Chapter 1

Introduction & Review of Literature
INTRODUCTION & REVIEW OF LITERATURE

INTRODUCTION 1
REVIEW OF LITERATURE 3
1.1 Phosphorylation events in eukaryotic signalling 4-11
   1.1.1 Protein kinases 4
   1.1.2 Mechanism of phosphorylation of kinases 5
   1.1.3 Regulation of kinases by phosphatases 10
1.2 Phosphorylation process in prokaryotic signal transduction 11-14
   1.2.1 Two component system in prokaryotes 11
   1.2.2 Regulation of two component system 13
1.3 Eukaryotic-type Ser/Thr or Tyr kinases and phosphatases in bacteria: an emerging concept in bacterial signal transduction 14-18
   1.3.1 Eukaryotic-type Ser/Thr kinases in bacteria 15
   1.3.2 Role of eukaryotic-type Ser/Thr kinases in bacteria 16
   1.3.3 Concept of reversible phosphorylation 17
1.4 Eukaryotic-type Ser/Thr protein kinase mediated phosphorylation and its regulation in mycobacteri 18-28
   1.4.1 Eukaryotic-type Ser/Thr protein kinases in mycobacteria 18
   1.4.2 Eukaryotic-type protein phosphatases in mycobacteria 26
   1.4.3 Interplay between protein kinases and phosphatases 28

SCOPE OF THE PRESENT STUDY 29-30
INTRODUCTION

Cells constantly face dramatic changes in their environment. Therefore, to be able to compete, survive and thrive, appropriate adaptive responses to changing environmental conditions need to be generated. The process of conversion of the extra cellular stimuli into intracellular response is referred to as signal transduction. Signalling pathways in both prokaryotes and eukaryotes often utilize protein phosphorylation as a molecular switch in regulating different cellular activities. Protein phosphorylation events in eukaryotes are mediated primarily by the activities of serine (Ser), threonine (Thr) or tyrosine (Tyr) kinases coupled with the opposing action of protein phosphatases (Hunter, 1995). On the contrary, the molecular system responsible for the stimulus-response coupling in bacteria involves a two-component system, comprising of histidine kinase and response regulator (West and Stock, 2001). However, analysis of different genome sequences revealed that, in addition to typical two-component systems, these regulatory activities in prokaryotes, especially in eubacteria, could also be coordinated by eukaryotic-type Ser/Thr and/or Tyr protein kinases and phosphatases.

The Ser/Thr and/or Tyr kinases of bacteria differ completely from the two component systems both in sequence and structure. Majority of the bacterial Ser/Thr protein kinase (STPK) genes encode receptors with the N-terminal intracellular kinase domain of about 270 residues consisting mainly of β-strands joined to the C-terminal sensor domain through the single predicted transmembrane helix (Av-Gay and Everett, 2000; Greenstein et al., 2005). The extra cellular C-terminal domain, composed predominantly of α-helices, presumably binds the signalling ligand and thereby plays a regulatory role. This unusual orientation is reverse to that seen in majority of receptor kinases in metazoans, but analogous to that seen in most of the receptor like kinases in plants (McCarty and Chory, 2000) and TGFβ receptor (Derynck and Feng, 1997). The radically different domain arrangement of these STPKs is suggestive of their diverse functional roles, distinct from that of eukaryotic Ser/Thr or Tyr kinases (Krupa and Srinivasan, 2005).

The protein kinases in eukaryotes are regulated through a multitude of mechanisms. However, very few studies have shed light on the structures of the
bacterial Ser/Thr kinases, mechanism of their autophosphorylation and hence regulation. The structure of bacterial protein kinase HPr kinase/phosphorylase from *Lactobacillus casei* exhibited similarity to kinases phosphorylating low molecular weight substrates (Fieulaine et al., 2001). In due course, the structures of few other bacterial STPKs from *Staphylococcus*, *Mycoplama* and *Mycobacterium tuberculosis* were also determined (Deutscher and Saier, 2005). The structural studies indicated that, while most eukaryotic type STPKs belong to a single family, there exist a large diversity in structural types in bacteria.

The eukaryotic-type Ser/Thr protein kinases from different bacteria have been biochemically characterized and suggested to be involved in various biological processes such as formation of thick pili and cell motility (Panichkin et al., 2006), iron metabolism (Cheng et al., 2006), antimicrobial resistance and intestinal persistence (Kristich et al., 2007), sporulation (Stein et al., 2006) and virulence (Wiley et al., 2006). In addition, the bacterial homologs of several eukaryotic-type phosphatases have been shown to regulate their cognate kinases (Gaidenko et al., 2002; Rajagopal et al., 2003).

