activate PknA. Autophosphorylation of PknA results in activation of PknA and downstream substrate phosphorylation occurs on proteasomal subunits. This affects holo-proteasome integrity and thereby leading to a state of proteasome depletion that somehow leads to increased resistance to H_2O_2 .

In prokaryotes, genes encoding putative 20S proteasome are found only in the phylum Actinobacteria that mycobacteria belong to. Recently, phosphorylation of proteasome subunits (both α - and β -subunits) have been reported in eukaryotes and archaeal proteasomes (96,97). Phosphorylation was determined to enhance proteolytic activity against peptide substrates in eukaryotes (98). Here, I report that phosphorylation of mycobacterial proteasomal subunits (pre-PrcB and PrcA) by PknA affects the integrity

of the proteasome complex, via inhibition of processing of pre-PrcB and thus assembly into the holo-proteasome. Therefore, it is suggested that *M. tuberculosis* is able to modulate the levels of holo-proteasome complex through PknA whose activation is stimulated by H₂O₂. Phosphorylation of proteasome by PknA mimics a state of proteasome depletion that may then contribute to the pathogen's higher resistance to H₂O₂ by possibly saving some unknown oxidative stress response proteins from proteolysis. I also present data to suggest that H₂O₂ activates PknA during H₂O₂ stress, and thus activated PknA become available to phosphorylate proteasome leading to rescue of bacilli in the presence of H₂O₂. Thus, these results could suggest a function of proteasome regulation by PknA to have a probable end result of establishing dormancy or persistence by *M. tuberculosis*.

The pathogenesis of TB has been widely recognized as a tug-of-war between the pathogen and the host immune system. It is believed that once endocytosed by host macrophage, the bacilli has to encounter a robust supply of immune system molecules to overcome the stress and maintain dormancy or lead to active infection. One major defense mechanism seen with *M. tuberculosis* is reduced growth rate as a survival mechanism to fool the host immune system. Darwin *et al.* (2003) and Gandotra *et al.* (2007) have independently shown that lack of proteasome aids survival of *M. tuberculosis* against H₂O₂ stress (71,72). Consistently, I have found that PknA diminished the proteasome assembly that in turn led to increased resistance to H₂O₂, and that the H₂O₂ stress, via activating PknA, reduced the formation of the proteasome. What remains to be answered is how lack of the proteasomal system results in higher resistance to H₂O₂. *M. tuberculosis* possibly relies on PknA as a mode to sense the H₂O₂ stress at this

stage and affects the integrity of the proteasome, which may then lead to accumulation of oxidative stress proteins such as catalase-peroxidase (KatG) (99). Thus, it would be interesting to see if the integrity of KatG can be affected by proteasome and PknA. Another question is how H_2O_2 affects the proteasome assembly through PknA. It is known that in eukaryotes, several protein kinases (ERK [extracellular signal-regulated kinases] and MAP [mitogen-activated protein] kinases) are activated by H_2O_2 that serves as a survival mechanism against H_2O_2 exposure (95,100). Tabet et al (2005) showed MAP kinase activation through phosphorylation in the presence of H_2O_2 to be also increasing in a time dependent manner (95,100). Thus my results are in agreement with the MAP kinase activation by H_2O_2 seen in eukaryotes, as PknA autophosphorylation level also seemed to increase when exposure time was increased in the presence of H_2O_2 . In eukaryotes, I κ B kinases (inhibitor of NF- κ B kinases) is known to be activated by H_2O_2 through phosphorylation of its activation loop at two serine residues (95). In this study I suggest a similar mechanism for the PknA activation in the presence of H_2O_2 . Although the residue known to be target of autophosphorylation on PknA are T172 and T174; it will be important to confirm whether H_2O_2 promote phosphorylation of same residues (95). Further experiments to show whether activation of PknA can be inhibited in the presence of KatG can provide clues to the H_2O_2 induced stimulation of PknA autophosphorylation shown here.

To better understand proteasome and its role in stress response, further studies focusing on determining how the phosphorylation of pre-PrcB and PrcA regulates the assembly of the proteasome complex would be crucial. We have yet to identify additional substrates of the PrcA-phosphorylated proteasome, and the molecular mechanisms by

which PrcA phosphorylation affect the substrate proteolysis.

Chapter 3

***In silico* approach to search for new substrates of PknA/PknB, and role of FbpB phosphorylation**

Abstract

The molecular switch between latency and active replication in *M. tuberculosis* is not greatly understood. It is speculated that precise regulation of cell wall biosynthesis must be a key mechanism to achieve transition between the two drastically varied growth stages. Therefore, to sense extracellular environment conditions protein-signaling cascades must act as messengers to relay information from outside to the inside of the cell. To investigate this possibility, I wanted to identify the role(s) of two eukaryotic-type Ser/Thr kinases, PknA and PknB by identifying and characterizing their substrates.

Here, I describe an *in silico* homology approach to identify new substrates of PknA/PknB using the predicted preferred substrate phosphorylation motifs of PknA/B (ATQXIP or TQXIP, where X is any amino acid), and a synthetic peptide screening technique. Through this approach, I identified seven candidate substrate proteins. Of those seven candidate proteins, FbpB appeared to be of great interest because it belongs to a complex known as antigen 85 complex involved in the synthesis of cell wall component mycolic acid, an important virulence factor of *M. tuberculosis*. My work suggests that FbpB is phosphorylated *in vivo* in *M. smegmatis* and in *M. tuberculosis*, and that the 30KDa-secreted form of FbpB is dephosphorylated in *M. smegmatis*. Mass spectrometry analysis of the *M. tuberculosis* FbpB protein purified from *M. smegmatis* revealed ²⁵⁵T as a single phosphorylation site. The synthetic mycolyl transferase assay employed for assessing FbpB's enzyme activity suggested that phosphorylation may

regulate its enzyme activity. The kinase responsible for FbpB phosphorylation is yet to be answered.

Introduction

Mycobacterium tuberculosis not only actively replicates during infection but also has a dormant intracellular phase where it has reduced metabolic activity and minimal cell division. This may account for the characteristic slow growth rate observed in *M. tuberculosis*, and could thus contribute to the ability of disease-causing members of mycobacteria to survive for long periods of time in animal hosts. While the molecular basis of the transition between these two completely different cell cycle phases is yet to be identified, it has been suggested that one key underlying mechanism must be in regulation of cell signaling in order to govern cell division and cell wall synthesis required for cell division.

Previously, our lab showed that PknA and PknB phosphorylated preferably at the phospho-acceptor threonine (T) residue followed by glutamine (Q) at +1 position and hydrophobic residues at the +3 and +4 positions. Subsequent identification of the *in vivo* substrates (Wag31 and Rv1422) of these kinases demonstrated that those substrate proteins have a phosphorylation site that matches very well with the previous finding of substrate specificity ($^{73}\text{TQAIP}$ in Wag31 and $^{325}\text{TQEIP}$ in Rv1422) (52). Further analyses of the preferred phosphorylation sequence for PknA/B against peptide library revealed that ATQXIP, (where X is any amino acid) is the consensus sequence preferred for the phosphorylation by PknA and PknB (52). In this chapter, I will describe an *in silico* method based on this consensus peptide sequence to screen for additional potential

substrate(s) of PknA and PknB, and investigate the role of phosphorylation of one such potential substrate, FbpB.

Results

1. Genomic search using a consensus peptide motif reveals 7 putative substrates of PknA/PknB

Based on the preferred phosphorylation motif (ATQXIP) identified for PknA and PknB, I hypothesized that additional substrates of PknA and PknB have similar amino acid sequences around their phosphorylation site. Homology search for additional proteins containing the ATQXIP sequence in the whole *M. tuberculosis* genome resulted in no new protein except for the already identified substrates, Wag31 and Rv1422 (Fig. 3.1).

