Serine/threonine phosphorylation in *Mycobacterium tuberculosis*: Identification of Protein Kinase B (PknB) substrates

by

Guinevere Kwun Wing Queenie Lee

B.Sc., University of British Columbia, 2004

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATED STUDIES

(Experimental Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)
April 2008

© Guinevere Kwun Wing Queenie Lee, 2008
ABSTRACT

Tuberculosis, caused by the intracellular pathogen *Mycobacterium tuberculosis*, is one of the most prevalent infectious diseases in our world today. In order to survive within the host the bacteria need to sense and respond to changes in the environment; however, signal transduction in this bacterium is poorly understood. PknB is a serine/threonine kinase essential for the *in vitro* survival of *M. tuberculosis* and therefore a potential drug target against the bacteria. It is the goal of the current study to elucidate downstream substrates of PknB. We have found that PknB shares *in vitro* substrates with another serine/threonine kinase, PknH, implying the potential complexity of the signaling pathways in the bacteria. We have also provided the first description of the coupling between serine/threonine kinases PknB and PknH with a two-component system response regulator DevR, and further proposed Ser/Thr phosphorylation as the negative regulator of DevR transcription activator activity based on LC-MS/MS analysis. Finally, we have identified a previously unknown phosphoprotein glyceraldehyde 3-phosphate dehydrogenase encoded by the ORF Rv1436, which demonstrates autophosphorylation activity and which phosphorylation is independent of PknB. Overall, the current study has contributed to advance our understanding of the signal transduction pathways and phosphoproteome in *Mycobacterium tuberculosis*.
Table of Contents

Abstract ........................................................................................................................................ iv
Table of Contents ........................................................................................................................ ivvii
List of Tables ................................................................................................................................. iv
List of Figures ................................................................................................................................. v
Abbreviations ................................................................................................................................. vii
Acknowledgements ......................................................................................................................... viii

Chapter 1: Introduction ...................................................................................................................... 1
  1.1 Tuberculosis: World Impact..................................................................................................... 1
  1.2 Active Versus Latent Tuberculosis ....................................................................................... 2
  1.3 Signal Transduction Systems in Mycobacterium tuberculosis ........................................... 3
    1.3.1 M. tuberculosis Two-component Systems ...................................................................... 3
    1.3.2 M. tuberculosis Serine/threonine Kinases ....................................................................... 7
  1.4 Thesis Objectives .................................................................................................................... 15

Chapter 2: Materials and Methods ................................................................................................. 16
  2.1 Bacterial Strains, Media, Growth Conditions and Cell Lysate Preparation ......................... 16
  2.2 Cloning, Expression and Purification of Mycobacterial Genes ........................................ 18
  2.3 In vitro Kinase Assays .......................................................................................................... 20
  2.4 Two-dimensional Gel Electrophoresis .................................................................................. 20
  2.5 Mass Spectroscopy ............................................................................................................. 21
  2.6 Enzyme Kinetics Calculation ............................................................................................. 21
  2.7 Phosphoamino Acid Analysis .............................................................................................. 22

Chapter 3: Results ............................................................................................................................ 23
  3.1 Cloning, Expression and Purification of pknB ...................................................................... 23
  3.2 PknB Phosphorylates At Least 12 Proteins in M. tuberculosis Soluble Cell Lysate .......... 25
  3.3 PknB and PknH Phosphorylate Rv2623 and DevR ............................................................ 27
  3.4 Cross-phosphorylation of PknB and PknH Substrates ........................................................ 29
    3.4.1. PknB Phosphorylates PknH Substrates Rv0681 and DacB1 ..................................... 29
    3.4.2. PknH Phosphorylates PknB Substrates PbpA and Rv1422 ...................................... 31
    3.4.3. PknG Does Not Phosphorylate PknB and PknH Substrates ..................................... 33
    3.4.4. Enzyme Kinetic Studies .............................................................................................. 38
  3.5 Characterization of DevR Phosphorylation by PknB .......................................................... 41
    3.5.1. Phosphorylation of DevR by PknB Is Time-dependent .............................................. 41
    3.5.2. Phosphorylation of DevR by PknB Is Mn²⁺ Dependent ............................................. 43
    3.5.3. PknB Phosphorylates DevR on Multiple Threonine Residues ................................ 45
  3.6 Identification of a Self-phosphorylating Protein Independent of PknB .............................. 49
    3.6.1. One Phosphoprotein Is Differentially Expressed in H37Rv Cell Lysate ................. 50
    3.6.2. LC-MS/MS Identified GAPDH Is the Phosphorylated Protein ............................... 55
    3.6.3. GAPDH Is Autophosphorylated in a Dose-dependent Manner .............................. 57
    3.6.4. GAPDH Autophosphorylation Is Independent of PknB ........................................... 59

Chapter 4: Discussion ...................................................................................................................... 61
  4.1 Challenges in the Study of PknB .......................................................................................... 61
  4.2 Identification of PknB Substrates ....................................................................................... 62
  4.3 Identification of PknH Substrates ....................................................................................... 64
  4.4 Cross-phosphorylation of PknB and PknH Substrates ........................................................ 66
  4.5 Characterization of DevR Phosphorylation by PknB ......................................................... 75
    4.6 GAPDH Is a Self-phosphorylating Protein ...................................................................... 78

General Conclusion ....................................................................................................................... 82
References ......................................................................................................................................... 83
List of Tables

Table 1.3.1. *M. tuberculosis* two-component signal transduction systems..................13

Table 1.3.2.1. Predicted roles of *M. tuberculosis* STPKs.................................17

Table 1.3.2.2. *In vitro* substrates of *M. tuberculosis* STPKs............................18

Table 2.1. Strains and cloning primers............................................................25

Table 2.2. Expression vectors used in the current study....................................27

Table 3.4.4. Michaelis-Menten analysis of substrates phosphorylation by PknB........48

Table 4.4.2. Percentage identity of *M. tuberculosis* STPKs domains....................77
List of Figures

Figure 1.3.2.1. Domains organization and phosphorylation sites of PknB........20
Figure 1.3.2.2. Domains organization and phosphorylation sites of PknH.........22
Figure 3.1.1. Cloning, expression and purification of PknB kinase domain........32
Figure 3.2. PknB phosphorylates multiple proteins in *M. tuberculosis* cell lysate.....34
Figure 3.3. PknB and PknH phosphorylate Rv2623 and DevR..........................36
Figure 3.4.1. PknB phosphorylates PknH substrates Rv0681 and DacB1.............38
Figure 3.4.2. PknH phosphorylates PknB substrates PbpA and Rv1422..............40
Figure 3.4.3.1. PknG does not phosphorylate Rv0681.................................42
Figure 3.4.3.2. PknG does not phosphorylate DacB1.................................43
Figure 3.4.3.3. PknG does not phosphorylate PbpA and Rv1422....................44
Figure 3.4.3.4. PknG does not phosphorylate Rv2623 and DevR....................45
Figure 3.4.4. Lineweaver-Burk plots of PknB substrates..............................47
Figure 3.5.1. Time dependent phosphorylation of DevR by PknB...................50
Figure 3.5.2. Divalent ions dependent phosphorylation of DevR by PknB.........52
Figure 3.5.3.1. Phosphoamino acid analysis of DevR phosphorylated by PknB....54
Figure 3.5.3.2. PknB phosphorylates DevR on multiple residues...................55
Figure 3.5.3.3. LC-MS/MS of trypsin-digested and phosphorylated DevR...........56
Figure 3.6.1.1. SDS-PAGE of standing cultures lysates with or without oil cover....59
Figure 3.6.1.2. 2-D gel electrophoresis of standing cultures lysates with oil cover.....60
Figure 3.6.1.3. 2-D gel electrophoresis of rolling cultures lysates....................61
Figure 3.6.1.4. 2-D gel electrophoresis of standing cultures lysates.................62
Figure 3.6.2. Amino acid sequence of GAPDH...........................................64

Figure 3.6.3. GAPDH autophosphorylates in a dose-dependent manner..............66

Figure 3.6.4. GAPDH autophosphorylation is independent of PknB......................68
Abbreviations

2-D  Two dimensional gel electrophoresis
ADS  Albumin dextrose saline
AIDS  Acquired immunodeficiency syndrome
BCG  *M. bovis* Bacillus Calmette-Guerin
BSA  Bovine serum albumin
CAMP  Christie, Atkin Munch-Peterson factor
Ci  Curie
CO  Carbon monoxide
CPM  Counts per minute
DPM  Disintegrations per minute
DTT  Dithiothreitol
FASTA  Fast Alignment Sequence Tools - All
FHA  Forkhead-associated domain
GBS  Group B *Streptococci*
GSNO  S-nitrosoglutathione
His-tag  Polyhistidine tag
HIV  Human immunodeficiency virus
IEF  Isoelectric focusing
IFN-γ  Interferon-gamma
IPG  Immobilized pH gradient
IPTG  Isopropyl-Beta-D-Thiogalactopyranoside
iTRAQ  Isobaric Tags for Related and Absolute Quantitation
kanR  Kanamycin resistance
kDa  kiloDalton
Km  Michealis-Menton constant
LB  Luria-Bertani broth
LC-MS/MS  Liquid chromatography-mass spectrometry/mass spectroscopy
MDR-TB  Multidrug resistant tuberculosis
NAD+  Nicotinamide adenine dinucleotide
ND  No data
Ni-NTA  Nickel-nitrilotriacetic acid agarose resin
NO  Nitric oxide
OADC  Oleic acid albumin dextrose complex
ORF  Open reading frame
PASTA  Penicillin-binding and serine/threonine kinase associated domain
PCR  Polymerase chain reaction
Pfam  Protein FAMilies database of alignments
pI  Isoelectric point
qRT-PCR  Quantitative real-time polymerase chain reaction
Rf  Relative front values
SCID  Severe combined immunodeficiency mice
SDS-PAGE  Sodium dodecyl sulphate polyacrylamide gel electrophoresis
STPK  Serine/threonine protein kinase
TLC  Thin layer chromatography
TM  Transmembrane domain
TNF-α  Tumor necrosis factor-alpha
Vmax  Maximum enzymatic rate at a given condition
WHO  World Health Organization
XDR-TB  Extreme drug resistant tuberculosis
Acknowledgements

Throughout the study, my supervisor Dr. Yossef Av-Gay has given me invaluable advices and has assisted me in my growth both personally and scientifically. I would like to extend my warmest thanks to such a wonderful mentor. I would also like to thank our post-doctoral fellow Dr. K.G. Papavinasasundaram for his guidance and support on experimental design and scientific ideas, Dr. Radha GopalaSwamy for her ideas and preparations in the Level 3 containment lab as well as all of my fellow lab members, especially our technician Ms. Mary Ko, all of whom have provided a supportive and inspiring environment in the laboratory, making the completion of this project both enjoyable and scientifically productive. Finally, I wish to thank my mother, father and all of my friends who have provided continuous support and encouragement to my passion in research.
Chapter 1
Introduction

1.1 Tuberculosis: World Impact

Tuberculosis is one of the leading causes of infectious diseases in the world today. The World Health Organization (WHO) estimated that approximately one-third of the world’s population is infected with the bacillus, *Mycobacterium tuberculosis*, which causes the disease [1]. In 2005 alone, 1.6 million deaths have been attributed to tuberculosis [1]. *M. tuberculosis* is an obligated aerobic, Gram-positive bacilli with an unusual cell wall composition high in lipid contents such as mycolic acids and arabinogalactan-lipid [2]. Its unusually thick cell wall accounts for its low permeability and resistance towards common antibiotics [3]. Current therapy against the disease involves treatment with antibiotics such as isoniazid and rifampin; however, this therapy requires a minimum 6-month continuous dosage and patient compliance is generally low [2]. This situation has given rise to drug resistant strains of *M. tuberculosis* including multidrug resistant tuberculosis (MDR-TB) and extreme drug resistant tuberculosis (XDR-TB) [4]. MDR-TB is resistant to all frontline drugs including isoniazid and rifampin, while XDR-TB is resistant to all frontline drugs as well as to second-line antibiotics such as fluoroquinolones and one injectable antibiotic such as kanamycin. This problem is escalated with TB/HIV coinfection, which remains the leading cause of death among AIDS patients worldwide [5]. As a result, continuous research effort around the globe is
devoted into developing new drugs and discovery of new drug targets effective against the tuberculosis bacilli.