The functions of Ser/Thr kinases and phosphatases depend critically on their natural substrates. Therefore, the identification of natural substrates of these kinases has been the focus of many recent studies. The phosphorylation of Ser/Thr kinases may also serve an additional purpose to assist binding of FHA domain containing proteins. As a consequence, several proteins harboring FHA domains have been identified to be the endogenous substrates of some of these kinases (Ponting et al., 1999; Krupa and Srinivasan, 2005). Few other substrates of bacterial Ser/Thr kinases from *Corynebacterium glutamicum*, *Bacillus subtilis*, *Myxococcus xanthus*, *Streptococcus*, and *Anabaena* have been identified utilizing several approaches. However, in contrast to the current picture provided by the handful of substrates recognized so far, identification of a large number of phosphoproteins would provide a scope for the regulation of a majority of the metabolic processes governed by the Ser/Thr kinases.

In this scenario, the present study focuses on *Mycobacterium tuberculosis*, which causes tuberculosis and is responsible for considerable human morbidity and mortality worldwide (Bloom and Murray, 1992; Ginsberg and Spigelman 2007). Induction of evasive mechanisms by mycobacteria involves the ability of the organism to adapt its responses to external signals, often utilizing reversible protein
phosphorylation, mediated by a regulated interplay of protein kinases and phosphatases (Schorey and Cooper, 2003). Previous studies from our laboratory have established the kinase activity of PknA, one of the eleven eukaryotic type Ser/Thr kinases present in the genome of M. tuberculosis (Chaba et al., 2002). PknA was found to exhibit a Mg\(^{2+}/\text{Mn}^{2+}\) dependent autophosphorylating ability, which could be inhibited by metal oxyanions, (e.g. molybdate, tungstate and vanadate), that prevent phosphotransfer reactions. Besides autophosphorylating predominantly at threonine residues, PknA has the ability to phosphorylate exogenous substrates, such as myelin basic protein and histone (Chaba et al., 2002). Interestingly, the constitutive expression of this kinase resulted in elongation of E. coli cells, supporting its regulatory role in cell division (Chaba et al., 2002). In addition, the only Ser/Thr protein phosphatase MstP, located in the same operon was found to dephosphorylate the kinase (Boitel et al., 2003; Chopra et al., 2003). The importance of PknA in regulating cell shape and possibly cell division in mycobacteria (Kang et al., 2005) via reversible phosphorylation of cellular proteins has also opened an arena for investigating the role of this kinase in mycobacterial signal transduction. The work embodied in this thesis, therefore, deals with PknA, especially emphasizing on structure-function studies. Additionally, along with the identification of its natural substrates, the work deals with the molecular mechanism of autophosphorylation of PknA and regulation of kinase by the only Ser/Thr phosphatase present in the genome.

**REVIEW OF LITERATURE**

The review presented here surveys various aspects of protein phosphorylation and its regulation in eukaryotes as well as in prokaryotes, emphasizing on the mechanism of phosphorylation of protein kinases and the role of protein phosphatases therein. Furthermore, an attempt has been made to encapsulate the current understanding of eukaryotic-type Ser/Thr kinases on the signalling pathways in different bacteria, followed by underscoring their importance in mycobacterial signal transduction.
Environmental signals are converted into appropriate cellular responses, principally through protein phosphorylation in both prokaryotes and eukaryotes. The phosphorylation events are materialized through a regulated interplay of protein kinases and phosphatases, in order to regulate and coordinate diverse aspects of cell metabolism, gene expression, cell growth, differentiation and division (Barford, 1996). Forthcoming sections will summarize the enormous diversity existing in the process of signal transduction in eukaryotes as well as in prokaryotes.

1.1 Phosphorylation events in eukaryotic signalling

Protein phosphorylation, first discovered in eukaryotes in the mid-1950s, is the most common mechanism of cellular regulation in eukaryotes (Hunter, 2000). Phosphorylation is mediated by tightly regulated protein kinases and phosphatases, and a large family of Ser/Thr and/or Tyr kinases by far, has been found to operate in eukaryotes.

1.1.1 Protein kinases

Kinases are highly abundant molecules, virtually involved in all the critical processes ranging from cell growth and cell division to memory. They are activated or inactivated through phosphorylation, and intracellular signals pass through several kinases in a linear phosphorylation cascade before reaching their destination (Hunter, 2000). Protein kinases are further classified based on the nature of acceptor amino acids. The detail in this regard is provided below.