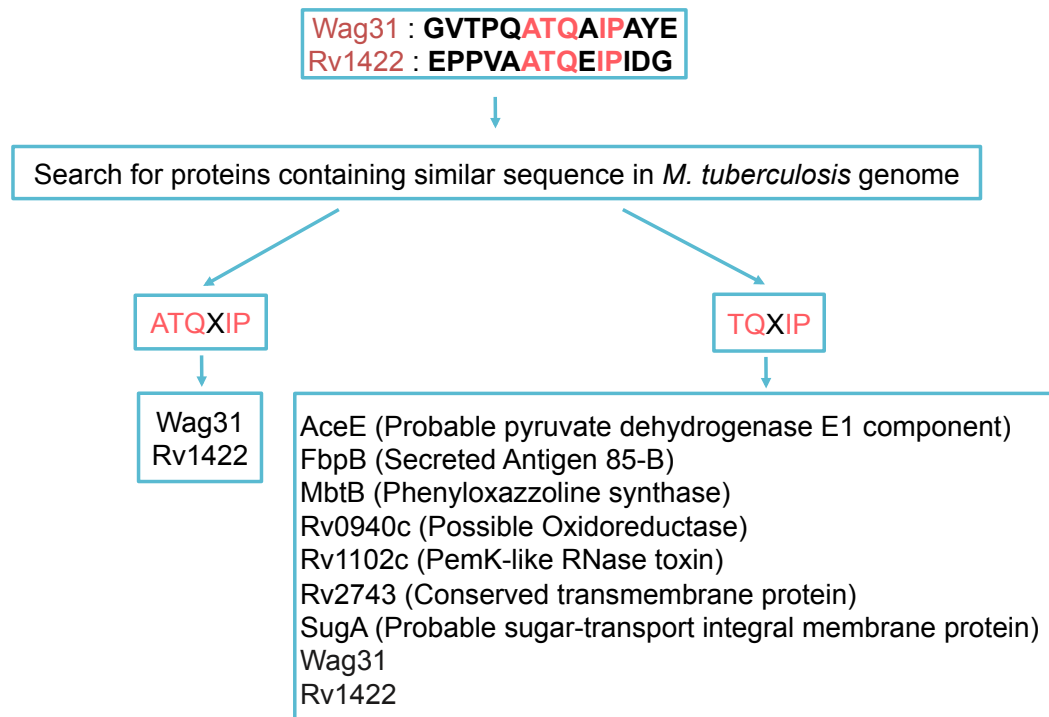


Fig. 3.1: In silico search for putative substrates of PknA/PknB. Initial search with ATQXIP identified only Wag31 and Rv1422, however reducing stringency to TQXIP motif resulted in identifying 7 putative substrates of PknA/PknB.

However, a second round of investigation done by reducing the stringency of the consensus using TQXIP sequence revealed seven new candidate proteins that contain one or more of the potentially phosphorylated TQ motif. The list of candidate substrate protein is described in Fig. 3.2. All the peptides generated had MA residues at the amino terminus as a way to tag the peptides while verifying their sequence. The poly-lysine (KKK) tail at the carboxyl-terminus adds a net positive charge to the peptides at low pH thereby helping in their adsorption onto p81 phosphocellulose paper (101).

1.	FbpB	MARNDP TQQIP KLKLVKKK
2.	AceE	MAEFRDT QRIPV SDKKK
3.	MbtB	MASVLAT QVVAGI RKKK
4.	Rv0904c	MATPAPT QPIPI LKLVKKK
5.	Rv1102c	MASCDNT QTIPV CDKKK
6.	SugA	MASAPLT TQQIP SLGKKK
7.	Rv2743c	MATIAVT QAIPA TLLKKK

Fig 3.2: List of candidate proteins obtained from the *in silico* search for substrates of PknA/PknB. Each of the seven putative substrate proteins of PknA/PknB containing preferred phosphorylation motif highlighted in blue (TQXIP) was custom synthesized as 17-mer peptides for initial screening by *in vitro* phosphorylation.

2. PknB maximally phosphorylates FbpB and Rv1102c *in vitro*

In order to determine whether PknA and/or PknB phosphorylate any of these new substrate candidates, I synthesized a series of peptides that include the TQ motif and the

surrounding residues of these proteins. All the peptides were synthesized as custom oligopeptides of 18mers with sequences spanning upstream and downstream of TQXIP motif (Fig. 3.2). To test if these peptides can be phosphorylated by PknA/B, I also needed to purify active PknA/B kinase proteins. The intracellular kinase domains of *M. tuberculosis* PknA and PknB were expressed and purified as fusion proteins with glutathione-S-transferase (GST) tag from *E. coli*. As described before, a preliminary *in vitro* kinase assay was performed with Rv1422 to confirm their catalytic activity. Each synthetic peptide (200ng) was then subjected to *in vitro* kinase assay with PknA or PknB (200ng) in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The peptide of Wag31 was included as a positive control, along with peptide-only and enzyme-only as negative controls. The reaction was performed for two different incubation periods (20 and 40 min), and the incorporation of ^{32}P into peptides was measured using a scintillation counter. Fig. 3.3 shows that PknA highly phosphorylates the peptides of Rv0940c and SugA while PknB strongly phosphorylates those of Rv1102c, FbpB, and SugA, suggesting that PknA and PknB show specificity even with these short synthetic peptides.

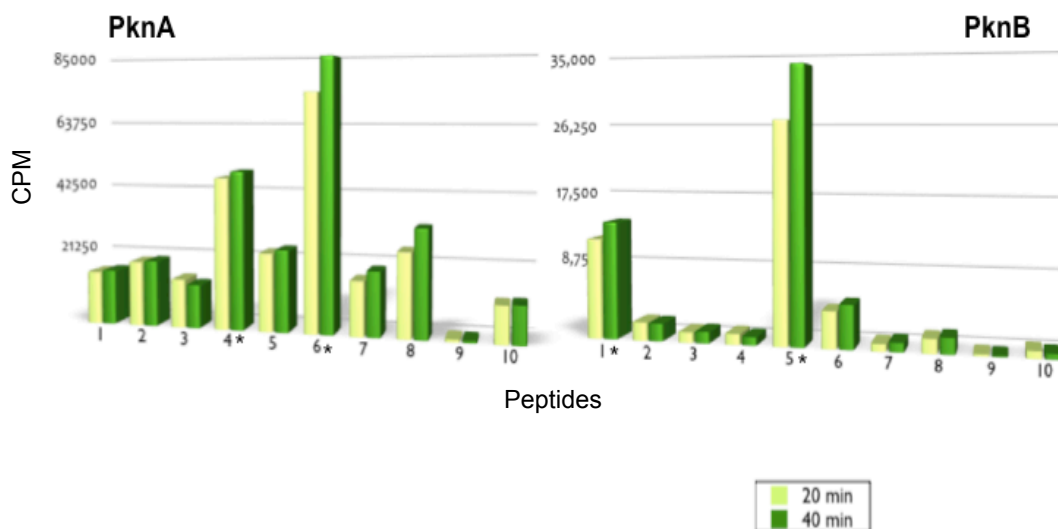


Fig. 3.3: *In vitro* kinase assay with synthetic peptide of substrate candidates in the presence of PknA/B and [γ - 32 P]ATP. The yellow bars indicate the levels of phosphorylation in counts per minute for 200 ng of each peptide for 20 minutes of reaction incubation time and green bars indicate the same for 40 minutes of reaction incubation time. X-axis: Peptides; Y-axis: Counts per minute (CPM). Kinase assay reactions run using peptides labeled as follows; 1.FbpB, 2.AceE, 3.MbtB, 4.Rv0940c, 5.Rv1102c, 6.SugA, 7.Wag31, 8.Rv2743c, 9.Peptide only control; and 10.Kinase only control.

Among these substrate candidates phosphorylated by PknA or PknB, I chose to further study FbpB (Fibronectin binding protein B) for the following reasons; 1) It is a member of a mycolyl transferase enzyme complex that is involved in the biosynthesis of trehalose dimycolate, a major cell wall components in mycobacteria, which contributes to the impermeable nature of mycobacterial cell wall and thereby their virulence, and 2) It interacts with extracellular matrix protein fibronectin and this interaction has been suggested to play a role in binding of tubercle bacillus cell to the mucosal surface and thus in the phagocytosis of the bacteria by the monocytes (80).