1.2 Active Versus Latent Tuberculosis

Transmission begins with the aerosolization of pulmonary secretions of a patient with active tuberculosis while coughing, sneezing, speaking or singing [6]. One to three tubercle bacilli is sufficient to establish an infection in the lung [6]. Once the bacilli are inside the host lung cavity, they bind to and get phagocytosed by lung alveolar macrophages. The bacteria escape intracellular killing by mechanisms such as the inhibition of phagosome-lysosome fusion and hence the acidification of the compartment, allowing it to survive and replicate within the macrophage [7]. Active tuberculosis results in a small percentage of infections, possibly due to the failure of the host to mount an appropriate immune response against the infection [8]. In fact, only 5% to 10% of infected individuals develop clinical disease [6]. In latent infections, *M. tuberculosis* is walled off by granulomas which consist of macrophages and giant cell, T cells, B cells and fibroblasts, thereby preventing its spread to extrapulmonary sites [8]. Within the granuloma, the bacteria survive in a persistent state for decades, until the host is immunocompromised due to age or other factors such as an HIV infection, which then the infection “reactivates” and the bacteria actively multiplies [2]. Gene expression profiles in actively multiplying bacteria and persisting bacteria have been shown to be very different. In persisting bacteria, genes such as the DevR-DevS regulon (also known as DosR and DosS) are upregulated, possibly serve to adapt to environmental changes
such as decrease in oxygen level, also known as hypoxia, and increase in environmental toxicity within the granuloma [7].

1.3 Signal Transduction Systems in M. tuberculosis

All living organisms sense their environments in order to adapt to changes and survive. The ability to sense the environment is especially important for M. tuberculosis because its infection cycle exposes it to the toxic intracellular environment within phagosomes of macrophages and the hypoxic environment inside granulomas. The ability for any cell to sense and respond to its environment is accomplished through signal transduction. In bacteria, signal transduction is mediated by two-component systems and eukaryotic-like serine/threonine kinases via protein phosphorylation-dephosphorylation reactions. M. tuberculosis has 11 two-component systems and 11 serine/threonine kinases PknA to L [9].

1.3.1 M. tuberculosis Two-component Systems

Two-component systems are nearly unique to the bacterial kingdom and are absent in animals [10]. A prototypical two-component system consists of a histidine sensor kinase, which, upon stimulation, becomes activated by autophosphorylation and phosphorylates an aspartate residue on its cognate response regulator that is a transcription factor or a regulator of other proteins [11]. Moreover, these two elements in the two-component signaling system are usually located in the same operon and are co-transcribed, with some exceptions ranging from orphaned sensor kinases or response regulator to complex
phosphorelay systems [11]. Two-component systems have been shown to control a range of regulatory processes including gene activation, direct effects on bacterial physiology such as chemotaxis, programmed developmental events in sporulation and bacterial virulence in pathogenic organisms [12]. A list of all M. tuberculosis two-component systems and experimental evidence giving hints to their functions are summarized in Table 1.3.1. In essence, most of the 11 two-component systems have been studied. mtrA-mtrB was found to be essential for growth [13] and others such as devR-devS [14, 15] were identified to be involved in various aspects of virulence. Recent mutational studies have shown that the knockout mutant of mprA, a response regulator of one of M. tuberculosis two-component systems, is attenuated for long-term persistence in the lungs of mice models [16]. Another mutant of a two-component system gene, phoP, is found to be attenuated but not essential for persistence in mice and guinea pigs [17]. Based on these observations, it is believed that two-component systems play important roles in M. tuberculosis virulence.
<table>
<thead>
<tr>
<th>Gene Combination</th>
<th>Clues to Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>devR-devS</td>
<td>ΔdevR mutant hypervirulent in SCID mice; devR-devS induced by hypoxia</td>
<td>[14, 15]</td>
</tr>
<tr>
<td>kdpD-kdpE</td>
<td>ΔkdpDE mutant hypervirulent in SCID mice</td>
<td>[14]</td>
</tr>
<tr>
<td>mprA-mprB</td>
<td>ΔmprA mutant attenuated for long-term persistence in mice lungs</td>
<td>[16]</td>
</tr>
<tr>
<td>mtrA-mtrB</td>
<td>Essential genes</td>
<td>[13]</td>
</tr>
<tr>
<td>narL-narS</td>
<td>ΔnarL mutant no phenotype in SCID mice</td>
<td>[14]</td>
</tr>
<tr>
<td>phoP-phoR</td>
<td>ΔphoP mutant attenuated in mice and guinea pigs</td>
<td>[17]</td>
</tr>
<tr>
<td>prrA-prrB</td>
<td>ΔprrA mutant no phenotype in BALB/c mice</td>
<td>[18]</td>
</tr>
<tr>
<td>senX3-regX3</td>
<td>ΔregX3 mutant no phenotype in BALB/c mice</td>
<td>[18]</td>
</tr>
<tr>
<td>tcrX-tcrY</td>
<td>ΔtcrX mutant hypervirulent in SCID mice</td>
<td>[14]</td>
</tr>
<tr>
<td>trcR-trcS</td>
<td>ΔtrcS mutant hypervirulent in SCID mice; no phenotype in BALB/c mice</td>
<td>[14]</td>
</tr>
<tr>
<td>tcrA-Rv601c-Rv600c</td>
<td>No Data</td>
<td>[19]</td>
</tr>
</tbody>
</table>

Table 1.3.1. *M. tuberculosis* two-component signal transduction systems
1.3.1.1 DevR-DevS

*M. tuberculosis* is a successful pathogen because it can survive and replicate in intracellular environment and granulomas that are hypoxic; yet, little is known about how the bacillus changes its gene expression in order to adapt to and survive hypoxia. Two of the best-studied hypoxia-related genes are the two-component system DevR-DevS operon, which is upregulated under hypoxic conditions [20, 21]. In addition, expression of devR-devS transcript is higher in the virulent H37Rv strain than the attenuated H37Ra strain, suggesting a possible role in virulence [22]. *ΔdevR* mutant has been generated and displayed a hypervirulent phenotype, killing SCID mice more rapidly than wildtype mice [14], further supporting DevR’s role in virulence. In this two-component system, DevR is the response regulator whereas DevS is its cognate sensor kinase located on the cellular membrane. DevR has been shown to be a transcription factor that binds to DNA in tetramers consisting of two dimers [21]. Recent reports have shown that the extracellular domain of DevS senses and binds to environmental signals NO and CO and autophosphorylates [23-26]. Autophosphorylated DevS transfers phosphate onto DevR Asp54 residue and the phosphorylation enhances DevR’s DNA-binding activity [27, 28]. Upon binding to DNA, DevR induces the expression of a range of approximately 170 genes unique to hypoxia including *hspX* and *Rv2623* [15, 20, 29-31].
1.3.2 *M. tuberculosis* Serine/threonine Kinases

Another class of signaling molecules in *M. tuberculosis* is the serine/threonine kinases (STPK), which are studied in detail in our laboratory and reviewed in multiple articles [9, 32, 33]. Unlike the phosphorylation events mediated by two-component systems, serine/threonine phosphorylations are stable at 37°C and acidic environments. They are coupled to dephosphorylation reactions carried out by protein phosphatases, suggesting the tightly regulated nature of this class of signaling molecules [34]. STPKs were previously thought to be unique to eukaryotes, but with the advances in complete genome sequencing projects, it has been shown that prokaryotes, including *M. tuberculosis*, also contain STPKs and phosphatases [19]. In other prokaryotes, STPKs have been shown to play roles in development regulation, stress response and pathogenicity [11]. Bacteria capable of differentiating into a new developmental state, including *Streptomyces*, *Anabaena* and *Myxococcus xanthus* contain a large number of STPK-encoding genes [11]. The *M. tuberculosis* genome contains 11 STPKs named PknA to PknL (without PknC). All of them contain the typical kinase domain characterized by Hanks [35]. 9 out of 11 are predicted to have one transmembrane domain (PknA, B, D, E, F, H, I, J and L), while the other two are predicted to be cytosolic (PknG and K) [9]. Like the two-component systems, very little is known about either the function or the signaling pathways of *M. tuberculosis* STPKs. Our current knowledge implies that these kinases are involved in diverse functions ranging from cell shape regulation (PknA and B) [36], amino acid transport (PknG) [37] to a possible role in virulence (PknH) [38]. A summary of predicted roles and mutant phenotypes of these STPKs are listed in Table 1.3.2.1. In
order to gain a further understanding on the \textit{M. tuberculosis} STPKs signaling pathways, recent effort focuses on identifying proteins which receive phosphates from STPKs, in other words the downstream substrates of these kinases. It has been shown previously that proteins with the forkhead-associated (FHA) domain and proteins with a XXXXTQXXX amino acids motif are preferentially phosphorylated by \textit{M. tuberculosis} STPKs [36, 39-41]. In fact, the majority of substrates are identified using these motifs. A summary of \textit{in vitro} substrates identified to date is listed in Table 1.3.2.2. It must be noted that multiple reports have shown the ability of one kinase to phosphorylate multiple substrates, as well as the sharing of one substrate between multiple kinases. This implies a high degree of complexity in the STPKs signal transduction process and future effort must focus on identifying true, \textit{in vivo} substrates of these STPKs.
**Table 1.3.2.1.** Predicted roles of *M. tuberculosis* STPKs

<table>
<thead>
<tr>
<th>STPK</th>
<th>Predicted role based on bioinformatics</th>
<th>Knockout mutant phenotype</th>
<th>Overexpression mutant phenotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>PknA</td>
<td>Cell elongation / division</td>
<td>ND (essential gene)</td>
<td>Broad and irregular cell shape</td>
<td>[9, 36]</td>
</tr>
<tr>
<td>PknB</td>
<td>Cell elongation / division</td>
<td>ND (essential gene)</td>
<td>Widened, bulging cells of nonuniform diameters</td>
<td>[9, 36]</td>
</tr>
<tr>
<td>PknD</td>
<td>Phosphate transport</td>
<td>ND</td>
<td>ND</td>
<td>[9]</td>
</tr>
</tbody>
</table>
| PknE  | Membrane transport                      | 1. Increased resistance to NO  
2. Decreased resistance to reducing agents  
3. Increased apoptosis of infected macrophages  
4. Decreased cytokine response in mice model | ND | [9, 42] |
| PknF  | Membrane transport                      | ND                        | (M. smegmatis data)  
1. Decreased growth rate  
2. Shorter and swelled cells | [9, 43] |
| PknG  | Amino-acid uptake, stationary-phase metabolism | 1. Decreased survival *in vitro* and in BALB/c mice  
2. Cells accumulate glutamate and glutamine | ND | [9, 37] |
| PknH  | Arabinan metabolism                    | 1. Increased resistance to acidified nitrite stress *in vitro*  
2. Hypervirulence in BALB/c mice | ND | [9, 38] |
| PknI  | Cell division                          | ND                        | ND                              | [9]        |
| PknJ  | Unknown                                | ND                        | ND                              | [9]        |
| PknK  | Transcription, secondary metabolites    | ND                        | ND                              | [9]        |
| PknL  | Transcription?                         | ND                        | ND                              | [9]        |

ND: No Data
Table 1.3.2.2. *In vitro* substrates of *M. tuberculosis* STPKs

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>EmbR</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>FHA</td>
</tr>
<tr>
<td>Rv0681</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
<td>+</td>
<td>TQXXX</td>
<td>[45], This Study</td>
</tr>
<tr>
<td>DacB1</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
<td>+</td>
<td>TQXXX</td>
<td>[45], This Study</td>
</tr>
<tr>
<td>Rv2623</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
<td>+</td>
<td>qRT-PCR</td>
<td>This Study</td>
</tr>
<tr>
<td>DevR</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
<td>+</td>
<td>qRT-PCR</td>
<td>This Study</td>
</tr>
<tr>
<td>PbpA</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
<td>+</td>
<td>Genomic location</td>
<td>[47]</td>
</tr>
<tr>
<td>Rv1422</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
<td>+</td>
<td>2-D</td>
<td>[36]</td>
</tr>
<tr>
<td>GarA</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>2-D</td>
<td>[48]</td>
</tr>
<tr>
<td>Rv1747</td>
<td>ND</td>
<td>+</td>
<td>+/-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>2+</td>
<td>3+</td>
<td>FHA</td>
<td>[39, 40]</td>
</tr>
<tr>
<td>Rv0020c</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>-</td>
<td>FHA</td>
<td>[40]</td>
</tr>
<tr>
<td>Rv0516c</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>Homolog</td>
<td>[49]</td>
</tr>
<tr>
<td>Rv3221A</td>
<td>-</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>Homolog</td>
<td>[49]</td>
</tr>
<tr>
<td>Wag31</td>
<td>+</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
<td>+</td>
<td>2-D</td>
<td>[36]</td>
</tr>
<tr>
<td>EmbR2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>CD1551</td>
<td>[50]</td>
</tr>
<tr>
<td>Rv1365c</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
<td>+</td>
<td>Homolog</td>
<td>[49]</td>
</tr>
<tr>
<td>Rv1904</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>Homolog</td>
<td>[49]</td>
</tr>
<tr>
<td>FtsZ</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>Function</td>
<td>[51]</td>
</tr>
<tr>
<td>MmpL7</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>potential</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
<td>3+</td>
<td>2-D</td>
<td>[52]</td>
</tr>
<tr>
<td>Rv2175c</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td>Genomic location</td>
<td>[53]</td>
</tr>
</tbody>
</table>