**Protein Ser/Thr kinases:** Ser/Thr specific protein kinases esterify a phosphate residue with the alcoholic group of Ser and Thr residues. Majority of these kinases are cytosolic, however, few transmembrane receptors are known. The largest subfamily of cytosolic Ser/Thr protein kinases is AGC subfamily, comprising of protein kinase A (cyclic-AMP dependent protein kinase), protein kinase G (cyclic-GMP dependent protein kinase) and protein kinase C (Ca\(^{2+}\)/diacylglycerol dependent protein kinase). Mitogen activated protein kinases (MAPK) constitute another important family of protein kinases in eukaryotes, involving phosphorylation of threonine as well as tyrosine residues (Cano and Mahadevan, 1995). The transmembrane receptor Ser/Thr kinases include TGF\(\beta\) family of cytokines, and consist of secreted peptides of
which TGFβs, activins and the bone morphogenic proteins are well known (Piek et al., 1999; Keah and Hearn, 2005).

**Protein Tyr kinases:** Tyrosine specific protein kinases create a phosphate ester with the phenolic OH group of tyrosine residues. Coupling of an extra cellular signal to tyrosine phosphorylation in the interior of the cell can take place by two means and hence, involves two different types of receptors as outlined below.

**Receptor tyrosine kinases:** These include transmembrane receptors with intrinsic tyrosine kinase activity. The ligand binding domain of the protein resides outside the cell, whereas the tyrosine kinase activity is localized in the cytoplasmic receptor domain (Alberts et al., 2002). The receptor tyrosine kinases are particularly involved in regulating cell division activity, differentiation and cell morphogenesis by activating the transcription of particular gene or a set of genes via a chain of reactions through phosphorylation of substrate proteins.

**Non-receptor tyrosine kinases:** The other class of tyrosine kinases referred to as non-receptor or cytosolic kinases is associated on the cytoplasmic side, with a receptor tyrosine kinase that is activated when the ligand binds to the extra cellular receptor domain. The extra cellular stimuli lead to activation of these kinases by both intramolecular and intermolecular mechanisms. Non-receptor Tyr kinases often contain several protein-protein or protein-lipid interaction modules, such as SH2, SH3 and plekstrin homology domains within the same polypeptide chain. These tyrosine kinases have further been categorized into three major classes namely, Src, Abl and Jak (Hubbard and Till, 2000).

### 1.1.2 Mechanism of phosphorylation of kinases

Protein kinases exhibit a myriad of mechanisms for their control, and the availability of structural information of the kinases has paved way for understanding the mechanism of activation and phosphorylation of the kinases. The identification of first kinase structure in 1991 for protein kinase A (PKA) advanced an understanding into mechanistic aspects of the kinase regulation (Knighton et al., 1991). Despite the presence of an overall conserved fold, the kinases display differences in the core sequence and flanking regions in order to allow them to respond to a unique set of
signals to turn their activity on or off. Although it is a complex process, this section gives an overview on the activation and signalling processes in eukaryotes in general.

**Universal activation mechanism**

Protein kinases typically have two domains/subunits namely, catalytic or kinase and regulatory domains and exhibit a multitude of mechanisms for their regulation. The best understood aspect of regulation reconciled in recent years is phosphorylation on a residue(s) located in a particular segment in the centre of the kinase domain, which is termed as the activation segment or T-loop (Nolen *et al.*, 2004). The activation loop in several kinases has been found to be highly disordered in the crystal structure and is capable of undergoing large conformational changes when the kinase switches between active (on) and inactive (off) states due to phosphorylation (Johnson *et al.*, 1996). The open or the inactive form is presumably necessary to allow access of ATP to the catalytic site and release of product ADP; however, the closed form is necessary to bring residues into correct conformation to promote catalysis. Sequence comparison has identified certain structural determinants explaining the reason behind their activation through phosphorylation. It has been suggested that kinases containing a catalytic aspartate preceded by an arginine residue, termed as RD kinases, are known to be activated by phosphorylation in the activation segment (Johnson *et al.*, 1996).

While protein kinases regulate the function of target proteins within the cell through the phosphorylation of serine, threonine and/or tyrosine residues, they are also regulated through phosphorylation in their core structure. The structure of a representative protein kinase, PKA, as shown in Fig. 1.1A highlights a small ATP binding domain and a large substrate binding domain along with an activation loop phosphorylated at Thr, making several key contacts with charged residues from the large and small lobes (Hanks *et al.*, 1988). Majority of kinases require phosphorylation at this position for the enhancement of its catalytic activity. The catalysis was increased by 3 orders of magnitude in PKA as a result of phosphorylation, whereas analogous residues in other kinases such as cdk2, ERK2, InRK, PKC, e-Src, and v-Fps enhanced the catalytic activity by 2-5 fold (Adams *et al.*, 1995; Adams, 2002). The crystal structure of insulin receptor kinase InRK, as shown in Fig. 1.1B, in the phosphorylated state highlights a well ordered activation loop and an open active site accessible for the substrate. However, in the
dephosphorylated state of this kinase, the loop moves considerably to cover the active site (Hubbard, 1997). A detailed kinetic analysis has sub-categorized the activation loop functionally as gated and non-gated, based upon the substrate binding and movement (Adams, 2003). Taken together, the activation loop functions as a "phosphorylation-sensitive switch" that facilitates the binding of substrate.