3. FbpB is highly phosphorylated *in vivo* in *Mycobacterium smegmatis*

Since PknB phosphorylated the synthetic peptide of FbpB, I decided to further test whether PknB could also phosphorylate the FbpB protein *in vitro*. To do this, I first attempted to express the 6His-tagged *fbpB* gene in *E. coli* but this was not successful, probably due to difference in codon usage between *E. coli* and *M. tuberculosis*. Consistent with this, Kravchenko *et al.* (2007) showed that altering a few amino acid codons of FbpB resulted in high expression levels of FbpB in *E. coli* (102). As an alternative approach, I decided to express and purify *M. tuberculosis* FbpB from *M. smegmatis*. The *M. tuberculosis fbpB* gene was amplified by PCR with the C-terminal strep-tag and cloned behind an acetamide-inducible promoter in an integration vector

pMH94A (pMH94A-P_{acet}-*fbpB*-*strep*). This plasmid was then transformed into *M. smegmatis* and the recombinant FbpB-strep was expressed by adding 0.2% acetamide, and purified by using the Strep-Tactin affinity purification system (Novagen). Activity of PknB was tested by an *in vitro* kinase assay with the Rv1422 protein, a known substrate of PknB, where Rv1422 showed a high level of phosphorylation, suggesting that this PknB was an active enzyme (In the work presented in this dissertation, activity of both PknA and PknB has been confirmed for every *in vitro* kinase assay by this technique, data not shown). When this FbpB protein was incubated with PknB and [γ -³²P]ATP, it showed very weak signal for phosphorylation (Fig. 3.4). The presence of other strong signals is a result of long exposure time for this autoradiograph.

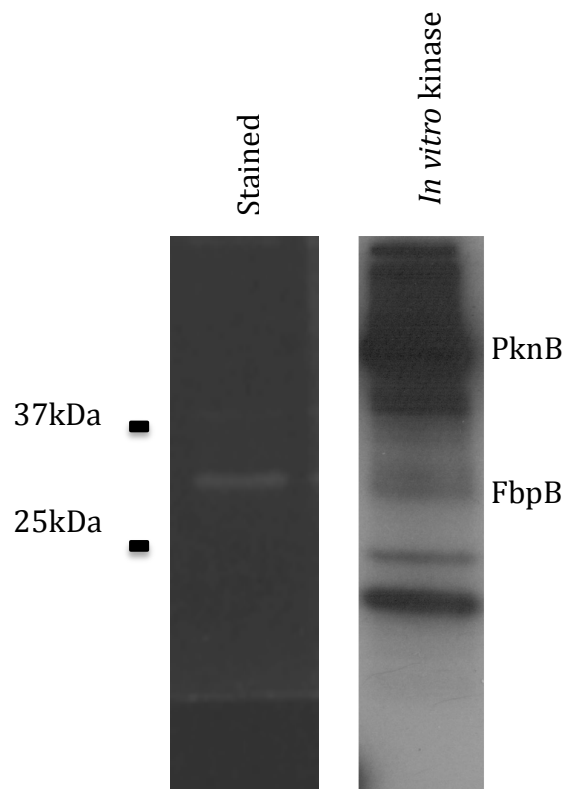


Fig. 3.4: Weak phosphorylation of FbpB purified from *M. smegmatis* by PknB in the presence of [γ -³²P]ATP. Left panel shows Gel Code stained gel. Right panel is the autoradiograph for *in vitro* kinase assay.

To obtain the signal intensity from FbpB shown in Fig. 3.4 (right column), I had to expose the gel for a long time and the signal from FbpB was even weaker than nonspecifically phosphorylated proteins (Left panel, Fig 3.4). One possible reason for this low level of radioactivity (phosphorylation) from FbpB was that FbpB was already phosphorylated in *M. smegmatis* before the purification. To test this possibility, I measured the phosphorylation of the FbpB protein without incubating with PknB by a Western blot analysis with a phospho-[S/T]Q antibody (Fig. 3.5). The FbpB protein revealed a strong Western signal, suggesting that FbpB had already been highly phosphorylated in *M. smegmatis*. Since there exists only one TQ motif in FbpB (T234), the synthetic peptide of FbpB was highly phosphorylated by PknB, and phospho-[S/T]Q antibody detected high level of phosphorylation in FbpB, I hypothesized that the ²³⁴T is a phosphorylation site.

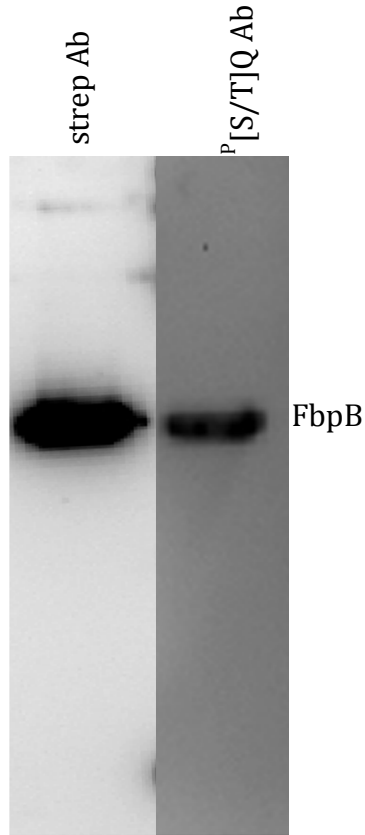


Fig. 3.5: FbpB has been phosphorylated in *M. smegmatis* before purification. Western blot with a phospho-[S/T]Q antibody (lane 2) followed by stripping and re-probing with a strep antibody against Strep-Tactin purified FbpB (lane 1),

4. Secreted FbpB is not phosphorylated in *M. smegmatis*

Since FbpB is the second major secreted protein in *M. tuberculosis* after ESAT-6, I also wanted to determine if the secreted form of FbpB is also phosphorylated (103). To test this possibility, *M. smegmatis* cells containing pMH94A-P_{acet}-*fbpB-strep* was cultured in the presence of 0.2% acetamide until late log-phase, and intracellular FbpB-strep was purified as described above. The secreted extracellular FbpB-strep was also purified by collecting the culture supernatant and precipitated total protein with ammonium sulfate (60%), and using the Strep-Tactin purification Kit. After dialysis,

approximately equal amount of the intracellular and extracellular FbpB-strep was subjected to a gradient SDS-PAGE (4 - 12%, Bio-Rad) to analyze by an immunoblot analysis with a strep antibody or a phospho-[S/T]Q antibody (Fig. 3.6).

FbpB undergoes signal peptide processing, which leads to the removal of 40 residues at the N-terminus during its secretion (77). In Fig. 3.6, we observed two major bands for FbpB-strep. While I plan to identify these bands by Edman degradation and mass spectrometry, predicted molecular weight of these bands are consistent with the processed form (the lower band with ~30 kDa) and the unprocessed form of FbpB (the upper band with ~34 kDa). Moreover, it is known that the secreted FbpB exists in two different forms, one as cell wall associated and the other as completely secreted into the extracellular environment (77). Consistent with this, I found both unprocessed and processed FbpB proteins from cell pellet (Intracellular, left column, Fig. 3.6) but mainly processed form of FbpB from supernatant (Extracellular, left column).

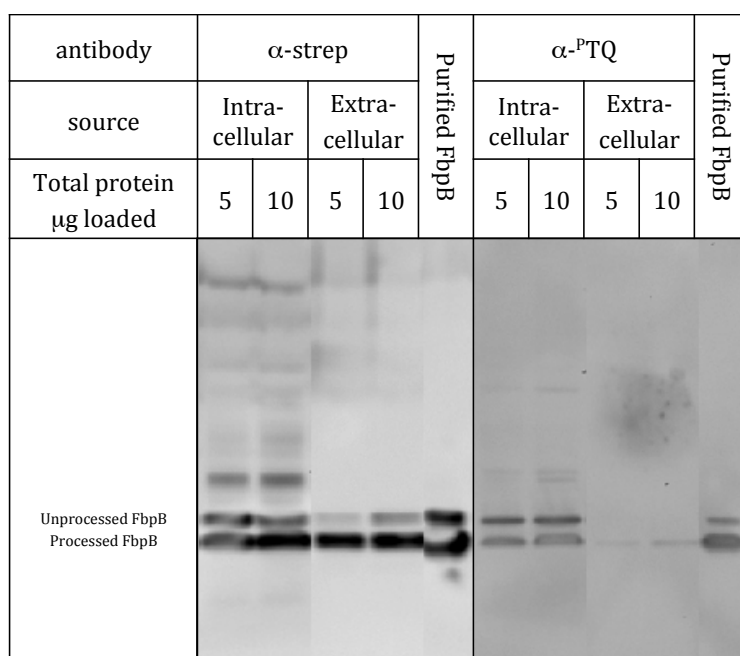


Fig. 3.6: Only intracellular FbpB is phosphorylated. Western blot with intracellular (CELL) and culture filtrate protein (CFP) of FbpB using a strep antibody followed by re-probing with a phospho-[S/T]Q antibody.