2-D: Two-dimensional gel electrophoresis coupled with LC-MS/MS; Homolog: homolog of known substrates; Genomic location: The genes encoding for the substrates are located close to, or adjacent to, the STPKs; FHA: Forkhead-associated domain; CD1551: A clinical isolate of *M. tuberculosis* different from the H37Rv laboratory strain used in this study.
1.3.2.1 PknB

PknB is an essential STPK for *M. tuberculosis* survival *in vitro* [54] and is located close to the origin of replication in the *M. tuberculosis* genome [9]. Due to its essential nature and the absence of a close homolog in human, it has been suggested to be a potential drug target [9]. Indeed, STPK inhibitor H7 has been shown to inhibit the growth of *M. bovis* BCG and *M. smegmatis* [55]. As a result, PknB is one of the most studied STPKs in *M. tuberculosis*. PknB is predicted to contain one N-terminal kinase domain, one transmembrane domain and four repeating PASTA domain (penicillin-binding and serine/threonine kinase associated domain) at the C-terminus [9]. It was reported that PknB purified from *Escherichia coli* demonstrates autophosphorylation activities on Ser166, Ser169, Thr171, Thr173, Thr294 and Ser295, which the first four phosphorylation sites correspond to the activation loop of the kinase domain and the latter two sites correspond to a linker between the kinase domain and the transmembrane domain (Figure 1.3.2.1) [56]. Crystal structure of PknB reveals that it forms dimers; however, function of dimerization has not been resolved [56]. Functions of PknB were suggested in a study which reported overexpression of PknB in *M. tuberculosis* produces widened, bulging cells of nonuniform diameter as noted in Table 1.3.2.1, implying that PknB has a role in cell shape regulation [36]. To date, eight *in vitro* substrates have been identified for PknB including GarA [48], PbpA [47], Rv1422 [36], Rv1747, Rv0020c [40], Rv0516c, Rv3221A [49] and EmbR [41], suggesting that PknB phosphorylates multiple proteins within the cell.
Figure 1.3.2.1. Domains organization and phosphorylation sites of PknB.
PknB demonstrates autophosphorylation activities on Ser166, Ser169, Thr171, Thr173, Thr294 and Ser295. PknB contains one N-terminal kinase domain, one transmembrane domain and four repeating PASTA domain (penicillin-binding and serine/threonine kinase associated) at the C-terminus. (reproduced from Young, 2003)
1.3.2.2 PknH

In contrast to PknB, PknH is a non-essential STPK for mycobacterial survival [38] and it consists one N-terminal kinase domain, one transmembrane domain and one C-terminal extracellular sensor domain of which the function has not been elucidated [9]. PknH expressed and purified from *E. coli* demonstrates autophosphorylation activities on Thr36, Thr41, Thr48, Thr174, Thr219, Thr222, Ser291, Thr307 and Thr311 of which the first seven phosphorylation sites correspond to the activation loop of the kinase domain and the latter two sites correspond to a linker between the kinase domain and the transmembrane domain (Figure 1.3.2.2) [57]. Our lab has shown that ∆pknH knockout mutant demonstrates a hypervirulent phenotype in BALB/c mice and is more sensitive to acidified nitrite stress, suggesting a potential role in virulence [38]. To date, three *in vitro* substrates have been identified for PknH including EmbR [58], Rv0681 and DacB1 [45]. Note that EmbR has also been identified as a substrate of PknB, suggesting potential cross-phosphorylation activities between substrates of PknB and PknH, which would be investigated in the current study.
Figure 1.3.2.2. Domains organization and phosphorylation sites of PknH. PknH demonstrates autophosphorylation activities on Thr36, Thr41, Thr48, Thr174, Thr219, Thr222, Ser291, Thr307 and Thr311. (Molle, 2006, figure derived from Young, 2003)
1.4 Thesis Objectives

**General Objective:** To gain understanding of PknB signaling events.

**Specific Objectives:**

1. Identify novel substrates for PknB.

2. Characterize these substrate(s) of PknB in terms of:
   a. Enzyme kinetics
   b. Phosphorylation time dependency
   c. Divalent-ions dependency
   d. Identification of phosphorylated residues
   e. Obtain further *in vitro* proof of phosphorylation with *M. tuberculosis* cell lysate
Chapter 2
Materials and Methods

2.1 Bacterial Strains, Media, Growth Conditions and Cell Lysate Preparation

Bacterial strains used in the current study are summarized in Table 2.1. Cloning and expression strains *E. coli* DH5α and BL21(DE3) were grown in LB broth with the addition of ampicillin (100µg/mL) or plated on solid LB media. *M. smegmatis* was grown in Middlebrook 7H9 broth (Difco) or plated as a lawn on 7H10 agar (Difco) supplemented with 10% ADS (5% w/v BSA, 2% w/v dextrose, 0.85% w/v sodium chloride to pH 7.0), 0.05% Tween-80 (BDH) and 0.2% glycerol (Fisher). *M. tuberculosis* strains were cultured under rolling conditions in Middlebrook 7H9 broth (Difco) supplemented with 10% OADC (Difco), and 0.05% Tween-80 (BDH). To prepare *M. tuberculosis* cell lysates, cells were harvested and pelleted. Pellets were washed twice, resuspended and lysed in 50mM HEPES (pH 7.2) in the presence of Complete EDTA-free protease inhibitor (Roche) with glass beads in Ribolyser with a speed setting of 6.5 for two cycles of 25 seconds each. Lysates were centrifuged at 13,000xg for 10 minutes at 4°C and the supernatants were filtered through a low-binding Durapore 0.22µm membrane filter (Millipore) to obtain the soluble fraction of cell lysates used in this study.
Table 2.1. Strains and cloning primers

<table>
<thead>
<tr>
<th>Strains</th>
<th>Characteristics / Relevant Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli DH5α</strong></td>
<td>supE44 ΔlacU169 (φ80 lacZ ΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</td>
<td></td>
</tr>
<tr>
<td><strong>E. coli BL21(DE3)</strong></td>
<td>hsdS gal (λclts857 ind1 Sam7 nin5 lacUV5-T7 gene 1)</td>
<td></td>
</tr>
<tr>
<td><strong>M. tuberculosis H37Rv</strong></td>
<td>Lab strain</td>
<td></td>
</tr>
<tr>
<td><strong>M. tuberculosis ΔdevR</strong></td>
<td>Targeted disruptions of devR with kanR determinant of H37Rv</td>
<td>[20]</td>
</tr>
<tr>
<td><strong>Cloning primers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pknB_kinase_domain-Forward</td>
<td>5'-CACC-TCCGACCCTACGGAATT-3'</td>
<td>This Study</td>
</tr>
<tr>
<td>pknB_kinase_domain-Reverse</td>
<td>5'-CTA-CTGGCCATCGGTGAGC-3'</td>
<td>This Study</td>
</tr>
</tbody>
</table>


### 2.2 Cloning, Expression and Purification of Mycobacterial Genes

Expression vectors used in this study are described in Table 2.2. The kinase domain of *pknB* encoding amino acid spanning from amino acid residues 8 to 292 was amplified by polymerase chain reaction (PCR) using primers as described in Table 2.1. *M. tuberculosis* H37Rv genomic DNA was used as the template for all PCR reactions. The PCR product was ligated to pET151D/TOPO N-terminal His-tag vector using pET151 Directional TOPO Cloning Kit (Invitrogen) to make expression vector pGL101 which was expressed in *E. coli* DH5α BL21(DE3) with 0.4mM IPTG incubated overnight at room temperature. After expression and sonication, the soluble fractions were purified by Ni-NTA columns (Qiagen) that trap His-tag proteins. Expression plasmid of ORF Rv1436 encoding for the *M. tuberculosis* glyceraldehyde 3-phosphate dehydrogenase was obtained from Dr. Michael James’ laboratory from the University of Alberta. Full-length Rv1436 was ligated into pDEST15 (Invitrogen) to create the expression plasmid and was CaCl$_2$ transformed into *E. coli* strains DH5α and subsequently into BL21(DE3) to make the expression strain. *E. coli* BL21(DE3) containing the plasmid was cultured to OD$_{600}$ = 0.6. Over-expression of the gene product was induced by the addition of 0.4mM IPTG. Cultures were left shaking overnight and harvested the next day. Cells were lysed using sonication. The soluble fractions were purified by Glutathione-Agarose (Sigma) suspended in a purification column according to the manufacturer’s protocol. Both PknB and Rv1436 elutions were dialyzed against 20mM Tris-HCl at pH 7.2 containing 1mM DTT and 5% glycerol. All other expression vectors of protein kinases and substrates used in this study were obtained from previous studies summarized in Table 2.2.
<table>
<thead>
<tr>
<th>Name of Plasmid</th>
<th>Domain(s)</th>
<th>Vector</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGL101</td>
<td>Kinase domain of PknB&lt;sub&gt;8-292&lt;/sub&gt;</td>
<td>pET151D/TOPO (Invitrogen)</td>
<td>This study</td>
</tr>
<tr>
<td>pWAB105</td>
<td>Kinase domain of PknH&lt;sub&gt;1-401&lt;/sub&gt;</td>
<td>pET22b (Novagen)</td>
<td>[45]</td>
</tr>
<tr>
<td>pYA410E</td>
<td>Full Length PknG&lt;sub&gt;1-750&lt;/sub&gt;</td>
<td>pET22b (Novagen)</td>
<td>[37]</td>
</tr>
<tr>
<td>pWAB110</td>
<td>Full Length Rv0681&lt;sub&gt;1-209&lt;/sub&gt;</td>
<td>pET22b (Novagen)</td>
<td>[45]</td>
</tr>
<tr>
<td>pWAB112</td>
<td>Soluble fragment of DacB1&lt;sub&gt;133-350&lt;/sub&gt; excluding two transmembrane domains</td>
<td>pET22b (Novagen)</td>
<td>[45]</td>
</tr>
<tr>
<td>pJBA202</td>
<td>Truncated fragment of PbpA&lt;sub&gt;316-491&lt;/sub&gt; containing PknB phosphorylation sites</td>
<td>pET28a (Novagen)</td>
<td>[47]</td>
</tr>
<tr>
<td>pRV1422</td>
<td>Full Length Rv1422&lt;sub&gt;1-342&lt;/sub&gt;</td>
<td>pET28a (Novagen)</td>
<td>[36]</td>
</tr>
<tr>
<td>pXW13-2</td>
<td>Full Length DevR&lt;sub&gt;3-216&lt;/sub&gt;</td>
<td>pET22b (Novagen)</td>
<td>[59]</td>
</tr>
<tr>
<td>pXW9-5</td>
<td>Full Length Rv2623&lt;sub&gt;2-297&lt;/sub&gt;</td>
<td>pET22b (Novagen)</td>
<td>[59]</td>
</tr>
<tr>
<td>pRV1436</td>
<td>Full Length Rv1436&lt;sub&gt;1-338&lt;/sub&gt;</td>
<td>pDEST15 (Invitrogen)</td>
<td>[60]</td>
</tr>
</tbody>
</table>
2.3 In vitro Kinase Assays

All reactions were performed under 20 mM PIPES at pH 7.2, 10mM MgCl$_2$ and 10mM MnCl$_2$ except the divalent ions dependency study. 0.6-8 µM of the kinase domain of PknB, 1.3 µM of the kinase domain of PknH, 0.2-1.63 µM of PknG and 0.06-18 µM of substrates or 10 to 100 µg cell lysate were added to each reaction. Reactions started with the addition of 5µCi γ-[32P]ATP (Perkin-Elmer). All reactions were incubated for 0-60 minutes at 37°C or room temperature. Reactions were stopped by the addition of SDS-sample loading dye, and then incubated at 95°C for 5 minutes. Samples were resolved by 10% or 12% SDS-PAGE gel, then the gels were silver or Coomassie blue stained and dried. Phosphorylation was detected using PhosphorImager SI (Molecular Dynamics). Phosphorylated samples were cut from gels and subjected to scintillation count (Beckman Coulter LS 6500).

2.4 Two-dimensional Gel Electrophoresis

In vitro kinase assay was performed according to the above buffer and γ-[32P]ATP conditions. Isoelectric focusing (IEF) and second-dimensional gel electrophoresis were carried out according to Amersham Bioscience 2-D electrophoresis manual by Berklman and Stenstedt [61]. Isoelectric focusing (IEF) was carried out using 8 M urea, 0.5% (w/v) CHAPS, 0.2% (w/v) DTT, 0.5% (v/v) IPG Buffer and 0.002% bromophenol blue. Immobline Drystrips pH 4-7 13cm (Amersham Pharmacia Biotech) and the IPGphor system (Amersham Pharmacia Biotech) were used. Strips were rehydrated for 14 hours and focused for 28,000 Volt hours (Vh). IPG strips were equilibrated prior to running the
second-dimensional gel electrophoresis with a 10% SDS-PAGE gel. Gels were silver stained and exposed using PhosphorImager SI.