![Ribbon diagram of X-ray structures of two eukaryotic protein kinases highlighting the activation loops.](image-url)

Figure 1.1. Ribbon diagram of X-ray structures of two eukaryotic protein kinases highlighting the activation loops. (A) cyclic-AMP dependent protein kinase A (PKA) cocrystallized with ATP and inhibitory peptide. (B) Insulin receptor kinase (InRK). (Adopted from Adams, 2002 with slight modifications)

**Cis/Trans autophosphorylation**

The autophosphorylation of the kinase, as a consequence of receptor oligomerization and kinase activation, can be explained by two distinct mechanisms. According to one model, receptor autophosphorylation may occur in an intramolecular or cis manner in which the catalytic domain of kinase phosphorylates directly the sites in the same molecule. Alternatively, autophosphorylation may follow an intermolecular or trans mechanism, wherein the catalytic domain of one receptor phosphorylates the sites of a neighbouring molecule. The binding of a ligand to the receptor protein kinase leads to dimerization of two monomeric kinases and hence bring about trans-autophosphorylation of the kinase as depicted in Fig. 1.2. Mechanistically, the autophosphorylation causes the two sub domains of the intrinsic
kinase to shift and hence open the kinase domain for ATP binding. In the inactive form, the kinase sub domains are aligned so that ATP cannot reach the catalytic centre of the kinase.

![Diagram of ligand binding, dimerization, and active receptors](image)

**Figure 1.2. Transphosphorylation of receptor kinases.** On occupation by the ligand, the receptor forms a dimer and this induces a change in conformation of the cytoplasmic domain that reveals its latent tyrosine kinase activity. This phosphorylates the tyrosine residues on the linked receptor molecule (interphosphorylation). The dimerized, phosphorylated molecule constitutes the catalytically active receptor (Adopted from Balasubramanyam and Mohan, 2001 with slight modifications).

In majority of receptor or cytosolic tyrosine kinases, receptor dimerization increases the local concentration of catalytic domains, which are either intrinsic (RTKs) or extrinsic (JAKs), enabling transphosphorylation of A-loop tyrosines (Kroihner *et al.*, 2001; Imada and Leonard, 2000). Slight variations of transphosphorylation seem to operate in some tyrosine receptor kinases. For instance, the platelet-derived growth factor receptor (PDGFR), can form heterodimers with other similar but not identical members of the same subfamily, and thus allow a highly varied response to the extra cellular signal (Heldin and Wastermark, 1999). On the other hand, both cis- and trans- mechanisms may prevail in some kinases having more than one phosphorylation sites, however the activation occurs through
transphosphorytion. A typical example is insulin-like growth factor receptor, which upon autophosphorylation at Tyr1158, Tyr1162, and Tyr1163 in the activation loop, undergoes conformational changes resulting in its full activation. The first phosphorylation is said to be a cis-autophosphorylation, switching the kinase from "off" to "standby" mode, followed by inter-receptor transphosphorylation and thus proposing a model of cis-inhibition or trans-activation of the receptor (Balasubramanyam and Mohan, 2001). The soluble receptors, for instance, epidermal growth factor receptor (EGFR), have also been shown to autophosphorylate by intermolecular cross-phosphorylation, probably facilitated by receptor oligomerization (Honegger, 1988). Additionally, the mechanism of trans-autophosphorylation has also been demonstrated for several histidine kinases such as ArcB, EnvZ (Iuchi, 1993; Cai and Inouye, 2003). Although the intermolecular mechanism of autophosphorylation has been found to be operative in a majority of kinases, cis autophosphorylation was reported for several of them. Such a mechanism has recently been observed in dual-specificity tyrosine-phosphorylation-regulated protein kinases, DYRK (Lochhead et al., 2005), TGF-βRII (Chen and Weinberg, 1995) etc.