Interestingly, while both processed and unprocessed forms of intracellular FbpB were highly phosphorylated, the secreted FbpB did not show any phosphorylation (Intracellular and Extracellular, right column, Fig. 3.6), suggesting that the phosphorylation status of FbpB may be important for determining whether FbpB is attached to cell wall or completely secreted, or for processing and secretion processes of FbpB.

To further confirm the phosphorylation status between the intracellular and the extracellular FbpB, I conducted a 2D western blot with protein samples used in Fig 3.6 (Fig. 3.7). 200 µg of purified FbpB-strep was first rehydrated in a 2D strip (pH 4.7-5.9), and subjected to isoelectric focusing and 2D SDS-PAGE. First, a western blot was performed with a phospho-[S/T]Q antibody, and the membrane was stripped before probing with a strep antibody. Fig. 3.7 shows that most of the purified FbpB protein in the cell lysate is phosphorylated (Intracellular, Fig. 3.7), but after secretion there seems to be a loss of phosphorylation (Extracellular, Fig 3.7), confirming that the extracellular form of FbpB is not phosphorylated.

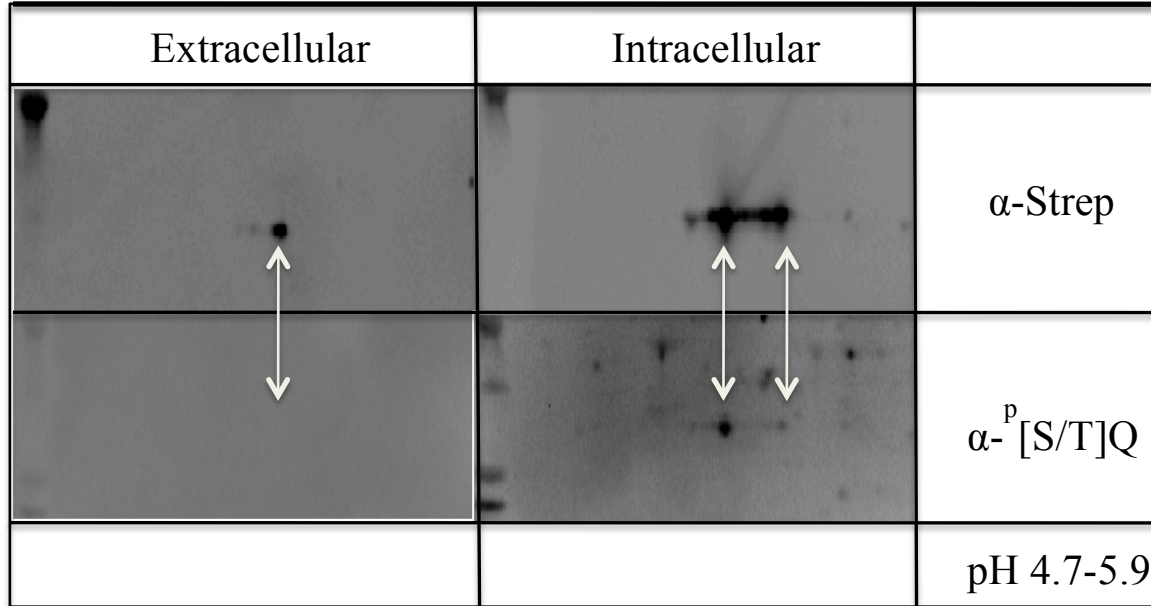


Fig. 3.7. Different phosphorylation pattern between intracellular and extracellular FbpB. 2D-Western blot of intracellular and extracellular FbpB with a strep (top panel) and a phospho-[S/T]Q (bottom panel) antibody.

5. FbpB is phosphorylated in *M. tuberculosis*

To examine whether FbpB is phosphorylated in *M. tuberculosis*, the pMH94A- $P_{\text{acet-}fbpB}$ -strep construct was transformed into an attenuated *M. tuberculosis* mc²6030 strain and cultured in the synthetic Sauton's minimal media for 3 weeks. Cells were further incubated for additional 3 days with 0.2% acetamide to induce the expression of FbpB-strep. Intracellular Strep-FbpB was purified using Strep-Tactin affinity column and subjected to western blot with a phospho-[S/T]Q and a strep antibody (Fig. 3.8). The FbpB-strep protein purified from *M. tuberculosis* showed processed and unprocessed FbpB, similar to those found in *M. smegmatis*. Western blot with a phospho-[S/T]Q antibody demonstrated the *in vivo* phosphorylation of FbpB purified from *M.*

tuberculosis. This experiment also suggested that processing/secretion process for FbpB in *M. smegmatis* is similar to that of *M. tuberculosis*.

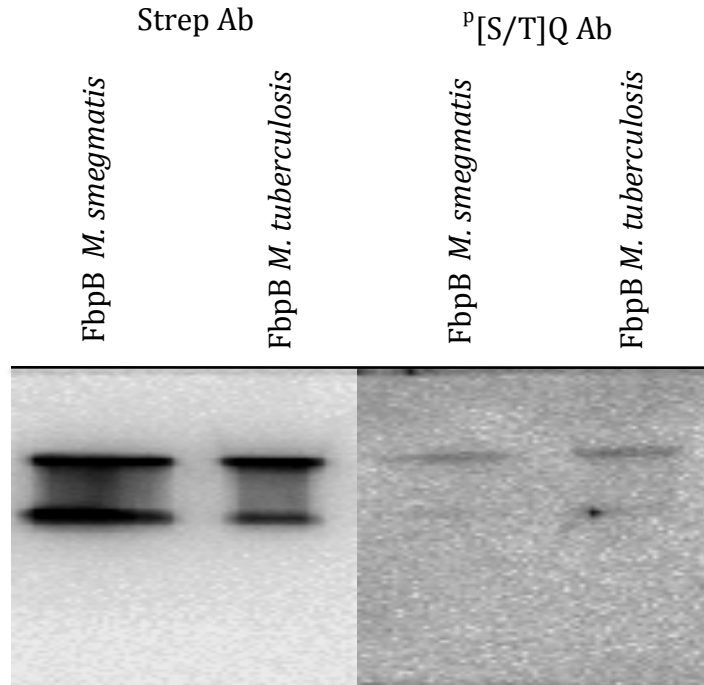


Fig. 3.8. FbpB is phosphorylated in vivo in *M. tuberculosis*. Western blot of intracellular FbpB from *M. tuberculosis* with a strep and a ^P[S/T]Q antibody. FbpB purified from *M. smegmatis* used as a positive control.

6. FbpB is phosphorylated at ²⁵⁵T

With *in vivo* phosphorylation of FbpB in *M. tuberculosis* identified, I wanted to determine the actual site(s) of phosphorylation in FbpB. To do this, FbpB-strep purified from *M. smegmatis* was resolved using 2D-PAGE and desired spots were analyzed by mass spectrometry. Mass spectrometry analysis revealed a weak phosphorylation signal

from ^{255}T , but not from the predicted ^{234}T (data not shown). This was disappointing, as ^{255}T did not represent the predicted phosphorylation site based on the motif TQXIP that was hypothesized to be the preferred phosphorylation motif for PknA/PknB. Upon analyzing the trypsin digest report for FbpB, I came to conclude that it's possible that the peptide length spanning the ^{234}T residue may not be the ideal ionization limit of mass spectrometry analysis. A second round of digest with chymotrypsin or other enzyme could be done to overcome this barrier and confirm the phosphorylation site in FbpB by mass spectrometry.