2.5 Mass Spectroscopy

LC-MS/MS for DevR phosphorylation was performed in the University of California, Berkeley by Dr. Tom Alber and Carl Mieczkowski. LC-MS/MS that identified GAPDH was performed in the Genome B.C. Proteomes Centre in the University of Victoria, B.C.

2.6 Enzyme Kinetics Calculation

Counts per minute (CPM) data obtained from scintillation counter were first converted to disintegrations per minute (DPM) using the formula CPM = DPM x efficiency of scintillation counter on the specific radioisotope. Efficiency of the scintillation counter on P-32 radioisotope was 1. DPM data were then converted to Curie (Ci) with a conversion factor 1 Ci = 2.2 x 10^{12} DPM. Specific activity of γ-[32P]ATP was calculated according to manufacturer’s guidelines (PerkinElmer). Using the specific activity (Ci/mmol), Curie was converted into molar units. Total volume of the reaction was then used to calculate the Molar concentration. The final Molar concentration indicates the concentration of radioactive phosphates incorporated into the protein under investigation. The Molar concentrations (ρM) obtained were divided by the reaction time (minutes) to obtain the velocity of the reaction (ρM/minute). Lineweaver-Burk plots were obtained by plotting the inverse of the enzymic velocities and the concentrations of protein substrates
used in the reactions. $K_m$ is the absolute value of the inverse of the X-intercept, and $V_{max}$ is the inverse of the Y-intercept.

2.7 Phosphoamino Acid Analysis

One dimensional phosphoamino acid analysis was performed according to a previously published protocol [62]. DevR is labeled with $\gamma$-[32P]ATP by PknB and the proteins were resolved by 10% SDS-PAGE. The proteins on the gel were transferred to Immobilon membrane (Millipore) for 4 h at 60 Volts, a protocol modified from Kamp et al. [63]. The membrane was then analyzed by the PhosphorImager SI and the corresponding DevR band was cut. This sample was hydrolyzed in 200uL of 5.7N HCl for 3 hours at 95$^\circ$C, dried in Speed-Vac and resuspended in 20uL H$_2$O. 1uL of the DevR sample, along with phosphoamino acid markers P-Ser, P-Thr and P-Tyr, were analyzed by ascending thin-layer chromatography using TLC Cellulose Plate (ANALTECH) using isobutyric acid and 0.5M NH$_4$OH (5:3, v/v). Positions of markers were detected by spraying the plate with 0.25% ninhydrin in acetone. Autoradiograms were detected by PhosphorImager SI. $R_f$ (relative front) values were obtained from dividing the distance traveled by the samples by the distance traveled by the solvent.
Chapter 3
Results

3.1 Cloning, Expression and Purification of pknB

In order to study the in vitro activity of PknB, it is necessary to obtain a purified sample of the protein. The full length PknB contains one transmembrane domain [9]. To avoid insoluble protein forming inclusion bodies during purification which would lead to loss of yield and/or loss of activity, only the kinase domain of PknB was cloned and purified in the current study. We cloned, expressed and purified the kinase domain of PknB corresponding to amino acid 8 to 292 using pET151D/TOPO E. coli cloning system (Invitrogen) as described in Materials and Methods. The construct was shown as a plasmid map in Figure 3.1.1A. PCR was performed and yielded a product that matches the size of the predicted product at 855bp (Figure 3.1.1B). The PCR product was ligated to expression vector pET151D/TOPO and expressed in E. coli BL21. The E. coli cell lysate was passed through Ni-NTA columns and was washed with 20mM of imidazole and then eluted with 250mM of imidazole (Figure 3.1.1C). Interestingly, we observed a large amount of protein being washed out at the 20mM fraction which matches the predicted size of PknB at approximately 35kDa. Both the 20mM and the 250mM fractions were subjected to dialysis and their autophosphorylation activities were tested using in vitro kinase assays. We observed that the 20mM imidazole wash fraction yielded a pure sample that was active, whereas the 250mM imidazole elution fraction
Figure 3.1.1. Cloning, expression and purification of PknB kinase domain. (A) Plasmid map of pknB kinase domain cloned into pET151D/TOPO vector. (B) PCR reaction was performed with M. tuberculosis genomic DNA as template with primers targeting PknB kinase domain. Lanes: 1, No DNA template; 2, No reverse primer; 3, no forward primer; 4, Complete reaction with PCR product at 855bp. (C) SDS-PAGE analysis of pknB expression in E. coli. Lanes: 1, column flow through; 2, 20 mM imidazole wash; 3 and 4, 250 mM imidazole elution. (D) Autoradiograph of an in vitro kinase assay showing PknB autophosphorylation activity. Lanes: 1, 10µg PknB purified from 20 mM imidazole wash; 2, 10µg PknB purified from 250 mM imidazole wash.
yielded a pure sample that was barely active (Figure 3.1.1D). Hence, it was decided that the 20mM imidazole wash fraction would be used for subsequent *in vitro* kinase assays.

### 3.2 PknB Phosphorylates At Least 12 Proteins in *M. tuberculosis* Soluble Cell Lysate

As mentioned in the introduction, previous studies have shown that PknB phosphorylates multiple substrates *in vitro*. To date, eight *in vitro* PknB substrates have been identified [36, 40, 41, 47-49]; however, it was estimated that approximately 500-1000 proteins in the *M. tuberculosis* proteome are modified by serine/threonine phosphorylation [32]. Since there are only 11 STPKs and 13 two-component system histidine kinases known in the bacteria [11], this implies that one kinase may phosphorylate an average of over 20 proteins within the cell. Therefore, it is possible that there are unidentified substrates for PknB remaining to be discovered so we hypothesized that PknB phosphorylates additional substrates in the current study. We tested our hypothesis by visualizing the *M. tuberculosis* phosphoproteome with $\gamma$-[32P]ATP, with or without incubation of purified PknB, using two-dimensional gel electrophoresis. These two reactions were subjected to two-dimensional gel electrophoresis and the gels were silver stained. Figure 3.2A shows the silver stained gel with the addition of PknB has successfully resolved individual proteins present in the cell lysate. Next, autoradiographs of the two gels were obtained. For the lysate incubated with $\gamma$-[32P]ATP without the addition of PknB, autoradiograph showed that it produces a very weak overall pattern of phosphorylation signal (Figure 3.2C). In contrast, in the autoradiograph of the gel with the addition of purified PknB, we observed at least 12 signals unobserved otherwise (Figure 3.2B). This result indicated
Figure 3.2. PknB phosphorylates multiple proteins in *M. tuberculosis* cell lysate. 2-D electrophoresis gel of 11 µg H37Rv cell lysate soluble fraction incubated with 47 µg PknB and γ-[32P]ATP showing PknB phosphorylates at least 12 proteins in the cell lysate. (A) Silver staining of (B). (B) Autoradiograph of (A) showing proteins phosphorylated by PknB indicated by arrows. (C) Autoradiograph of H37Rv cell lysate with γ-[32P]ATP without incubation with PknB.
that there were at least 12 proteins in the H37Rv cell lysate that received phosphates from PknB, suggesting there are indeed unknown substrates of PknB remaining to be discovered.

3.3 PknB and PknH Phosphorylate Rv2623 and DevR

Similar to PknB, PknH is also a transmembrane STPK with one extracellular sensor domain [9]. Previous studies in our laboratory have pointed to the possibility that a protein encoded by the ORF Rv2623 could be a potential substrate of PknH based on qRT-PCR and iTRAQ analysis of a ΔpknH knockout mutant showing that Rv2623 is upregulated in the knockout mutant as compared to the wildtype [46]. Based on these results, we hypothesized that Rv2623 is a downstream substrate of PknH. As Rv2623 expression has been suggested to be under the control of a two-component response regulator DevR [15] and our iTRAQ data showed an upregulation of proteins belonging to the DevR regulon [46], we hypothesized that DevR might also be phosphorylated by PknH. Since a previous report has shown that PknB and PknH shares EmbR as their in vitro substrate [41], PknB phosphorylation was tested alongside with PknH. Rv2623, DevR and the kinase domain of PknH were cloned and purified by other members from our laboratory [45, 46]. In vitro kinase assay as shown in Figure 3.3 indicates that both Rv2623 and DevR were indeed phosphorylated by the kinase domain of PknH and PknB. In contrast to PknH phosphorylation, the phosphorylation of DevR by μM concentration of PknB yielded a very strong signal.
Figure 3.3. PknB and PknH phosphorylates Rv2623 and DevR. Autoradiograph showing in vitro phosphorylation of 9 µM Rv2623 and 5 µM DevR by 1 µM PknH and 1 µM of PknB with γ-[32P]ATP. Arrows indicates phosphorylation bands corresponding to Rv2623 and DevR.
3.4 Cross-phosphorylation of PknB and PknH Substrates

Results from section 3.2 suggested that there are multiple unidentified protein substrates for PknB remained to be discovered. As mentioned previously, PknB and H share the same in vitro substrate EmbR [41], implying possible cross-phosphorylation activities between PknB and PknH substrates by the two STPKs. Indeed, our results from the previous section showed that PknB and PknH share Rv2623 and DevR as in vitro substrates. Therefore, we hypothesized that PknB phosphorylates other known substrates of PknH including DacB1 and Rv0681 [45], while PknH phosphorylates published substrates of PknB including PbpA [47] and Rv1422 [36].

3.4.1. PknB Phosphorylates PknH Substrates Rv0681 and DacB1

Cloned and purified Rv0681 and DacB1 were obtained from the previous study [45] and we tested the ability of PknB to in vitro phosphorylate these proteins. Myelin basic protein (MBP) is a known artificial substrate for PknB [64] and served in this experiment as the positive control. As seen in Figure 3.4.1, autoradiographs showed that both DacB1 and Rv0681 were phosphorylated by PknB.
Figure 3.4.1. PknB phosphorylates PknH substrates Rv0681 and DacB1. Autoradiograph of *in vitro* kinase assays showed PknB phosphorylation of PknH substrates. Artificial substrate MBP was the positive control. 5 µM Rv0681 and 2 µM of DacB1 were incubated with 5 µM PknB for 30 minutes at 37°C. Reactions were loaded into 10% SDS-PAGE. Arrows indicates phosphorylation bands corresponding to MBP, Rv0681 and DacB1.
3.4.2. PknH Phosphorylates PknB Substrates PbpA and Rv1422

Expression plasmids of PbpA and Rv1422 were obtained from the research groups which have initially published the interactions between PknB and these two substrates [36, 47] and the proteins were expressed and purified according to the published protocols. *In vitro* kinase assays were performed with or without recombinant PknB and PknH. The autoradiograph presented in Figure 3.4.2 shows that PknH phosphorylates both PbpA and Rv1422. Phosphorylation of Rv1422 by PknB produced a stronger signal compared to the phosphorylation by PknH while PknH produces a stronger signal with PbpA as compared to PknB.
Figure 3.4.2. PknH phosphorylates PknB substrates PbpA and Rv1422. Autoradiograph of *in vitro* kinase assays showed the phosphorylation of PbpA and Rv1422 by both PknB and PknH. 5 µM PbpA and 1 µM Rv1422 were incubated with 4 µM PknB and 1 µM PknH for 30 minutes at 37°C. Reactions were loaded into 12% SDS-PAGE. Arrows indicates phosphorylation bands corresponding to PbpA and Rv1422.
3.4.3. PknG Does Not Phosphorylate PknB and PknH Substrates

To test the specificity of PknB and PknH substrates cross-phosphorylation, *in vitro* kinase assays were performed with PknG and the above substrates. As opposed to PknB and PknH which are transmembrane kinases, PknG is a soluble kinase studied in detail in our laboratory [37]. Because PknG lacks a transmembrane domain and would presumably localize at different cellular locations than PknB and PknH, we expected that PknG will have a different substrate specificity than PknB and PknH. We tested whether PknB and PknH recombinant proteins can be phosphorylated by PknG. Autoradiographs indicated that Rv0681 and DacB1 cannot serve as substrates for PknG, as shown in Figure 3.4.3.1 and 3.4.3.2. In Figure 3.4.3.3 and 3.4.3.4, PbpA, Rv1422, DevR and Rv2623 showed weak phosphorylation by a very active sample of PknG and the phosphorylation level was equivalent with an extremely low autophosphorylation activity of PknB. We concluded that phosphorylation of these substrates by PknG is non-specific.
Figure 3.4.3.1. **PknG does not phosphorylate Rv0681.** PknG phosphorylation of MBP is the positive control. 1.6 µM PknG and/or 0.8, 1.7, 3.7, 7.5 µM Rv0681 were incubated for 15 minutes at room temperature with γ-[32P]ATP.
Figure 3.4.3.2. PknG does not phosphorylate DacB1. 1.6 µM PknG and/or 0.2, 0.4 and 2.3 µM DacB1 were incubated for 15 minutes at room temperature with $\gamma$-[32P]ATP. PknH was incubated with DacB1 and PknG with MBP as the positive control.
Figure 3.4.3.3. PknG does not phosphorylate PbpA and Rv1422. 0.2 µM PknG, 2.8 µM PknB, 3.9 µM PbpA and/or 0.4 µM Rv1422 were incubated for 15 minutes at room temperature with γ-[32P]ATP. PknG phosphorylation of PbpA and Rv1422 is non-specific.
Figure 3.4.3.4. PknG does not phosphorylate Rv2623 and DevR. 0.2 µM PknG, 2.8 µM PknB, 6.7 µM Rv2623 and/or 3.8 µM DevR were incubated for 15 minutes at room temperature with γ-[32P]ATP. PknG phosphorylation of Rv2623 and DevR is non-specific.
3.4.4. Enzyme Kinetic Studies