**Substrate phosphorylation**

Kinases are catalysts that transfer the γ-phosphate of ATP to target protein substrates and therefore, it is essential to study the substrates in order to have a true understanding of a specific signal transduction pathway. Binding of substrate to the kinase can be simply divided into two components: binding occurring at the active site or interactions at a site distal to the active site. Thus, substrates, in general, contain at least two binding motifs: the active site peptide and a distal docking motif. Sometimes, even though the protein substrate may not have an optimal peptide recognition sequence, the tethering of the protein substrate in close proximity to the kinase can render it a physiological substrate. In several kinases for instance, PKR, p21 PAK, ROCK and EGF, dimerization leads to allosteric changes in the substrate-binding site as well as in catalytic efficiency in a manner similar to that recognized by the activation loop phosphorylation (Taylor and Ghosh, 2006). On the other hand, most of the kinases have been reported to phosphorylate multiple substrates. A classical example in this regard is the casein kinase II (CK2), a ubiquitous eukaryotic
Ser/Thr protein kinase, which is present in the nucleus as well as cytoplasm and phosphorylate more than hundred substrates, many of them being involved in the regulation of cell division (Allende and Allende, 1995; Yoon and Seger, 2006).

1.1.3 Regulation of kinases by phosphatases

Reversible phosphorylation of proteins play pivotal role in regulating different aspects of metabolism, gene expression, cell growth, division and differentiation. The action of protein phosphatases lead to removal of stable esters formed by protein kinases and the process essentially reverse the phosphorylation of proteins. Based upon the residues dephosphorylated, the phosphatases may be classified as Ser/Thr or Tyr phosphatases. The phosphoserines or phosphotyrosines function to maintain an active kinase state until dephosphorylation by Ser/Thr or Tyr phosphatases restore the low activity basal state. To determine the specificity of signalling network, two possible strategies employed by protein kinases and phosphatases are outlined below.

Modular protein-protein interaction domains: Kinases and phosphatases utilize protein–protein recognition domains to establish interaction with other signalling protein partners. In fact, majority of the non-receptor protein kinases and phosphatases utilize these interaction modules. Typical examples are SH2, SH3 and PDZ domains, such as the Src, Abl, Hck and Csk protein kinases and the SHP protein phosphatases (Miller, 2003).

Docking interactions: Most of the kinases and phosphatases utilize docking interactions, which involve the binding surfaces of the catalytic domain distinct from the active site. These interactions are particularly prevalent in Ser/Thr kinases and phosphatases, which confer a high degree of specificity and, in some cases, allosteric regulation. A majority of Ser/Thr phosphatases (e.g. PP1 and calcineurin) and protein kinases (e.g. CDK–cyclins, MAP kinases, PDK1 and GSK3) have been shown to utilize these interactions (Espanel et al., 2003; Espanel and Huijsduijnen, 2005). Interestingly, at least one protein tyrosine kinase (Csk) has recently been shown to utilize docking (Remenyi et al., 2006). Most of the docking interactions are weak and are mediated through short linear recognition elements spanning only a few residues.

So far we recapitulated our understanding of the eukaryotic protein kinases. Since this thesis deals with the signalling processes in bacteria, subsequent discussion
will primarily be focussed on the prokaryotes with a special emphasis on eubacteria to put the work carried out in proper perspective.

1.2 Phosphorylation process in prokaryotic signal transduction

The key players in bacterial phosphorylation are the histidine kinases constituting the part of widely documented two-component system. The forthcoming sections will therefore deal with the role of two-component signal transduction system in bacteria.