7. Mycolyl transferase activity of FbpB is affected by phosphorylation

Next, because FbpB is a mycolyl transferase, I wanted to test whether phosphorylation has any effect on its enzymatic activity. To measure the mycolyl transferase activity, I collaborated with Dr. Don Ronning group (at the University of Toledo, OH) by using an assay system they developed (104). I incubated the purified FbpB with *p*-nitrophenyl 6-*O*-octanoyl- β -d-glucopyranoside and β -glucosidase at room temperature and the rate of *p*-nitrophenolate production was measured as the absorbance at 405nm. A negative control was a reaction without FbpB. In addition, FbpC purified in Dr. Ronning group was included as a positive control since they used it to standardize the assay system.

In Fig. 3.9 (A), wild-type FbpB purified from *M. smegmatis* shows mycolyl transferase activity, suggesting that this assay system can be used to test whether the phosphorylation of FbpB is involved in regulating the mycolyl transferase activity of FbpB. The right panel (B) in Fig. 3.9 shows the positive control assay performed with the

standardized FbpC enzyme (blue line) along with a negative control having no enzyme/blank reaction (red line).

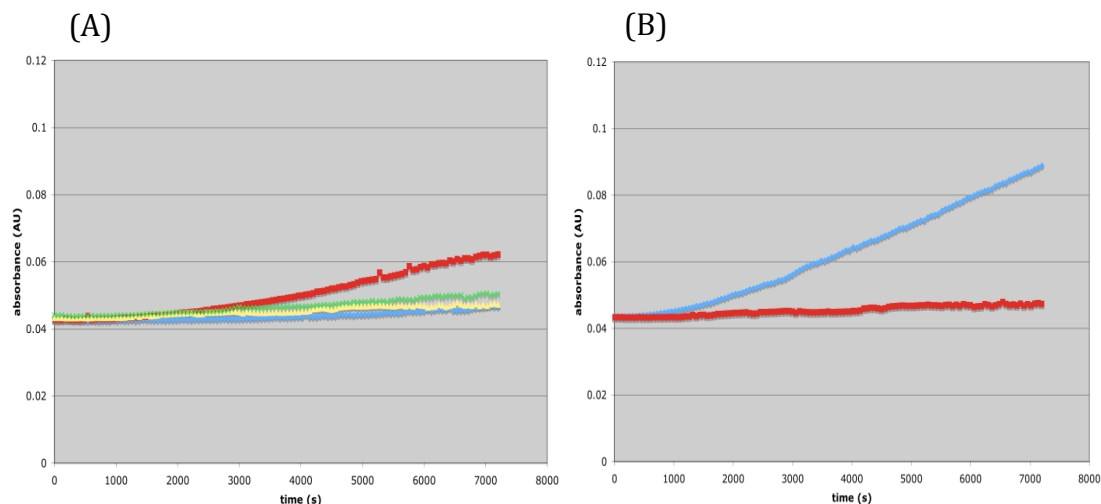


Fig. 3.9. Mycolyl transferase assay. (A) Wild-type FbpB purified from *M. smegmatis* was incubated with the synthetic substrate and samples tested include; (blue line) WT FbpB purified from *M. smegmatis* and dialyzed against a buffer containing glycerol; (red line) WT FbpB purified from *M. smegmatis* and dialyzed against a buffer without glycerol; (yellow line) Blank; (green line) WT FbpB purified from culture filtrate protein and dialyzed against a buffer without glycerol. (B) FbpC purified from *E. coli* used as a positive control for enzyme assay. Samples tested include (red line) No enzyme blank; (blue line) FbpC purified from *E. coli*.

As I confirmed that wild-type FbpB-strep had mycolyl transferase activity in Fig. 3.9, I wanted to test whether phosphorylation has any effect on its enzymatic activity using wild-type FbpB, phospho-ablative FbpBT234A and phospho-mimetic FbpBT234E. by using the assay system used in Fig.3.9. Fig. 3.10 (left panel) shows that FbpBT234A has lower mycolyl transferase activity than wild-type FbpB. However, a similar experiment with FbpBT234E and wild-type FbpB activity on right panel suggested almost similar activity of both these proteins, suggesting that phosphorylation could be enhancing mycolyl transferase activity of FbpB. In summary, although ²³⁴T is yet to be confirmed as a phosphorylation site in FbpB, it still seems to be an important residue in determining the mycolyl transferase activity in FbpB.

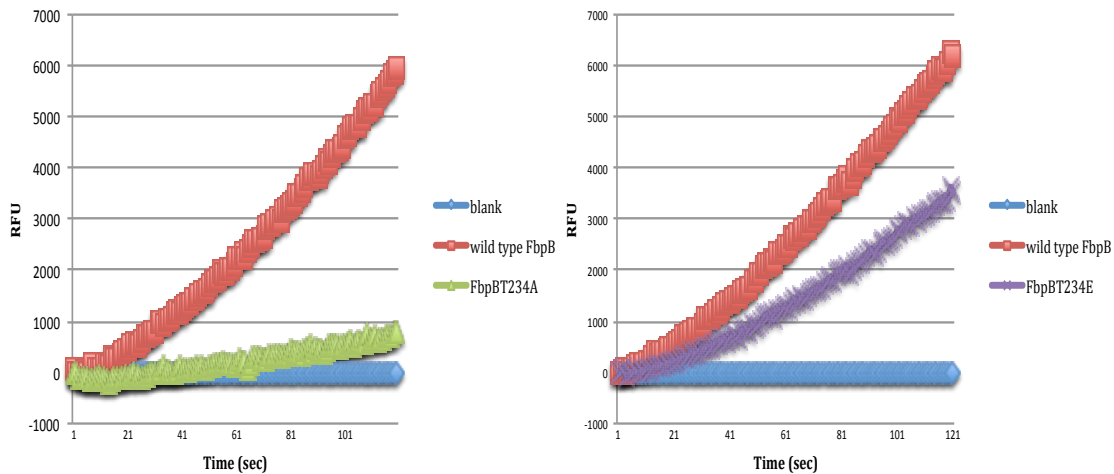


Fig. 3.10: Phosphorylation might enhance mycolyl transferase activity. Wild type and FbpBT234A purified from *E. coli* subjected to enzyme assay. Left panel: FbpBT234A mutant showing lower activity than wild type FbpB. Right panel: FbpBT234E purified from *E. coli* shows higher activity as compared to FbpBT234A mutant. RFU value indicated for each sample here represents average of triplicate reactions.

Discussion

In this study, I demonstrated an *in silico* method to screen for potential substrates of PknA/PknB. This approach provides an opportunity to identify potential targets of PknA/PknB phosphorylation, and also to later characterize the substrate proteins so as to determine their regulatory function thereby identifying the role(s) of PknA/PknB in *M. tuberculosis*. The peptide library screen employs *in vitro* kinase assay using the STPKs-PknA and PknB that catalyze the transfer of the γ -phosphate of adenosine triphosphate (ATP) to substrate peptide's thr residues.

Previously, Kang *et al.* (2005) have shown that PknA and PknB predominantly phosphorylate at thr residues, with a strong phosphorylation motif preference for ATQXIP. In this work, it is interesting to note that PknA and PknB phosphorylate

different sets of peptides thereby suggesting their slightly different substrate specificity (52). Among the seven candidate peptide substrates, four of the peptides namely, FbpB, Rv0940c, Rv1102c and SugA showed highest level of phosphorylation by PknA/PknB. Rv0940c whose peptide was maximally phosphorylated by PknA, is predicted to be an oxidoreductase. Rv1102c is an mRNA interferase, with its peptide showing highest phosphorylation by PknB when compared to PknA. Further investigations on this protein led us to the finding that Rv1102c is part of a toxin-antitoxin system (Rv1102c-Rv1103c system) (105). Additionally, Dr. Jeong-Sun Han and I were able to demonstrate that 1) Rv1102c interacts with Rv1103c, 2) Rv1103c antagonizes the mRNA interferase activity of Rv1102c and thus acts as its cognate antitoxin, and lastly 3) the expression of Rv1102c is coupled to that of Rv1103c. The research paper titled ‘Characterization of chromosomal toxin-antitoxin, Rv1102c-Rv1103c system in *Mycobacterium tuberculosis*’ was second authored by me and published in 2010 (105). SugA is part of an ABC transporter complex, whose role in virulence is highlighted by its specific function in trehalose uptake.