In order to assess the specificity and the reaction rate of each of the above substrates against PknB, we performed dose-dependent *in vitro* kinase assays of PknB against serially diluted DevR, PbpA, Rv0681, DacB1, Rv2623 and Rv1422. Samples were loaded into SDS-PAGE and the phosphorylated substrates were subjected to scintillation counting. Results were analyzed using the Michaelis-Menten equation and plotted as Lineweaver-Burk plots to obtain Km and Vmax values (Figure 3.4.4). Our results indicated that all of the substrates tested were phosphorylated by PknB in a dose-dependent manner. Km and Vmax values of the reactions are summarized in Table 3.4.4.
Figure 3.4.4. **Lineweaver-Burk plots of PknB substrates.** Lanes in the autoradiograph on top of each graph corresponds to the data points on the graph. Reactions were performed with 0.6 µM PknB and \( \gamma^{32P} \)ATP. (A) DevR (B) PbpA (C) Rv0681 (D) DacB1 (E) Rv2623 and (F) Rv1422. Samples were analyzed in triplicates.
<table>
<thead>
<tr>
<th>Substrate</th>
<th>Km (µM)</th>
<th>Vmax(ρM/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DevR</td>
<td>8.22±3.71</td>
<td>0.11±0.026</td>
</tr>
<tr>
<td>PbpA</td>
<td>0.81±0.11</td>
<td>6.88±0.32</td>
</tr>
<tr>
<td>Rv0681</td>
<td>29.91±12.27</td>
<td>29.12±9.23</td>
</tr>
<tr>
<td>DacB1</td>
<td>5.75±1.51</td>
<td>15.40±5.46</td>
</tr>
<tr>
<td>Rv2623</td>
<td>20.83±1.14</td>
<td>33.68±1.20</td>
</tr>
<tr>
<td>Rv1422</td>
<td>1.73±0.39</td>
<td>7.50±0.54</td>
</tr>
</tbody>
</table>
3.5 Characterization of DevR Phosphorylation by PknB

Among all substrates tested in the current study, DevR is of particular interest because it is the response regulator of the DevR-DevS two-component system [22]. The coupling between STPKs and a two-component system response regulator was previously unknown. Our previous study in section 3.3 provided the first description of the coupling between \textit{M. tuberculosis} STPKs and a two-component system protein. The fact that both PknB and PknH, two STPKs, can phosphorylate DevR and Rv2623 suggests that serine/threonine phosphorylation controls the DevR regulon. In the following section we characterized the phosphorylation of DevR by PknB. We further elucidated that PknB phosphorylates DevR on threonine residues by phosphoamino acid analysis and the phosphorylated residues were identified by LC-MS/MS. Finally, we tested the phosphorylation state in \textit{M. tuberculosis} wildtype H37Rv cell lysate as compared to \textit{ΔdevR} knockout mutant cell lysate.

3.5.1. Phosphorylation of DevR by PknB is Time-dependent

\textit{In vitro} kinase assays were performed and reactions were sampled at 0.5, 5, 15 and 30 minutes, loaded into SDS-PAGE and subjected to scintillation count. Scintillation count per minute (CPM) was plotted against reaction time in minutes. Our result indicates that PknB phosphorylated DevR in a time-dependent manner with reaction saturation at approximately 30 minutes (Figure 3.5.1).
Figure 3.5.1. Time dependent phosphorylation of DevR by PknB. 5 µM of DevR (Δ) was incubated with 5 µM PknB (□) and γ-[32P]ATP. Reactions were sampled at 0.5, 5, 15 and 30 minutes and loaded into 10% SDS-PAGE. Gels were dried and bands corresponding to DevR and PknB were cut and subjected to scintillation count. Scintillation count per minute (CPM) was plotted against reaction time (minutes).
3.5.2. Phosphorylation of DevR by PknB is Mn\textsuperscript{2+} Dependent

Previous studies have shown that PknB autophosphorylation was enhanced by Mn\textsuperscript{2+} ions [64, 65]. PknB phosphorylation of the artificial substrate MBP was demonstrated to be dependent on Mn\textsuperscript{2+} not Mg\textsuperscript{2+} ions [65]. No previous study tested the combined effect of Mn\textsuperscript{2+} and Mg\textsuperscript{2+} on PknB phosphorylation reactions. In the current study, \textit{in vitro} kinase assays with γ-[32P]ATP were performed with various concentrations and combinations of Mg\textsuperscript{2+}, Mn\textsuperscript{2+} and Ca\textsuperscript{2+} ions in the kinase buffer. Our results showed that 10mM of Mn\textsuperscript{2+} alone strongly promoted the phosphorylation of DevR by PknB whereas 10mM of Mg\textsuperscript{2+} or Ca\textsuperscript{2+} alone resulted in very weak phosphorylation signals (Figure 3.5.2A and B). However, when 10mM of Mg\textsuperscript{2+} was used in addition to 10mM of Mn\textsuperscript{2+} ion, phosphorylation signal decreased about 0.8 fold. This suggests Mg\textsuperscript{2+} may be mildly inhibitory. We further examined this reduction in phosphorylation by keeping Mn\textsuperscript{2+} ion concentration constant at 10mM while varying Mg\textsuperscript{2+} ion concentration from 0, 1, 10 and 15mM (Figure 3.5.2C). Our result showed that an increase concentration of Mg\textsuperscript{2+} ion indeed resulted in reduced phosphorylation signals of both the kinase and the substrate in a dose-dependent manner, with sharp decrease even at the lowest concentration of 1mM Mg\textsuperscript{2+} and a constant but slow reduction as the Mg\textsuperscript{2+} concentration increased to 15mM. Next, the reverse was attempted. Mg\textsuperscript{2+} ion concentration was kept constant at 10mM while 0, 1, 10 and 15mM of Mn\textsuperscript{2+} ions were tested (Figure 3.5.2D). Our result showed that in the presence of 10mM Mg\textsuperscript{2+} ion, 10mM of Mn\textsuperscript{2+} gave the highest phosphorylation signal but at 15mM phosphorylation decreased in both the kinase and the substrate. Lastly, we tested the effect of the addition of Ca\textsuperscript{2+} ion into a 10mM Mg\textsuperscript{2+} and Mn\textsuperscript{2+}
Figure 3.5.2. Divalent ions dependent phosphorylation DevR by PknB. 8 µM of DevR (Δ) was incubated with 6 µM of PknB (□) and γ-[32P]ATP in various buffers. Reactions were loaded into 10% SDS-PAGE. Gels were dried and bands corresponding to DevR and/or PknB were cut and subjected to scintillation count. (A) Autoradiograph of in vitro kinase assays with 10 mM of divalent ions in kinase buffers. (B) DevR scintillation count per minute (CPM) corresponding to lanes in the autoradiograph. (C) In vitro kinase assays were performed with kinase buffers consisting varying Mg\(^{2+}\) concentration 0, 1, 10, 15 mM and constant 10 mM Mn\(^{2+}\). (D) In vitro kinase assays were performed with kinase buffers consisting varying Mn\(^{2+}\) concentration 0, 1, 10, 15 mM and constant 10 mM Mg\(^{2+}\). (E) In vitro kinase assays were performed with kinase buffers consisting varying Ca\(^{2+}\) concentration 0, 1, 10, 15 mM and constant 10 mM Mn\(^{2+}\) and Mg\(^{2+}\). In (C), (D) and (E) DevR was subjected to scintillation count and CPM verses varying Mn\(^{2+}\), Mg\(^{2+}\) or Ca\(^{2+}\) concentrations were plotted.
buffer (Figure 3.5.2E). Our results showed the addition of Ca$^{2+}$ ion over a concentration of 0, 5, 15, 25mM only slightly reduced phosphorylation signals.

### 3.5.3. PknB Phosphorylates DevR on Multiple Threonine Residues

One-dimensional phosphoamino acid analysis revealed that both PknB and DevR were phosphorylated predominantly on threonine residues (Figure 3.5.3.1). Next, we rationalized that since each additional phosphate added to a protein shifts the isoelectric point of the protein and thus the number of phosphorylation states of DevR can be visualized using two-dimensional electrophoresis [53]. We performed an *in vitro* kinase assay with purified PknB and DevR and resolved the phosphorylated proteins using two-dimensional gel electrophoresis. Our result showed that phosphorylated DevR possesses at least six different phosphorylation states (Figure 3.5.3.2). To further identify the specific amino acid residues phosphorylated on DevR, a sample of the protein was analyzed by LC-MS/MS. We identified five peptide fragments (Figure 3.5.3.3). Out of the five fragments, two fragments were identified to contain phosphates and one phosphate was found on each fragment. The first phosphate-containing fragment (peptide 3 in Figure 3.5.3.3) consists of the sequence QDPLSGLTDQER and contains two possible sites of phosphorylation, Ser148 and Thr151. The second phosphate-containing fragment (peptide 5 in Figure 3.5.3.3) consists of the sequence TQAAVFATELK and also contains two possible sites of phosphorylation, Thr198 and Thr205.
Figure 3.5.3.1. Phosphoamino acid analysis of DevR phosphorylated by PknB. (A) Control TLC Cellulose lanes with phospho-serine $R_f = 0.32$, phospho-threonine $R_f = 0.38$ and phospho-tyrosine $R_f = 0.42$. (B) Autoradiograph of DevR sample $R_f = 0.38$ and PknB sample $R_f = 0.39$. 
Figure 3.5.3.2. PknB phosphorylates DevR on multiple residues. 3 µM of DevR was incubated with 3 µM of PknB and γ-[32P]ATP. The reaction was subjected to 2-D electrophoresis, showing at least 6 phosphorylation states of DevR.
Figure 3.5.3.3. LC-MS/MS of trypsin-digested and phosphorylated DevR. PknB-phosphorylated DevR was subjected to LC-MS/MS analysis. 5 peptides were identified (underlined). Fragments (3) and (5) have one phosphate. Arrows point to potential phosphorylation sites: Ser148 or Thr151 and Thr198 or Thr205. Triangles below the sequence point to all the potential phosphorylation sites at threonine residues in the sequence.
3.6 Identification of a Self-phosphorylating Protein Independent of PknB

In order to find evidence to prove that PknB phosphorylates DevR in a cellular environment, we studied the phosphorylation of cell lysate proteins from the wildtype bacteria versus the ΔdevR knockout mutant by PknB. We hypothesized that the addition of purified PknB to the ΔdevR mutant cell lysate will produce an electrophoresis gel autoradiograph pattern that is different in its phosphorylation signal when compared to the addition of purified PknB to the wildtype cell lysate, and this might be our way to identify DevR-dependent substrates of PknB.