1.2.1 Two component system in prokaryotes

In bacteria, two-component systems are widely used signal transduction processes, engaged in a multitude of gene regulatory mechanisms that respond to wide variety of alterations in physiological conditions (Grebe and Stock, 1999; Koretke et al., 2000). The two-component signal transduction systems are constituted by a membrane localized sensor histidine kinase that perceives environmental stimuli, and a cytoplasmic response regulator which affects gene expression. The typical histidine protein kinase (HPK) is a transmembrane receptor with an amino terminal extra cellular signal input domain that often possesses sub-domains with recognized signalling functions, and a carboxy-terminal cytosolic signalling domain (Galperin et al., 2001; Wolanin et al., 2002). By and large, in response to specific signals, the sensor kinase catalyzes an ATP-dependent autophosphorylation reaction on a conserved histidine residue of the autokinase domain. The phosphate moiety is subsequently transferred from the sensor kinase to a conserved aspartate residue in the N-terminal regulatory domain of its cognate response regulator, which further activates the DNA-binding activity of its C-terminal output domain (Fig. 1.3). Currently, more than 4000 two component systems have been identified in 145 sequenced bacterial genomes, demonstrating the enormous impact of these signalling events on the adaptation of bacteria to changing microenvironment (Ulrich et al., 2005).
The role of two component systems has been implicated in several processes such as osmosensing, chemotaxis, sporulation and virulence as reviewed by Saito (2001). The EnvZ-OmpR signal transduction pathway of *E. coli* comprising of only two proteins, the sensor histidine kinase EnvZ and the response regulator OmpR has been shown to be involved in osmoregulation. A phosphoryl group at His-243 of EnvZ is transferred to a conserved aspartyl group in the receiver domain of OmpR, which is composed of an N-terminal receiver domain and a C-terminal DNA-binding domain. The receiver domain has been shown to modulate the DNA binding properties of the output domain, which either activate or repress the expression of the major outer membrane proteins OmpC and OmpF to fit optimally to the environmental osmotic conditions (Kondo *et al.*, 1997; Park *et al.*, 1998). Another well studied two component system CheA-CheY has been shown to be involved in chemotaxis in *E. coli*. CheA serves as the histidine kinase component of this pathway, transfers the phosphoryl group to the response regulator protein CheY and hence regulates the rotation of the flagellar motor (Eisenbach, 1996). A more complex example of the two-component system is the regulation of sporulation in the
Gram-positive bacterium \textit{B. subtilis}, where multistep phosphorelay occurs. In this series of reaction, the phosphoryl group is first transferred from histidine (H1) in a sensor histidine kinase (KinA or KinB) to the conserved aspartate in the receiver domain of Spo0F followed by transfer to a specific histidine residue (H2) in the intermediate protein Spo0B. This phosphoryl group is finally transferred to aspartate residue in another receiver domain protein Spo0A which possess a DNA-binding domain (Burbulys \textit{et al.}, 1991).

Recently, Beier and Gross (2006) have reviewed the contribution of the two component system in bacterial virulence. The regulation of \textit{S. aureus} virulence involving the AgrA-AgrC two component system has been shown to control the transcription of the regulatory RNA III, as well as three additional two component systems named SaeR-SaeS, SsrA-SsrB and ArlR-ArlS (Novick, 2003; Bronner \textit{et al.}, 2004). Similarly, the BvgA-BvgS two component system has been demonstrated to be the master regulator of virulence in \textit{Bordetella pertussis}. In addition, the control of virulence via two component system has also been established in several other bacteria, such as PhoP-PhoQ, PmrA-PmrB, OmpR-EnvZ, RcsC-YojN-RcsB in \textit{Salmonella enterica} (Garcia \textit{et al.}, 1996; Altier, 2005); PhoQ-PhoP, DevR-DevS, MprA-MprB, PrrA-PrrB in \textit{M. tuberculosis} (Saini \textit{et al.}, 2004; Malhotra \textit{et al.}, 2004); and CiaR-CiaH, MicA-MicB, RR04-HK04, RR06-HK06 in \textit{S. pneumoniae} (Calva and Oropeza, 2006).

\subsection*{1.2.2 Regulation of two component system}

The response regulator is the key to regulate activity of any two component system. The phosphorylation of the regulatory domain of the response regulator activates the DNA-binding activity of its C-terminal output domain, resulting in the activation or repression of genes under its control. Additional levels of control are achieved by phosphatase activity of the sensor kinase itself (Ninfa and Magasanik, 1986). It is interesting to note that, if the kinase state predominates, the net result is the phosphorylation of the response regulator. On the other hand, when the phosphatase activity prevails, the net outcome is its dephosphorylation. In this way, the regulation of these dual function receptor histidine kinases involve the balance between "kinase on" and "phosphatase off", or "kinase off" and "phosphatase on" states. Another mode of regulation is through aspartyl phosphatases, which also limit the activation of the response regulator by the removal of phosphoryl groups (Ohlsen
et al., 1994; Perego et al., 1994). The genes encoding sensor kinases and their response regulators are often transcriptionally linked and located in the same operon (Stephenson and Hoch, 2002). However, cross-regulation of response regulators by additional histidine kinases have also been reported. A classical example is the OmpR response regulator, which could receive phosphate in vivo not only from its cognate HPK EnvZ, but also from ArcB histidine kinase, despite being present in different operon (Matsubara et al., 2000).