Of the newly identified candidate substrate proteins, FbpB seemed to be of great interest as it belongs to a complex known as antigen 85 complex involved in the synthesis of cell wall component mycolic acid, which is one of the major virulence factors of *M. tuberculosis*. I have shown that FbpB is phosphorylated *in vivo* in *M. smegmatis* as well as in *M. tuberculosis* and that the 30 KDa secreted form of FbpB is not phosphorylated. Additionally, I also determined that in *M. smegmatis* intracellular FbpB is phosphorylated while the extracellular is not, suggesting a possible role of phosphorylation in its processing and/or secretion (Fig. 3.5). Based on this analysis, I investigated the role of

FbpB phosphorylation in its secretion by purifying secreted form of FbpB from *M. smegmatis* culture-filtrate and compared it with intracellular FbpB by western blot with phospho-[S/T]Q antibody. Furthermore, mass spectrometry analysis of FbpB purified from *M. smegmatis* revealed ^{255}T as a single phosphorylation site in FbpB and currently this phosphorylation site needs to be confirmed. The synthetic mycolyl transferase assay employed for assessing FbpB's enzyme activity suggested phosphorylation may reduce the mycolyl transferase activity of FbpB. Even though, mass spectrometry did not identify ^{234}T as the phosphorylation site, phospho-mutants analysis suggests that the residue ^{234}T is important for mycolyl transferase activity of FbpB. This data could thus suggest the reason for the apparent difference in level of mycolyl transferase activity seen between FbpB and the other members of the antigen 85 family; FbpA and FbpC. FbpB's reduced mycolyl transferase activity could be due to the probable lack of ^{234}T phosphorylation upon secretion since we know that secreted form and FbpBT234A had the least enzyme activity.

The hydrophobic nature of mycobacterial cell wall confers passive resistance against chemicals, dehydration, and degradation by host factors, inactivation of reactive oxygen intermediate (ROI) and reactive nitrogen intermediate (RNI), and prevents entry of toxic anti-TB compounds and antibiotics into the cell (106,107). Careful regulation of the STPKs enables precise cellular responses to external signals, allowing bacteria to survive in a variety of changing environmental conditions. As discussed earlier, FbpB is a predominantly secreted protein forming the bulk of culture filtrate protein in mycobacteria that is predicted to play a role in internalization and adaptation of *M. tuberculosis*. The kinase responsible for FbpB phosphorylation and the role of FbpB

phosphorylation in host-pathogen interaction, for example in fibronectin binding, and pathogenesis are yet to be answered.

In summary, I provide an *in silico* peptide library screening method to fish for PknA and PknB targets in *M. tuberculosis*, which could possibly be applied to screen for substrates of other kinases. Future work focusing on other two candidate substrates; SugA and Rv0940c could be tested in order to provide insight into the way these protein act as molecular switches that regulate diverse cellular event such as growth, development and pathogenesis by signaling molecules. The global urgency related to TB, which is currently the leading infectious disease causing the highest mortality, with the added potential of these proteins in regulating vital metabolic pathways, provide strong rationales for investigating the functions and mechanisms of the *M. tuberculosis* STPK signaling systems.

APPENDIX A

EXPERIMENTAL PROCEDURES

A.1. Culture conditions.

H37Rv, mc²6230 *M. tuberculosis*, and *M. smegmatis* MC²155 cultures were grown at 37 °C in Middlebrook 7H9 liquid medium (Difco) supplemented with 10% ADC [ADC = 5% (w/v) BSA fraction V powder, 2% (w/v) glucose and 0.85% (w/v) NaCl], and 0.05% (w/v) Tween-80, or on Middlebrook 7H9-ADC agar plates supplemented with 0.05% (w/v) glycerol. Kanamycin (50 µg/ml), hygromycin (50 µg/ml) or apramycin (50 µg/ml) was added to culture media where required. *Escherichia coli* DH5α (Invitrogen) was used as a cloning host, and *E. coli* BL21 (DE3) for expression of recombinant proteins. Cultures were grown at 37 °C in LB broth or solid medium with apramycin (50 µg/ml), kanamycin (50 µg/ml), chloramphenicol (34 µg/ml) or ampicillin (50 µg/ml) where appropriate.

A.2. Plasmid construction

For *in vitro* kinase assay of the chimeric proteasome complex, *strep-prcA* was PCR amplified using the genomic DNA of *M. tuberculosis* H37Rv and cloned into an integrating plasmid pCK202 driven by the acetamide-inducible promoter. All the primers used in Chapter 2 are listed in Table 2. This construct, named as pCK206 (All the constructs made are listed in Table 1), comprises the attachment site (*attP*) and the integrase gene (*int*) of mycobacteriophage L5 for integration (108,109). For *in vivo* phosphorylation analysis of PrcA in *DprcBA M. smegmatis*, *prcBA-strep* was PCR amplified by using primers TBprcBA pUAB100-F and TBprcBA-C-strep3', and cloned

behind a constitutive *hsp60* promoter in a replicating plasmid, pCK315 (and named as pCK322). For testing the phosphorylation of WT and mutant forms of PrcA in *E. coli*, *prcBA-strep* was PCR amplified by using primers C-StrepTBprcB-5 and TBprcBA-C-strep3', and cloned under T7 promoter in pACYCDeut-1 (Novagen) (named as pCK343). To construct a $\Delta prcBA$ *M. smegmatis* strain (KMS98), 5'- and 3'-flanking regions of the *prcBA_{Ms}* genes was amplified by PCR by using primers Dn-PrcA-F, Dn-PrcA-R, Up-PrcB-F, and Up-PrcB-R, and the genomic DNA of WT *M. smegmatis* MC²155. The hygromycin resistance gene was inserted between the two flanking regions in pRH1351 and named as pCK260.

For *in vivo* and *in vitro* kinase assay of the FbpB, *strep-fbpB* was PCR amplified using the genomic DNA of *M. tuberculosis* H37Rv. All the primers used in Chapter 3 are listed in Table 3, and cloned into an integrating plasmid pCK202 carrying an acetamide-inducible promoter, and named as pCK206 (All the constructs are listed in Appendix A, Table 1). Plasmids expressing *fbpBT234A* and *fbpBT234E* were generated by using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) with primers indicated in Table 3.

A.3. Protein expression, purification and analysis of recombinant proteins

To purify the chimeric proteasome complex, *M. smegmatis* KMS65 was grown to mid-log phase where expression of *strep-prcA* was induced by 0.2% acetamide for 4 hr. Strep-PrcA was purified by using the Strep-Tactin Affinity Purification Kit (Novagen). The *M. tuberculosis* proteasome complex for the *in vivo* phosphorylation was purified from $\Delta prcBA$ *M. smegmatis* carrying pCK322 plus pCK5 (KMS109), pCK7 (KMS108), or pMH94 vector (KMS157) by overexpressing *pknB* at mid-log phase with 0.2%

acetamide for 4 and 16 hr. A parallel flask without acetamide was also cultured for 16 hr. PrcA-strep was purified by using the Strep-Tactin Affinity Purification Kit (Novagen) as described above. To test the effect of PknA on proteasome complex formation in *E. coli*, pCK343 was transformed into *E. coli* BL21 (DE3) containing pCK3 or pCK4, which were cultured in Overnight Express Autoinduction medium at 37 °C for 10 hr, and PrcA-strep was purified as described above. For 2-D PAGE, immunoblot, and mass spectrometry, total protein extraction from *M. tuberculosis* harboring pMH94 plasmid alone, pCK5, or pCK7 and subsequent 2-D SDS-PAGE were performed as described previously. For the *in vivo* phosphorylation of PrcA, 30 µg of each purified PrcA-Strep was similarly analyzed in 2-D SDS-PAGE with IPG strips of pH 4-7 (Bio-Rad). Then, Western blots were performed by a phospho-T antibody, and subsequently with a strep-II antibody (Novagen) after stripping off the first antibody by using a stripping buffer (100 mM 2-mercaptoethanol, 2% (w/v) SDS, 62.5 mM Tris-HCl, pH 6.7) at 50 °C for 30 min. Mass spectrometry for the identification of the phosphorylation sites in PrcA was performed as previously described. To analyze the proteasome complex in *M. smegmatis* overexpressing *pknA*, 15 µg of PrcA-strep plus PrcB complex used in Fig. 3.5 was analyzed in 2-D SDS-PAGE with Flamingo Stain (Bio-Rad).