3.6.1. One Phosphoprotein Is Differentially Expressed in H37Rv Cell Lysate

A ΔdevR knockout mutant strain was obtained from Dr. David Sherman (University of Washington, Seattle). Three growth conditions were included in this study to obtain three different cellular protein profiles. The first condition was a standing culture because DevR was known to be induced in standing cultures [15]. In this condition, wildtype *M. tuberculosis* H37Rv was grown along with the ΔdevR mutant strain to log phase. Then based on a study reporting that the expression of *devR* peaks at 2-4 hours under hypoxic condition [66] the two cultures were left standing for 4 hours in order to induce *devR* expression. The second growth condition ensured a complete cut off of oxygen source and involved growing the two strains to log phase, then overlay the cultures with mineral oil and leave them standing for 4 hours. The third growth condition involved growing the cells to log phase as rolling cultures for 4 hours. The cells were
lysed and the soluble fraction of the cell lysate was obtained according to Materials and Methods. In vitro kinase assays were performed with purified PknB and the cell lysates. First, we resolved the standing culture samples with and without oil cover using one-dimensional gel electrophoresis. Our results showed that incubation with soluble PknB induced at least three differential phosphorylation signals present in the \(\Delta\text{devR}\) mutant lysate when compared to the wildtype lysate, as indicated with arrows in Figure 3.6.1.1. Next, in order to better visualize individual proteins, we resolved the samples from all three growth conditions using two-dimensional gel electrophoresis. Silver staining and autoradiographs of all six gels showed that protein expression patterns and the phosphorylation patterns were identical between wildtype and \(\Delta\text{devR}\) knockout mutant lysate in both the standing cultures overlaid with oil and the rolling cultures (Figure 3.6.1.2 and 3.6.1.3); one significant difference in both silver stain and in the phosphorylation pattern was observed in the standing cultures gels, indicated with arrows in Figure 3.6.1.4. The arrows point to the same spot at approximately 40 kDa with a pI of about 5.5 in the wildtype cell lysate which was barely detectable in silver stain and decreased in autoradiograph intensity in the \(\Delta\text{devR}\) knockout mutant cell lysate, implying that the presence of this protein is dependent on DevR and that this candidate protein is phosphorylated. This protein does not match the molecular weight of DevR at 25 kDa and pI of 6.4 so we decided to find out the identity of the protein presented in this spot using LC-MS/MS.
Figure 3.6.1.1. SDS-PAGE of standing culture lysates with or without oil cover. One-dimensional SDS-PAGE resolved 10 µg of ΔdevR mutant cell lysate soluble fraction as compared to wildtype H37Rv, with or without the addition of 2.8 µg purified PknB incubated with γ-[32P]ATP. (A) Coomassie Blue Staining. (B) Autoradiograph of (A). Arrows indicate differential phosphorylation signals.
Figure 3.6.1.2. 2-D gel electrophoresis of standing cultures lysates with oil cover. Two-dimensional SDS-PAGE resolved 10 µg of ΔdevR mutant cell lysate soluble fraction as compared to wildtype H37Rv grown as standing cultures overlaid with oil with the addition of engineered PknB incubated with γ-[32P]ATP. No significant difference is observed between the wildtype and the mutant lysate in both silver staining and in autoradiographs.
Figure 3.6.1.3. 2-D gel electrophoresis of rolling cultures lysates. Two-dimensional SDS-PAGE resolved 10 µg of ΔdevR mutant cell lysate soluble fraction as compared to wildtype H37Rv grown as rolling cultures with the addition of purified PknB incubated with γ-[32P]ATP. No significant difference is observed between the wildtype and the mutant lysate in both silver staining and in autoradiographs.
Figure 3.6.1.4. 2-D gel electrophoresis of standing cultures lysates. Two-dimensional SDS-PAGE resolved 10 µg of ΔdevR mutant cell lysate soluble fraction as compared to wildtype H37Rv grown as standing cultures with the addition of engineered PknB incubated with γ-[32P]ATP. Arrows indicate one difference observed in wildtype H37Rv lysate but barely visible in the ΔdevR. Inserts at the top right corner of each gel zoomed into the area of difference.
3.6.2. LC-MS/MS Identified GAPDH Is the Phosphorylated Protein

In order to identify the protein present in the spot mentioned in the previous section, we re-ran the *in vitro* kinase assay. The spot was cut from the gel and sent to the University of Victoria Genome B.C. Proteomes Centre for LC-MS/MS analysis. Peptides identified from the spot matches the sequences of *M. tuberculosis* glyceraldehyde 3-phosphate dehydrogenase (GAPDH), encoded by the ORF Rv1436, with high confidence (Figure 3.6.2). 15 of all the identified peptides matched the sequence of GAPDH with a score of 624 (-10logP, P=possibility that the observed match is a random event). One of the 15 peptides identified was of Rank 2 (VLDDEFGIVK), meaning it was assigned to another protein, a homologue of GAPDH in *Mesostigma viride*, with a higher confidence. We failed to identify any phosphorylated residue in all of the peptides found.
Figure 3.6.2. Amino acid sequence of GAPDH. LC-MS/MS identified the protein present in the spot from Figure 3.6.1.4 to be GAPDH, encoded by the M. tuberculosis ORF Rv1436. Peptides identified by LC/MS-MS are underlined with a score of 624 (-10logP, P=possibility that the observed match is a random event). A total of 13 matches were found.

```plaintext
1   - MTVKINGF GRIGRFYRA LLAQQEQGTA DVEVVAHNI TDNSTLHLL KFDSILGRLP
61  - CDVLEGDOT IVVGRAKIKA LAVREGPAAL PWGDGVDVV VESTGLFTHA AKAKGHDAG
121 - AKKVIISAPA TDEDITVLG VNDDKYGGSQ NIISNASCTT NCLAPLAKVL DDEFGIVKGL
181 - RITIHAYTQD QNLQDGPHE KRRARRAALN IVPTSTGAAK AIGLVMQOLK GKLGDGYALRV
241 - PIPTGSVTDL TVDLSTRASV DEIHAAPKAA AEGLKKGILK YYDAPIVSSD IVTDPHSSIF
301 - DSGLTKVIDD QAKVSVSYDN EWGYSNRLVD LVTLVGKSL
```
3.6.3. GAPDH Is Autophosphorylated in a Dose-dependent Manner

In section 3.6.1, we observed an increased level of phosphorylation of the protein spot corresponding to GAPDH in the wildtype lysate absent in the $\Delta$devR knockout mutant cell lysate. This might be due to a higher level of protein or that it is a substrate for PknB missing in the mutant lysate. We hypothesize that GAPDH either autophosphorylates and/or GAPDH is phosphorylated by PknB.

An expression plasmid of the full-length $Rv1436$ which encodes for GAPDH cloned into pDEST15 (Invitrogen) was obtained as a courtesy from Dr. Michael James’ lab at the University of Alberta [60]. The protein was expressed as a GST-fusion protein and purified according to the steps outlined in materials and methods. *In vitro* kinase assays were performed and our results indicated that GAPDH is indeed a self-phosphorylating protein and phosphorylation behaved in a dose-dependent manner (Figure 3.6.3).
Figure 3.6.3. GAPDH autophosphorylates in a dose-dependent manner. Autoradiograph of GAPDH incubated with γ-[32P]ATP at 37°C for 1 hour.
3.6.4. GAPDH Autophosphorylation Is Independent of PknB

To investigate whether GAPDH is a substrate of PknB \textit{in vitro}, we performed \textit{in vitro} kinase assays with purified PknB and GAPDH. Samples were resolved with SDS-PAGE and scintillation count was performed on bands corresponding to both PknB and GAPDH in order to quantify phosphorylation levels. GAPDH incubated with or without PknB shows a similar level of phosphorylation (Figure 3.6.4). Our result suggested that the autophosphorylation of GAPDH is independent of PknB.
Figure 3.6.4. GAPDH autophosphorylation is independent of PknB. Scintillation count per minute (CPM) verses concentration (µM) plot of GAPDH and γ-[32P]ATP incubated with and without 0.2 µM of PknB. Reactions were loaded into 10% SDS-PAGE. Gels were dried and bands corresponding to GAPDH and PknB were cut and subjected to scintillation count. GAPDH CPM when incubated without PknB (□). GAPDH CPM when incubated with PknB (Δ). PknB CPM when incubated with GAPDH (O).
Chapter 4
Discussion

4.1 Challenges in the Study of PknB

PknB was identified as an essential protein needed for in vitro growth of *M. tuberculosis* [54] as such it is an excellent drug target against tuberculosis. Indeed, inhibitors against PknB have been previously shown to inhibit the growth of *M. tuberculosis*, *M. bovis BCG* and *M. smegmatis* [54, 55, 67] but very little is currently known about the signaling events mediated by PknB. Hence, it is crucial to gain understanding of the signaling pathways of PknB for the benefit of future drug discovery. Studies on PknB has been difficult because (i) PknB is an essential gene, meaning knockout mutants cannot be generated to give insights to its function; (ii) PknB has one transmembrane domain and is embedded across the cell membrane, posing challenges to the expression and purification of a cloned protein because overexpression of the gene would result in inclusion bodies and no active kinase; in addition, having a transmembrane domain also means that PknB would not be present in any soluble cell lysates and methods used to solublize insoluble cell fractions generally lead to the deactivation of enzymes and thus is not fit for experiments; (iii) due to the infectious nature of *M. tuberculosis*, manipulation of the insoluble membrane fraction outside a Level 3 containment facility which may contain live bacteria is prohibited; (iv) PknB, like any other kinase, is extremely low in abundance within the cell which challenges the detection limit of two-dimensional gel electrophoresis and mass spectroscopy, and antibody detection of such minute amounts of
PknB is not feasible; additionally, antibodies currently available have low specificity; and (v) more generally, *M. tuberculosis* is an extremely slow growing bacteria with a doubling time of 18-24 hours [6] meaning that generation of cultures for experimental use requires a big investment of time and effort. Current research groups have tried to overcome these problems by (i) using the less infectious *M. bovis* BCG or the faster-growing, non-infectious *M. smegmatis* as experimental models; (ii) clone, express and obtain the purified kinase domain of PknB instead of the full length protein from non-pathogenic expression models such as *E. coli* to overcome solubility issues in purification and to ensure the yield of active kinase; and (iii) instead of directly visualizing PknB on two-dimensional eletrophoresis gels, researchers look at the phosphoproteome resulted with the addition of purified PknB to *M. tuberculosis* cell lysates in order to identify downstream substrates. In this thesis, we attempted to elucidate PknB signaling pathways using the above strategies.

4.2 Identification of PknB Substrates

*In vitro* downstream substrates identification has been one of the recent foci in the study of *M. tuberculosis* STPK, and commonly used methods include (i) visualization of the *M. tuberculosis* cell lysates two-dimensional phosphoproteome incubated with purified STPK and γ-[32P]ATP using autoradiography in order to amplify phosphorylation signals in cellular proteins that receive phosphates from the STPK under study [48], (ii) comparison of the two-dimensional phosphoproteome of STPK in wildtype versus overexpression or knockout mutant strains incubated with or without the additional of
purified STPK and \( \gamma \)-[32P]ATP [53], (iii) it has been reported that proteins with forkhead associated (FHA) domain(s), which are known to bind to eukaryotic proteins with phospho-serine and phospho-threonine residues, also preferentially binds to \( M. \) tuberculo\textit{sis} STPK; therefore FHA-domains containing proteins have been selected from the \( M. \) tuberculo\textit{sis} genome, cloned and tested for phosphorylation against STPKs [40, 48, 68], (iv) another motif, XXXXTQXXXX, was also reported to be preferentially phosphorylated by PknA, B and H so proteins with this motif have been selected for substrates screening [36], and finally (v) substrates were identified based on genomic location because it is believed that genes involved in the same pathways tend to cluster in the genome. Indeed, using genomic locations, other groups have identified a number of proteins including PbpA, which is genomically located in the same operon and two genes upstream of PknB, as an \textit{in vitro} substrate for PknB [47]; EmbR, genomically located directly upstream of PknH, as an \textit{in vitro} substrate for PknH [68]; and Rv2175c, genomically located directly upstream of PknL but coded in the opposite strand of DNA, as a substrate for PknL [53]. In fact, most groups have utilized a combination of these methods in order to identify novel substrates for \( M. \) tuberculo\textit{sis} STPKs [36, 47, 53, 68].

In the current study, we attempted to use method (i) mentioned above to find evidence that PknB phosphorylates more than the eight currently known protein substrates. Indeed, our results showed that at least 12 proteins are possibly phosphorylated by PknB, as shown in Figure 3.2B. In order to gain insights to the identities of novel PknB substrates, we used a novel approach, cross-phosphorylation, and found that PknB phosphorylates
proteins previously identified as substrates for another STPK, PknH. This approach will be discussed in section 4.4.

**4.3 Identification of PknH Substrates**

Like PknB, very little is known about the signaling events mediated by PknH. To date, only three *in vitro* PknH substrates have been identified including EmbR [58], Rv0681 and DacB1 [45]. We have identified two proteins from the *M. tuberculosis* two-component system and its regulon, DevR and Rv2623, as novel substrates for PknH in the current study. This is the first description of the coupling of signaling molecules between STPK and two-component system in *M. tuberculosis*.

Coupling of signal transduction molecules between two-component systems and eukaryotic-like serine/threonine kinase is a new area of research in signal transduction and few reports are available. One such report describes that the Gram-negative soil bacterium *Myxococcus xanthus* serine/threonine kinase Pkn14 phosphorylation of MrpC, essential for *M. xanthus* fruiting body formation, has a negative impact on the auto-regulation of MrpC which production is controlled by a two-component system MrpAMrpB [69]. Another report involves the eukaryotic-like serine/threonine kinase Stk1 in Gram-positive *Streptococcus agalactiae* which phosphorylates CovR, a two-component response regulator *in vitro* and negatively regulates the expression of GBS cytotoxin CAMP (Christie, Atkin Munch-Peterson) factor in a CovR dose-dependent manner [70]. Such reports imply that the coupling between two-component systems and
serine/threonine kinases leads to functional changes within the cell and may be important for bacteria virulence in the case of pathogenic bacteria.