1.3 Eukaryotic-type Ser/Thr or Tyr kinases and phosphatases in bacteria: an emerging concept in bacterial signal transduction

It has been discussed in preceding sections of this review, that Ser/Thr or Tyr kinase mediated protein phosphorylation is the predominant regulatory mechanism in the eukaryotic signal transduction process, while in prokaryotes it is two component systems. However, two component systems have also been detected in several eukaryotes including plants, yeasts, fungi and protozoa. Most of the eukaryotic two-component systems involve a multistep phosphorelay from His to Asp to His to Asp residues. The genome of the budding yeast Saccharomyces cerevisiae encodes only one histidine kinase, Sln1, involved in sensing of high osmolarity stress, whereas three histidine kinase genes CaSLN1, CaNIK1 (also called COS1), and CaHK1 have been identified in Candida albicans (Saito, 2001). Numerous sensor histidine kinases and response regulators from higher plants have also been reported (Oka et al., 2002). Similarly, in prokaryotes Ser/Thr or Tyr kinase mediated protein phosphorylation is also evident with the identification of first bacterial Ser/Thr kinase pkn1 from M. xanthus (Munoz-Dorado et al., 1991). Studies on these kinases in different bacteria have not only implicated their involvement in growth and development, but also in virulence/pathogenicity. The advent of bacterial genome sequencing in recent years has indicated the presence of cognate phosphatases in many bacterial species, most of which have been shown to regulate the kinases and hence different cellular processes (Shi et al., 1998; Bakal and Davies, 2000). Since the work embodied in this thesis deals with eukaryotic-type Ser/Thr protein kinase and the phosphatase in bacteria, the remaining section of this review will focus on this aspect in detail.

1.3.1 Eukaryotic-type Ser/Thr kinases in bacteria
A defining feature of the Ser/Thr protein kinases (STPKs) is the presence of an N-terminal kinase domain highly conserved with eukaryotic protein kinases, connected through a transmembrane helix to a putative regulatory domain. Comprehensive phylogenetic analysis of the bacterial and eukaryotic kinases indicated that STPKs of both kingdoms were evolved from a common ancestral kinase (Inouye et al., 2000; Petrickova and Petricek, 2003). However, it is noteworthy that even though phylogenetic analyses suggested a common ancestor for prokaryotic and eukaryotic STPKs, the predatory behaviour of bacteria on eukaryotic cells leaves open the possibility that the kinase cascades could have been obtained through horizontal gene transfer.

The first bacterial Ser/Thr protein kinase Pkn1 was discovered from *M. xanthus* (Munoj-Dorado et al., 1991), and was found to play a key role in cellular differentiation. A later study revealed the presence of many more STPKs and the organism is presently believed to have ~100 of them, the largest number of potential eukaryotic like kinases (Zhang et al., 1992; Inouye et al., 2000). In a separate series, Inouye’s group demonstrated that the Ser/Thr kinase *pkn4* exists in an operon with the gene encoding its target protein, the myxobacterial 6-phosphofructokinase Mx-PFK. The activity of Mx-PFK is regulated through phosphorylation by a STPK system similar to pathways in eukaryotic organisms (Nariya and Inouye; 2002, 2003). Recently, Nariya and Inouye (2005) identified MkapB as the modulator of Pkn4 activity, and progressively, several other regulators have been identified by yeast-two hybrid approach. All these studies suggested the existence of fine-tuned control of gene expression and protein activities in the life cycle of the organism.

Another extremely diverse group of gram-negative bacteria revealing the presence of more than 90 genes encoding for Ser/Thr or Tyr kinases and phosphatases is Cyanobacteria, with Anabaena having the largest number. A family of four such proteins were initially reported in this filamentous cyanobacteria, (Zhang, 1993), and the number increased to 52 kinases and 12 phosphatases gradually (Ohmori et al., 2001; Wang et al., 2002). Majority of these kinases possess a putative catalytic domain along with a presumed regulatory domain either at the N or at the C-terminus. Recently, experimental evidences have demonstrated that both the transmembrane and extra cellular domains promote dimerization of STPK (Pallova et al., 2007).

### 1.3.2 Role of eukaryotic-type Ser/Thr kinases in bacteria
**Introduction & Review of Literature**

*M. xanthus* is the first organism for which functional STPK cascade has been identified and fine-tuned regulation of developmental processes achieved through combination of Ser/Thr kinase cascade and a two-component system. The two component system comprising of histidine kinase MrpA and response regulator MrpB positively controlled expression of mrpC. The activity of the MrpC in turn was controlled via the Pkn8/Pkn14 STPK cascade. Phosphorylations of MrpC abolished the binding to its own promotor as well as to that of FruA, a response regulator-like protein and hence controlled developmental gene expression (Ogawa *et al.*, 1996). This was the first case reported in bacteria where both STPK and two-component systems integrated into same signalling event. Further insights into regulation of Ser/Thr kinases has recently been provided by Stein *et al.* (2006) where two STPKs, PktA5 and PktB8, have been shown to be essential for efficient aggregation and spore morphogenesis, since the deletion of any of them led to the formation of translucent mounds and yielded low spore.