For purification of FbpB-strep, and all the phosphomutants of FbpB used in Chapter 3 cells were grown to mid-log phase where expression of *FbpB-strep* was induced by 0.2% acetamide until stationary phase. FbpB-strep including all its phosphomutant proteins were purified by using the Strep-Tactin Affinity Purification Kit (Novagen). To purify extracellular FbpB, culture filtrate protein was isolated using 40% ammonium sulfate (AS) precipitation method. Mycobacterial cells expressing FbpB were

inoculated in modified Sauton's medium and incubated at 37°C until early stationary phase. The cultures were centrifuged at 16,000 g for 30 min at 4°C and filtered through 0.22µm Millex GV PVDF membrane (Millipore). The filtrate was later used for a 40% AS precipitation to isolate culture filtrate protein and purification of recombinant FbpB was carried out using strep affinity column (Profinia) as described before.

A.4. In-gel proteasome assay

3 µM proteasome complex purified from *E. coli* containing pCK343 was incubated with 1 µM GST-PknA or GST-PknB in the presence of 1 mM ATP in kinase assay buffer (50 mM HEPES, 50 mM NaCl, 10 mM MgCl₂, 2 mM MnCl₂) for 2 hr at 37°C. Reaction was stopped by adding Native Sample buffer (Bio-Rad), and proteins were separated on two native PAGE gels; one was used to visualize proteins by staining with Flamingo Fluorescent stain (Bio-Rad) and the other was used for an in-gel proteasome assay by incubating the gel in proteasome assay buffer (50 mM HEPES pH 7.4, 5 mM EDTA) and 100 µM Suc-LLVY-Amc (Boston Biochem) for 30 minutes at 30°C. Fluorescence from Amc released was analyzed by the ChemiDoc XRS system and Quantity one 1-D analysis software (Bio-Rad). Activity of PknA and PknB was tested in a parallel reaction that included 2 µM 6His-Rv1422, a known substrate of PknA and PknB, and its phosphorylation was analyzed in immunoblotting with a phospho-T antibody.

A.5. H₂O₂ susceptibility assays

To examine the effect of the proteasome on *M. smegmatis* survival in the presence of H₂O₂, wild-type (KMS197) and Δ *prcBA* *M. smegmatis* strains (KMS128 and

KMS116) were cultured overnight in 7H9 medium (50 µg/ml hygromycin and 50 µg/ml apramycin), diluted to OD₆₀₀=0.01 in 7H9 medium, treated with 4 mM H₂O₂, and incubated at 37 °C for 45 min. The cultures were serially diluted into a buffer containing 0.8% NaCl and 0.01% Tween-80 and plated on 7H9 plates for CFU determination. The influence of *pknA*-expression on the survival in the presence of H₂O₂ was tested by using the *ΔprcBA M. smegmatis* strain constitutively expressing *prcBA* with induction of *pknA*, *pknB*, or vector (KMS108, KMS109, and KMS157) overnight in 7H9 containing hygromycin, apramycin, and kanamycin. As a control, *ΔprcBA M. smegmatis* carrying *pknA*-expression cassette alone (KMS201) was included. To induce the expression of kinases, overnight culture was diluted to initial OD₆₀₀=0.05 and incubated with 0.1% acetamide for 5 hrs. To test for sensitivity to H₂O₂ cultures were further treated with 4 mM H₂O₂ and incubated at 37° C for 45 min, followed by serial dilution to determine CFU.

A.6. Effect of H₂O₂ on proteasome assembly and PknA phosphorylation

ΔprcBA M. smegmatis that constitutively expresses *prcBA* and contains *pknA*- (KMS109) or *pknB*-expression cassette (KMS108) was cultured with 0.2% acetamide for 6 hr. Cells were then treated with 2 mM H₂O₂ for 1.5 and 4 hr. 20 µg total lysate protein purified with TRIzol was used for SDS-PAGE followed by Western blot with a PrcB polyclonal antibody. Autophosphorylation of PknA in response to H₂O₂ was tested in *E. coli* BL21 (DE3) containing a pGEX-4T-3-*pknA* plasmid (pCK3) cultured in the presence of 0.5 mM IPTG for 6 hr, and then exposed to 2 mM H₂O₂ for 0, 60, and 120 min. The levels of the PknA phosphorylation and the GST-PknA were determined by Western blottings with phospho-T and GST antibodies.

A.7. *In vitro* kinase assay

To test the phosphorylation of FbpB *in vitro*, FpbB-strep, FbpBT234A, GST-PknB, or Rv1422-his protein was incubated with strep-FbpB in 1X kinase buffer (50 mM Tris-HCl at pH 7.5, 50 mM NaCl, 10 mM MgCl₂, 10 mM MnCl₂) along with 1 μCi of [γ -³²P]ATP, and incubated at room temperature for 1 hour. Reactions were terminated by adding SDS gel-loading buffer and the proteins were resolved by 8% SDS-PAGE, followed by staining with GelCode Blue (Pierce). Gels were dried under vacuum with the help of Hoefer slab gel dryer (Model GD2000) at 65 °C for 3 hours before exposing to CL-XPosure film (Thermo Scientific). Phosphorylation of the phosphoablative forms of FbpB was examined by incubating of the proteasome complex containing FbpBTA with GST-PknB and 1 mM regular ATP. The relative phosphorylation levels of each protein were determined by immunoblot with either a phospho-[S/T]Q or phospho-T antibody and, stripped to be re-probed with anti-strep antibody or GelCode staining of parallel reactions.

A.8. Mycolyl transferase assay

To test the mycolyl transferase enzyme activity of FbpB and its mutant forms, a mycolyl transferase assay system developed by Dr. Ronning group (University of Toledo, OH) was used. For this, I collaborated with the laboratory of Dr. Donald Ronning (University of Toledo, Ohio) that has established an assay system to measure mycolyl transferase activity using synthetic substrates. Briefly, the system uses a synthetic substrate (100 μm), *p*-nitrophenyl 6-*O*-octanoyl-β-d-glucopyranoside that undergoes a nucleophilic attack on the ester linkage of the octanoyl moiety by the enzyme to release *p*-nitrophenyl β-d-glucopyranoside. This product of the first catalysis then serves as a

substrate for β -glucosidase that releases glucose and *p*-nitrophenolate the chromogenic product that is measured as absorbance at 405nm. For the mycolyl transferase assay, each protein sample used was approximately 1 μ m.

APPENDIX B

Table 1. Strains and Plasmids used in Chapter 2

Strain	Description	Reference
<i>E. coli</i>		
<i>E. coli</i> Top10	<i>E. coli</i> host for molecular cloning	Invitrogen
<i>E. coli</i> BL21 (DE3)	<i>E. coli</i> host for protein expression	Invitrogen
<i>M. smegmatis</i>		
mc ² 155	WT strain of <i>M. smegmatis</i>	This study
KMS65	<i>M. smegmatis</i> mc ² 155 carrying pCK206	This study
KMS98	$\Delta prcBA_{Ms}$ <i>M. smegmatis</i> mutant (<i>prcBA::hyg</i>)	This study
KMS108	KMS110 carrying pCK322	This study
KMS109	KMS111 carrying pCK322	This study
KMS110	KMS98 carrying pCK7	This study
KMS111	KMS98 carrying pCK5	This study
KMS116	KMS98 carrying pCK322	This study
KMS128	KMS98 carrying pCK315	This study
KMS153	KMS98 carrying pMH94	This study
KMS157	KMS153 carrying pCK322	This study
KMS179	<i>M. smegmatis</i> mc ² 155 carrying pCK435 and pCK5	This study
KMS183	<i>M. smegmatis</i> mc ² 155 carrying pCK435 and pMH94	This study
<i>prcba</i>	$\Delta prcBA_{Ms}$ <i>M. smegmatis</i> mutant constructed independently by Barry group	
Plasmids		
pMH94	mycobacterial integration vector (Kan ^r)	(52)
pUAB100	mycobacterial protein fragment complementation vector	(52)
pCK3	pGEX-4T-3 (Amp ^r) carrying <i>pknA</i> under P _{T7}	(52)
pCK4	pGEX-4T-3 carrying <i>pknB</i> under P _{T7}	(52)
pCK5	pMH94 carrying <i>pknA</i> under P _{acet} (Kan ^r)	(52)
pCK7	pMH94 carrying <i>pknB</i> under P _{acet} (Kan ^r)	(52)
pCK21	pGEX-4T-3 carrying <i>pknBK40M</i> under P _{T7}	(52)
pCK22	pGEX-4T-3 carrying	(52)