DevR and Rv2623 were selected to be tested against PknH in this study because our previous study showed that $\Delta$pknH knockout mutant showed an increased level of Rv2623 transcript as well as shared the same hypervirulent phenotype with $\Delta$devR knockout mutant [46]. As mentioned in the introduction, DevR is a response regulator that mediates hypoxic response by inducing a spectrum of gene expression unique to hypoxia, and one of the genes well-known to belong to the DevR regulon is Rv2623 [20, 21]. Rv2623 encodes for a putative ATP-binding protein and its expression is known to upregulate in multiple stress models including macrophages model [71], Wayne dormancy model [72], standing cultures [73], low oxygen conditions [74] and infected mouse lungs [75], non-proliferating conditions [15], GSNO/ethanol stress [30], artificial granuloma in mice [29]. It is also known that Rv2623 requires DevR for induction in hypoxic conditions [20] but other than that Rv2623 has not been characterized. Serine/threonine phosphorylation of DevR and Rv2623 was previously unknown and was shown for the first time in the current study.

The current study does not provide in vivo evidence or functional insights to the role of the phosphorylation of DevR and Rv2623 by PknH. Furthermore, in order for PknH to phosphorylate the two proteins, they must be present in the same cellular location and be expressed at the same time. Indeed, like DevR and Rv2623 which is expressed in hypoxic condition, transcription of PknH also increased 24, 48 and 72 hours post-
infection in macrophage model [76]. Furthermore, DevR is the cognate response regulator for the membrane-bond DevS and therefore should localize to the cellular membrane while PknH has a transmembrane domain which also renders it to the cellular membrane. Based on these observations, it is likely that these proteins may have access to each other during infection and PknH or other STPKs with kinase domain homology to PknH may act as an additional regulator to the DevR-DevS signaling. Future experiments may use antibodies to study whether PknH and DevR interact with each other and to provide in vivo evidence of the interaction as well as elucidate the serine/threonine phosphorylated residues on DevR by PknH using LC-MS/MS.

A manuscript of the above study was prepared along with the iTRAQ and qRT-PCR data for ΔpknH knockout mutant and has been submitted for publication [46].

**4.4 Cross-phosphorylation of PknB and PknH Substrates**

In this study, we showed that *M. tuberculosis* serine/threonine kinase PknB phosphorylated proteins which were previously shown to be substrates of PknH, whereas PknH was shown to phosphorylate known PknB substrates. We further described PknB phosphorylation of DevR and Rv2623 belonging to the DevR-DevS two-component system and its downstream regulon respectively.

The ability of *M. tuberculosis* serine/threonine kinases to share substrates in vitro is consistent with previous publications. For instance, previous reports have shown that
Rv1422 is phosphorylated by both PknA and B [36]; Rv1747 is phosphorylated by PknB, D, E and F [40]; Rv0020c is phosphorylated by PknB and F [40]; EmbR is phosphorylated by PknB and H [41]; Rv0516c is phosphorylated by PknB, D and E [49]; and Rv3221A is phosphorylated by PknB and E [49].

*In vitro* substrates-sharing can be explained by the presence of certain recognition motifs on the substrates. One widely known example is the FHA domain which recognizes and binds to phosphor-serine/threonine residues. Previous studies have identified 4 of the 6 FHA domain-containing proteins in *M. tuberculosis* as substrates for serine/threonine kinases [39-41, 48, 77]. The remaining two proteins have not been studied. This suggests that if a protein contains the FHA domain, it may bind to and receive phosphates from any serine/threonine kinase non-specifically. However, in the current study, none of the substrates tested possessed the FHA domain (Table 1.3.2.2). As a result, the FHA domain cannot explain our observation of substrates-sharing. Another known motif for substrate phosphorylation is the XXXXTQXXX motif. In a previous study, PknA and PknB were screened against a peptide library and were found to preferentially phosphorylate peptides with the XXXXTQX-hydrophobic-hydrophobic motif [36]. In a later study, two novel PknH substrates were identified by screening for proteins containing one of PknH autophosphorylation sites motif TQLGT [45]. These two studies suggested that the TQ motif seems to be a preferred site for PknA, B and H phosphorylation. Upon searching for the TQ sequences in all of the substrates we tested, we found that all of them do contain one TQ sequence. Rv0681 (TQLGT) and DacB1 (TQIGT) were identified as a potential PknH substrate because of their sequence.
homology to TQLGT. Rv2623 (TQSMA), DevR (TQAAV), PbpA (TQVFT) and Rv1422 (TQEIP) each contained one TQ motif (Table 1.3.2.2), although the amino acids that come after Q vary. Moreover, our mass spectroscopy data has shown that PknB potentially phosphorylates DevR on the threonine of the TQAAV sequence, further suggesting that phosphorylation events may have occurred on the TQ sequence. Yet, it should be noted that not all published PknA and PknB substrates contain the TQ sequence. For instance, both PknB substrates GarA and Rv0020c have the FHA domain but no TQ in their sequences. All protein sequences of the substrates we tested were fed into ClustalW search to locate possible conserved residues and no conserved residues except TQ were found. In summary, the presence of the TQ sequences on all of the substrates we tested may offer an explanation to why they are phosphorylated by both PknB and PknH.

*In vitro* substrates-sharing may also occur because of the homology between the kinase domains of PknB and PknH as all of the kinase assays in the current study were performed with only the purified kinase domains of PknB and PknH. FASTA analysis has shown that the kinase domains of PknB and PknH share 40% identity (Table 4.4.2). However, the kinase domain of PknH is only 25% identical to PknG, while the kinase domain of PknB is only 28% identical to PknG. Although phylographic analysis suggested that PknA and PknB belong to the same clade [78] their kinase domains are only 43% identical. The highest percentage identity observed between *M. tuberculosis*
Table 4.4.2. Percentage identity of *M. tuberculosis* STPKs domains. Protein sequences were analyzed by FASTA (lower triangle) and ClustalW2 percentage score (upper triangle). *M. tuberculosis* STPKs domains were defined by Pfam.

<table>
<thead>
<tr>
<th></th>
<th>PknA</th>
<th>PknB</th>
<th>PknD</th>
<th>PknE</th>
<th>PknF</th>
<th>PknG</th>
<th>PknH</th>
<th>PknI</th>
<th>PknJ</th>
<th>PknK</th>
<th>PknL</th>
</tr>
</thead>
<tbody>
<tr>
<td>PknA</td>
<td>-</td>
<td>41</td>
<td>37</td>
<td>43</td>
<td>35</td>
<td>23</td>
<td>43</td>
<td>31</td>
<td>36</td>
<td>36</td>
<td>43</td>
</tr>
<tr>
<td>PknB</td>
<td>43</td>
<td>-</td>
<td>41</td>
<td>40</td>
<td>40</td>
<td>24</td>
<td>40</td>
<td>34</td>
<td>47</td>
<td>36</td>
<td>46</td>
</tr>
<tr>
<td>PknD</td>
<td>40</td>
<td>41</td>
<td>-</td>
<td>69</td>
<td>43</td>
<td>21</td>
<td>72</td>
<td>38</td>
<td>42</td>
<td>37</td>
<td>36</td>
</tr>
<tr>
<td>PknE</td>
<td>40</td>
<td>40</td>
<td>66</td>
<td>-</td>
<td>43</td>
<td>23</td>
<td>72</td>
<td>38</td>
<td>40</td>
<td>35</td>
<td>38</td>
</tr>
<tr>
<td>PknF</td>
<td>32</td>
<td>33</td>
<td>29</td>
<td>27</td>
<td>28</td>
<td>-</td>
<td>15</td>
<td>22</td>
<td>22</td>
<td>23</td>
<td>26</td>
</tr>
<tr>
<td>PknH</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>27</td>
<td>25</td>
<td>-</td>
<td>39</td>
<td>41</td>
<td>35</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>PknI</td>
<td>37</td>
<td>57</td>
<td>58</td>
<td>37</td>
<td>52</td>
<td>42</td>
<td>38</td>
<td>72</td>
<td>38</td>
<td>54</td>
<td>38</td>
</tr>
<tr>
<td>PknJ</td>
<td>37</td>
<td>45</td>
<td>42</td>
<td>41</td>
<td>52</td>
<td>41</td>
<td>38</td>
<td>37</td>
<td>41</td>
<td>35</td>
<td>37</td>
</tr>
<tr>
<td>PknK</td>
<td>37</td>
<td>37</td>
<td>37</td>
<td>37</td>
<td>36</td>
<td>31</td>
<td>36</td>
<td>37</td>
<td>36</td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>PknL</td>
<td>43</td>
<td>54</td>
<td>56</td>
<td>53</td>
<td>56</td>
<td>53</td>
<td>53</td>
<td>31</td>
<td>36</td>
<td>38</td>
<td>40</td>
</tr>
</tbody>
</table>
serine/threonine kinase domains is 73% between PknE and PknH. These figures suggested that most of the serine/threonine kinases of *M. tuberculosis* possess kinase domains that differ to a large extent. Despite the low sequence identity (40%), PknB and PknH can phosphorylate the same substrates. The extremely low percentage identity between PknG and PknB/H (25% and 28%) offers a possible explanation to why PknG fails to phosphorylate *in vitro* substrates of PknB and PknH.

It should be noted that in Figure 3.4.4.1 to 3.4.4.4, although PknG clearly shows no phosphorylation of Rv0681 and DacB1, faint bands were observed with PbpA, Rv1422, DevR and Rv2623. As mentioned in the results section, we have concluded that the weak bands correspond to non-specific phosphorylation because PknG autophosphorylation was highly active whereas PknB autophosphorylation signal was extremely low while still producing substrate phosphorylation bands with the same intensities as PknG does. In fact, one of the major variability and limitation in this study is the activity of the purified kinases. Common factors we observed that affect kinase activities include (i) the identity of the kinase, for instance, our PknG purification batches are always more active than PknH followed by PknB, (ii) the concentration of imidazole used during purification, for instance, when PknB was purified with 250 mM imidazole, it became less active than when purified with 20 mM imidazole as shown in Figure 3.1.1D, (iii) the time elapsed since purification to when kinase assays were performed, for instance, we observe a decrease in PknB kinase activity when the purified protein was stored for over 1 year and (iv) the buffer used for dialysis, for instance, it is generally known that glycerol in dialysis buffer increases kinase activity. With all these factors affecting kinase activity, it
is extremely difficult to perform experiments over a span of time while ensuring the kinase activities are standardized. The best examples are shown in Figure 3.4.4.3 and Figure 3.4.4.4 in which a higher concentration of PknB was used in the kinase reaction than PknG but the autophosphorylation signal of PknG was clear and strong whereas the autophosphorylation signal of PknB was extremely low. Due to this observation, we conclude that PknG is non-specific against PknB and PknH substrates because even a not-so-active PknB manages to phosphorylate the substrates to the same level as the extremely active PknG.

In order to compare the specificity of PknB and PknH against each of the substrates in this study, we performed enzyme kinetics analysis of PknB against the substrates and compared the results with the published enzyme kinetics data of PknH. Since the absolute values of the kinetics data cannot be compared due to different concentrations and activity of the STPKs used, relative data will be used in the following discussion. The specificity of PknH against EmbR, DacB1 and Rv0681 has been reported and was represented as Km and Vmax values [45]. In that report, substrates with Km values ranked from lowest to highest are EmbR<DacB1<Rv0681, indicating EmbR has the lowest Km and thus binding affinity to PknH among the substrates. Substrates with Vmax values ranked from highest to lowest are DacB1>Rv0681>EmbR, indicating DacB1 has the fastest reaction rate among the substrates. In the current study, we used an identical experimental approach as the previous study to find Km and Vmax values for each substrate against PknB. Comparison with the previous PknH kinetics data shows that in both the previous study and the current study, Km values are all in the µM range.
and Vmax values are all in the ρM/min range, indicating that results between two experiments are agreeing with each other. As indicated in Table 3.4.5, our results show that when incubated against PknB, substrates with Km values ranked from lowest to highest are PbpA<Rv1422<DacB1<DevR<Rv2623<Rv0681, indicating PbpA has the lowest Km and thus binding affinity to PknB among the substrates tested. Genetically, \(pbpA\) is located in the same operon as \(pknB\), while in comparison \(embR\), which was demonstrated to have the lowest Km among the substrates, is also located in the same operon as \(pknH\). Based on their genomic locations, it has been speculated that PbpA is a natural substrate of PknB [47] and EmbR is a natural substrate of PknH [68]. Indeed, looking at our enzyme kinetics results, PbpA has the lowest Km value amongst all substrates tested for PknB. Similarly, EmbR has the lowest Km value amongst substrates tested for PknH. A low Km value means a low concentration of substrate is needed for the reaction to reach its maximum velocity; thus, a substrate with a low Km has a high affinity to its enzyme. Therefore, we can conclude that among all the substrates tested, PbpA has the highest affinity to PknB, providing an additional support that PbpA is a true \(in\ vivo\) substrate of PknB. In the same manner, the previous report has also been shown that EmbR has the highest affinity to PknH, giving additional support that EmbR is a true substrate of PknH. Next, our results show that when incubated against PknB, substrates with Vmax values ranked from highest to lowest are Rv2623>Rv0681>DacB1>Rv1422>PbpA>DevR, indicating Rv2623 has the fastest reaction rate among the substrates tested, while PbpA was one of the slowest. Similarly, EmbR also has the lowest Vmax among all the substrates tested in the previous study. In both studies Vmax is represented as the amount of radioactive phosphate transferred to
substrate per minute. So, our results indicate that phosphate transfer from PknB to PbpA is one of the slowest among all of the substrates. The same goes for the phosphate transfer rate from PknH to EmbR, which was the slowest compared to all the substrates tested in that study. In conjunction with the Km data, we can see that although the reaction rate is slower for both of these putative natural substrates, they are always the ones which require the least concentration in order to achieve the maximum velocity. Our results see a general trend that a substrate with a lower Km tends to have a lower Vmax and vice versa. It is also important to note that the only two published PknB substrates investigated in this study, PbpA [47] and Rv1422 [36], have similar low Km and low Vmax values, suggesting that these two proteins behave similarly as substrates of PknB in vitro. Overall, these enzyme kinetics data together provided an additional proof that PknB is indeed interacting with the respective substrates in a dose-dependent manner in vitro, and the results are agreeing with the kinetics data of PknH substrates.