	<i>pknAK42M</i> under P _{T7}	
pCK40	pET28a carrying 6His-Rv1422 under P _{T7}	(52)
pCK202	pMH94A (Apra ^r) carrying P _{acet} The Km ^r gene in pMH94 vector was replaced with Apra ^r gene from pCKACEI.	This study
pCK206	pCK202 carrying <i>strep-prcA</i> under P _{acet}	This study
pCK260	pRH1351 carrying a Hyg ^r gene cassette in flanking regions of <i>prcBA</i> of <i>M. smegmatis</i> .	This study
pCK315	pUAB100A (Apra ^r). The Km ^r gene in pUAB100 vector was replaced with Apra ^r gene from pMV261.	This study
pCK322	pUAB100A carrying <i>prcBA-strep</i> under P _{Hsp60}	This study

Table 2. Strains and plasmids used in Chapter 3

Strain	Description	Reference
<i>E. coli</i>		
<i>E. coli</i> Top10	<i>E. coli</i> host for molecular cloning	Invitrogen
<i>E. coli</i> BL21 (DE3)	<i>E. coli</i> host for protein expression	Invitrogen
<i>M. smegmatis</i>		
mc ² 155	WT strain of <i>M. smegmatis</i>	(52)
KMS65	<i>M. smegmatis</i> mc ² 155 carrying pCK206	(52)
KMS98	Δ <i>prcBA</i> _{Ms} <i>M. smegmatis</i> mutant (<i>prcBA::hyg</i>)	(52)
KMS108	KMS110 carrying pCK322	This study
KMS109	KMS111 carrying pCK322	This study
KMS110	KMS98 carrying pCK7	This study
KMS111	KMS98 carrying pCK5	This study
KMS116	KMS98 carrying pCK322	This study
KMS128	KMS98 carrying pCK315	This study
KMS153	KMS98 carrying pMH94	This study
KMS157	KMS153 carrying pCK322	This study
KMS179	<i>M. smegmatis</i> mc ² 155 carrying pCK435 and pCK5	This study
KMS183	<i>M. smegmatis</i> mc ² 155 carrying pCK435 and pMH94	This study
Plasmids		
pMH94	mycobacterial integration	(52)

	vector (Kan ^r)	
pCK3	pGEX-4T-3 (Amp ^r) carrying <i>pknA</i> under P _{T7}	This study
pCK4	pGEX-4T-3 carrying <i>pknB</i> under P _{T7}	(52)
pCK7	pMH94 carrying <i>pknB</i> under P _{acet} (Kan ^r)	(52)
pCK21	pGEX-4T-3 carrying <i>pknBK40M</i> under P _{T7}	(52)
pCK22	pGEX-4T-3 carrying <i>pknAK42M</i> under P _{T7}	(52)
pCK40	pET28a carrying 6His-Rv1422 under P _{T7}	(52)
pCK202	pMH94A (Apra ^r) carrying P _{acet} The Km ^r gene in pMH94 vector was replaced with Apra ^r gene from pCKACEI.	This study

APPENDIX C

Table 3. Primers used in Chapter 2

Primer Name	Sequence
TBprcBA pUAB100-F	5'-TGCAGATCTTTCGAACATGACCTGGCCGTTGCCCGATC-3'
TBprcBA-C-strep3'	5'-AGTTTCCAGCTGTCACTTCTCGAACTGGGGGTGCGACCA GCCCGACGATTTCGCCGTC-3
Dn-PrcA-F	5'-GCGACAAACCCACCGAATAG-3'
Dn-PrcA-R	5'-TCATCTAGAATCCACGAGACGCTGGAC-3'
Up-PrcB-F	5'-TCCTCTAGAGCTGCAACGCATCTACCTC-3'
Up-PrcB-R	5'-CTATTCGGTGGGTTTGTGCTTAATTAAGTTCGGAGAAC GAGGA3'
C-StrepTBprcB-5	5'-GACTAGCATATGACCTGGCCGTTGCCCGATC-3'
Δ prcBA-F	5'-CTGGAGACCACGCTCAAAC-3'
Δ prcBA-R	5'-GGGAGTCAGGCAACTATGGA-3'
a712g_sense (T234A)	5'-ACGACTCCGCCCGCCGGTCCG-3'
a712g_antisense (T234A)	5'-CGGACCGCCGGCGGGAGTCGT-3'
a583g_sense(T195A)	5'-GGCAGTGCCGACGCCTCGGGTGGTG-3'
a583g_antisense (T195A)	5'-CACCACCCGAGGCGTCGGCACTGCC-3'
RV1422F	5'-CCAGGATCCGATGACCGATGGCATCGTC-3'
RV1422R	5'-CCTATCGATTCACTTCTCGAACTGGGGGTGCGACCATCG CCACGCGTCGTCAC-3'

Table 4. Primers used in Chapter 3

Primer Name	Sequence
FbpB-5'	5'-AGTCTAGCATATGACAGACGTGAGCCGAAAG-3'
FbpB-3'	3'-TAGTCTAGATCACTTCTCGAACTGGGGGTGCGACCAGCCGGCGC CTAACGAACT-3'
A700gTAFbpBF	5'-GGGAGCGCAACGACCCTGCGCAGCAGA-3'
A700gTAFbpBR	5'-TCTGCTGCGCAGGGTCGTTGCGCTCCC-3'
FbpBTEF	5'-GGGAGCGCAACGACCCTGAGCAGCAGATCCC-3'
FbpBTER	5'-GGGATCTGCTGCTCAGGGTCGTTGCGCTCCC-3'
FbpBSA5	5'-CCTGCTGGACCCCGCCAGGGGATGGGGC-3'
FbpBSA3	5'-GCCCCATCCCCTGGGCGGGTCCAGCAGG-3'

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ABSTRACT

ROLE OF THE PKNA AND PKNB KINASES IN *MYCOBACTERIUM TUBERCULOSIS*

by

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Advisor: Dr. Choong-Min Kang**Major:** Biological Sciences**Degree:** Doctor of Philosophy

To respond to environmental changes, *M. tuberculosis* possesses eleven "eukaryotic-type" Ser/Thr protein kinases. The aim of the study described in this dissertation was to identify role of two of these kinases; PknA and PknB that are essential in *M. tuberculosis*. Two approaches are described to screen for potential *in vivo* substrates of PknA/PknB. First approach is based on proteomic search by over-expressing PknA/PknB in *M. tuberculosis*. Proteomic search led to identification of proteasome to be a substrate of PknA and PknB in *M. tuberculosis*. Furthermore, I demonstrate that the phosphorylation of PrcA and PrcB by PknA regulates processing of Pre-PrcB. Additionally, I show that PknA phosphorylation of pre-PrcB and PrcA reduces the assembly of the proteasome complex and thereby enhances mycobacterial resistance to H₂O₂. Thus, H₂O₂ stress diminishes the formation of the proteasome complex in a PknA-dependent manner. The second approach to identify potential *in vivo* substrates is by a homology search using the preferred phosphorylation motif ATQXIP identified based on already known *in vivo* substrates of PknA/PknB. This screen identified 7 new putative substrates of PknA/PknB and I chose to investigate the candidate substrate FbpB.

This study has confirmed the role of PknA and PknB in regulating holo-proteasome assembly, function, and also highlighted the possible role of *M. tuberculosis* proteasome in oxidative stress response. The phosphorylation of proteasome is important for increasing resistance to H₂O₂ has been reported for the first time in this study. This study was also the first time that identified secreted form of FbpB has reduced mycolyl transferase activity when compared to intracellular FbpB and that the residue ²³⁴T is important for FbpB enzyme activity possibly due to their different phosphorylation status.

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