Although our results showed that the kinase domains of PknB and PknH can phosphorylate the same substrates in vitro, it does not imply that these two kinases have the same function in mycobacterial growth. The function of any kinase would be determined by their extracellular signals, growth phases in which the kinases are expressed and their cellular localization. Both PknB and PknH possess extracellular domains [9]. The extracellular domain of PknB contains four repeating PASTA domains which were shown in Streptococcus pneumoniae to bind β-lactam antibiotics and their peptidoglycan analogues and was proposed to target the PASTA-containing protein to sites of cell growth [79, 80]. Overexpression of PknB in M. smegmetis and M. bovis
BCG resulted in a slower growth rate, diminished viability as well as a widened and bulging morphology, implicating PknB’s effect on cell wall synthesis or cell division [36]. *In vitro* growth experiments have shown that *pknB* is expressed predominantly in early to late log phase while expression decreased in stationary phase [36]. qRT-PCR results have shown that *pknB* expression increased in a macrophage infection model [81]. On the other hand, the extracellular domain of PknH has never been studied but PknH has been shown to localize mostly to the membrane fraction [58]. qRT-PCR result has shown that *pknH* expression also increased in macrophage infection model [76]. Our lab has shown that a Δ*pknH* knockout mutant has no phenotypic differences as compared to wildtype under *in vitro* growth; however it produces a hypervirulent phenotype in BALB/c mice, implicating PknH’s role in virulence and chronic infection [38]. Together, these previous studies suggested that PknB is involved in normal cell growth while PknH is involved in infection, and that they may detect different extracellular signals. Since our results suggest that PknB and PknH phosphorylates the same substrates, this may be an indication that the two kinases respond to different environmental signals to regulate the same signaling pathways under different growth conditions. PknB, PknH and the substrates tested in this study may be expressed at different levels in different growth phases and may localize at different sites on the membrane/cytosol, conferring different substrate specificity.

In summary, our results showed PknB and PknH share substrates *in vitro* and may implicate a complicated signaling network that involve coupling of different signaling molecules under different growth phase. Future studies should include localization
studies of the kinases and substrates under various growth phases and compare the phosphorylation sites of the substrates to find out whether PknB and PknH phosphorylate their substrates on the same residues *in vitro* and *in vivo*.

A manuscript of the above study was prepared and will be submitted for publication [82].

**4.5 Characterization of DevR Phosphorylation by PknB**

Among all the substrates tested, DevR is of particular interest because of its role as a response regulator in the DevR-DevS two-component system. The current study characterized the phosphorylation of DevR by PknB. Although we have found that DevR is phosphorylated by both PknH and PknB, the reason we have chosen to characterize the PknB-DevR kinase-substrate pair instead of the PknH-DevR kinase-substrate pair is because PknB produces a significantly stronger phosphorylation signal in DevR than PknH did. Our results showed that PknB-DevR phosphorylation reaction is time-dependent (Figure 3.5.1), is Mn$^{2+}$ but not Mg$^{2+}$ nor Ca$^{2+}$ dependent (Figure 3.5.2), and is the slowest among substrates tested and has a Km of 8.22µM (Figure 3.4.5 and Table 3.4.5). The enzyme kinetics results indicate that PknB-DevR phosphorylation happens very slowly in comparison to other substrates. It is 62 times slower than PbpA, which resides in the same operon as PknB and is thought to be PknB’s natural substrate as discussed in the previous section. This suggests that PbpA is a much better substrate for PknB than DevR. However, the binding affinity of DevR to PknB as indicated by their Km value ranges in the middle in comparison with the other substrates, suggesting that
DevR has an average affinity to PknB among the substrates tested. Despite the reaction rate and its affinity to PknB, our enzyme kinetics data provided additional evidence that DevR interacts with PknB in vitro in a dose-dependent manner. Previous studies by phosphoamino analysis have shown that PknB autophosphorylates on serine and threonine residues [56, 83] but in the current study we have failed to resolve spots corresponding to serine phosphorylations on the TLC plate. Instead, our phosphoamino acid analysis data shows that DevR, like PknB, is phosphorylated predominantly on threonine residues (Figure 3.5.3.1).

Most importantly, our study has shed light on the potential function of this serine/threonine phosphorylation of a two-component system response regulator in M. tuberculosis. Since previous reports have shown that pknB expression is upregulated in macrophage infection model [81] and devR expression is upregulated in multiple stress conditions [15, 29-31], these studies imply that both pknB and devR are expressed and coexist in an infection. The fact that we found PknB can phosphorylate DevR may suggest an additional level of regulation of the DevR regulon via serine/threonine phosphorylation. Our two-dimensional gel electrophoresis and mass spectroscopy data show that PknB phosphorylates DevR on at least six residues and two of the residues are Ser148 or Thr151 and Thr198 or Thr205 (Figure 3.5.3.2 and 3.5.3.3). Interestingly, we observed that two of these potential phosphorylation sites Thr198 and Thr205 coincide with a previous report on the key residues for DevR dimerization [21]. As mentioned previously, DevR binds to DNA in tetramers consisting of two dimers and the same report have shown that Thr198, Val202 and Thr205 are the key contacting residues for
dimerization. Moreover, Thr198 is immediately adjacent to a glutamine residue, which constitutes the TQ motif (TQAAV) hypothesized to be a putative phosphorylation site as mentioned above. Phosphorylation on this threonine residue by serine/threonine kinase would change the charge of the dimerization interface, prevent dimerization and ultimately prevent DevR from binding to DNA, thereby prevent DevR from inducing genes important for hypoxic response. This result implies that PknB or any serine/threonine phosphorylation may negatively regulate the DNA-binding activity of DevR and provides the first insight into how coupling between serine/threonine kinases and two-component system molecules can alter cellular functions in *M. tuberculosis*. Future studies should focus on investigating the dimerization and DNA-binding activity of serine/threonine-phosphorylated DevR.

A manuscript of the above study was prepared and will be submitted for publication in conjunction with the results in section 4.4 [82].
**4.6 GAPDH Is a Self-phosphorylating Protein**

GAPDH is an enzyme involved in the sixth step of glycolysis, catalyzing the conversion of glyceraldehyde 3-phosphate into 1,3-bisphosphoglycerate (1,3-BPG) and is NAD$^+$ and orthophosphate-dependent [84]. By comparing wildtype and $\Delta$devR knockout mutant cell lysate, we have identified GAPDH, which was present in silver staining of the wildtype but barely detectable in the mutant lysate. The difference in silver staining intensity is potentially due to the presence of an increased expression of GAPDH. Although no study has shown that Rv1436, the gene that encodes for GAPDH, belongs to the DevR regulon, a previous study has shown that GAPDH is present in *M. tuberculosis* standing culture but is undetectable in shaking culture. The authors of that report suggested since GAPDH is a key enzyme in glycolysis, upregulation of GAPDH serves to regenerate the pool of NAD$^+$ and would allow glycolysis to occur under anaerobic conditions to satisfy the energy demands of the bacteria during adjustment to growth under reduced oxygen tension in standing cultures. Since DevR is also involved in hypoxic response and our result shows that GAPDH is upregulated in the wildtype cell lysate and is almost undetectable using silver staining in the $\Delta$devR mutant cell lysate, we propose Rv1436 to be a gene regulated under DevR transcriptional activator activity which detection was probably missed in previous studies. Future studies should use qRT-PCR to compare the transcription level of Rv1436 between wildtype *M. tuberculosis* and $\Delta$devR knockout mutant grown as standing cultures to test our hypothesis.
Our autoradiograph comparison of the wildtype and \(\Delta\text{devR}\) knockout mutant cell lysate has shown GAPDH undergoes phosphorylation. We have further concluded that GAPDH autophosphorylation is independent of PknB. Consistent with our result, literature research has revealed that GAPDH in eukaryotes is also a self-phosphorylating protein [85]. The same report also showed that the rabbit skeletal muscle GAPDH behaves as a kinase catalyzing phosphorylation of proteins found in skeletal muscle terminal cisternae/triad preparation and is dephosphorylated by the incubation with NAD\(^+\), NADH and glyceraldehyde 3-phosphate. Surprisingly, no further research has been performed over the years to elucidate either the role of GAPDH autophosphorylation or its role as a kinase in eukaryoties, although autophosphorylation of a protein has been suggested to regulate its specificity and affinity for substrates and/or regulatory ligands [86].

Our results are also consistent with a previous report in \textit{Corynebacterium glutamicum}, a close relative of \textit{M. tuberculosis} which also belongs to the order Actinomycetales [87]. This report used radioactive labeling and immunostaining and showed that the GAPDH of \textit{C. glutamicum} is also phosphorylated. The study also showed that phospho-GAPDH appeared on two different spots on two-dimensional gel electrophoresis of \textit{C. glutamicum} cytoplasmic proteins, suggesting in \textit{C. glutamicum} GAPDH has at least two different modifications giving it two molecular weights with two different pIs. This indicates that GAPDH is either differentially phosphorylated or covalently modified in the cell. No functional studies have been performed on GAPDH in prokaryotes.
Furthermore, our result is in accordant with bioinformatics prediction of the phosphorylation state of *M. tuberculosis* GAPDH. NetPhos 2.0 server (http://www.cbs.dtu.dk/services/NetPhos/) predicted that the protein has 8 serine, 4 threonine and 2 tyrosine phosphorylation sites.

Although previous studies in eukaryotic cells have also shown that GAPDH is phosphorylated by multiple serine/threonine/tyrosine kinases [88-93], our result indicates that GAPDH phosphorylation is independent of PknB. However, this does not rule out the possibility that GAPDH may be phosphorylated by other kinases in *M. tuberculosis*. Previous reports have shown that phosphorylation can be a potential mechanism of regulation of glycolytic enzymes [88], along with other non-glycolytic activities. One of such report shows that PKC$_{\iota}$ serine-phosphorylates rabbit muscle GAPDH and that membrane association of $\beta$-tubulin is dependent on the phosho-GAPDH [92]. The same report also shows GAPDH interacts with PKC$_{\iota}$ regulatory domain. Although this research claims no autophosphorylation activity of GAPDH was detected, the author also justified that autophosphorylation might be masked by the huge phosphorylation signal resulting from PKC$_{\iota}$. The same group also demonstrated that GAPDH is tyrosine-phosphorylated by Src in the context of vesicles trafficking [93]. Other studies have also shown GAPDH to be tyrosine phosphorylated by pp60 c-Src, pp60 v-Src and epidermal-growth-factor-receptor kinase [89, 90]. Furthermore, rabbit GAPDH have been shown to be phosphorylated by $\text{Ca}^{2+}$/calmodulin-dependent protein kinase II [91]. In light of these reports, we conclude although our result shows that PknB does not affect the
phosphorylation level of GAPDH, future studies should test the phosphorylation using other *M. tuberculosis* STPKs.
**General Conclusion**

This study has given important insights to the phosphorylation events mediated by PknB and PknH and the phosphoproteome of *M. tuberculosis*. We have provided the first description of the *in vitro* coupling of signaling molecules between STPKs and a two-component system response regulator in *M. tuberculosis*, and proposing the STPK PknB to be a negative regulator of the transcriptional activity mediated by the response regulator DevR. We have also provided additional evidence to support previous reports that PknB and PknH phosphorylate multiple proteins within the cell and cross-phosphorylation occurs between PknB and PknH substrates, indicating a potentially intricate signaling network between the STPKs. Finally, we have identified one previously unknown phosphoprotein, GAPDH, in *M. tuberculosis*. Signal transduction and protein phosphorylation are the key events mediating *M. tuberculosis* survival and adaptation to environmental changes. Due to their indispensable roles, future endeavors should continue to elucidate the signaling pathways and phosphoproteome of *M. tuberculosis* in order to find potential drug targets against tuberculosis and to advance our understanding of the *M. tuberculosis* physiology.
References


59. Chao, J. and e. al., 2008.

60. James, M., Cloning of Mycobacterium tuberculosis glyceraldehyde 3-phosphate dehydrogenase. 2006, University of Alberta: Edmonton.