The role of several STPKs has been identified in cyanobacteria. The well studied STPK, Pkn22 has been shown to play a role in iron limitation (Xu *et al.*, 2003) as well as in oxidative stress (Latifi *et al.*, 2005). Another Ser/Thr kinase PknE, although exists in cluster with the phosphatase PrpA, is transcribed independently of the later. However, both the kinase as well the phosphatase play a role in the formation of heterocyst envelope as well as nitrogenase activity. Apart from this, several other STPKs such as PknA and PknD have also been shown to be involved in nitrogen fixation and heterocyst development (Zhang, 1993; Zhang and Libs, 1998).

Anabaena comprises of a family of 13 genes encoding proteins with both a Ser/Thr kinase domain and a His kinase domain, however their functions are largely unknown. They possess a Ser/Thr kinase catalytic domain at the N-terminus and a His kinase catalytic domain as the C-terminus, separated by at least one GAF domain or other regulatory domains. Hence, it was proposed that the two systems might be coupled in the signal transduction pathways (Wang *et al.*, 2002). Recently, two such kinases Pkn41 and Pkn42 have been shown to regulate iron content within the cells and required for growth when the demand for iron is high or in presence of an iron chelator. The expression of the two genes pkn41 and pkn42 was found to be directly regulated by NtcA, a global regulatory factor, suggesting that NtcA is involved in the coordination of nitrogen metabolism and iron homeostasis (Cheng *et al.*, 2006).
The genome of another cyanobacterium, *Synechocystis* sp. strain PCC 6803, contains 12 genes for Ser/Thr kinases (Kaneko et al., 1996; Leonard et al., 1998). The kinase SpkA has been shown to regulate the expression of genes in the three putative operons, namely, pilA1-pilA2, pilA5-pilA6, and pilA9, leading to the formation of thick pili and cell motility (Panichkin et al., 2006). Apart from SpkA, another kinase SpkB has also been predicted to be involved in cell motility. However, other kinases SpkC, E and F did not seem to play a role in motility, as the deletion mutants did not show any defect in the motility (Kamei et al., 2002). One of the putative Ser/Thr kinase, SpkJ, has been implicated in yhr signalling pathway of hyperosmotic stress response (Paithoonrangsarid et al., 2004). Besides, the kinases and phosphatases from *Synechocystis* have also been implicated in various other processes such as control of carbon metabolism (Wang et al., 2004), nitrogen control and cell differentiation (Rodriguez et al., 1994). The STPK and its substrate have also been characterized from *S. pneumoniae* (Novakova et al., 2005).

1.3.3 Concept of reversible phosphorylation

A key aspect of regulation of biological processes in both prokaryotes and eukaryotes is protein phosphorylation and dephosphorylation mediated by protein kinases and phosphatases, respectively. Studies have shown the involvement of Ser/Thr kinase-phosphatase pair PrkC and PrpC in the sporulation of *B. subtilis* (Madec et al., 2002; 2003). Similarly, stkl and its neighbouring gene stpl in *Pseudomonas aeruginosa* and YopH and YpkA of *Yersinia pseudotuberculosis* demonstrated the regulation via kinase phosphatase pair.

The Ser/Thr protein phosphatase Pph1 in *M. xanthus* was reported for the first time by Treuner-Lange et al., (2001). The protein belonged to PP2C family of protein phosphatases and its expression was observed under both vegetative and developmental conditions, but peaked during early aggregation. Using disruption studies, the role of phosphatase in vegetative growth and fruiting body formation was demonstrated. Further, a strong interaction between Pph1 and the protein kinase Pkn5, a negative effector of development as seen by yeast two hybrid, had put forward a functional link between a Pkn2-type protein kinase and a PP2C phosphatase.

The eukaryotic type Ser/Thr kinase and phosphatase from Group B Streptococcus, *S. agalactiae* was found to reversibly phosphorylate an inorganic
pyrophosphatase affecting cell growth, cell segregation and virulence. Later on, Jin and Pancholi (2006) identified a Ser/Thr kinase and the cognate phosphatase from Group A Streptococcus, *S. pyogenes*. Phosphorylated STPK as well the kinase domain alone were dephosphorylated in the presence of co-transcribed phosphatase, thus regulating the intracellular networks that affect cell division/septation, shape, size, expression and virulence. Further, the appearance of the histone like protein as the major phosphorylated protein in phosphatase depleted cell lysate indicted this putative transcriptional regulatory protein to be the natural substrate of reversible phosphorylation by the kinase and phosphatase pair.

1.4 Eukaryotic-type Ser/Thr protein kinase mediated phosphorylation and its regulation in mycobacteria

The genome of *M. tuberculosis* contains genes encoding eleven STPKs, one Ser/Thr phosphatase (PstP) and two protein tyrosine phosphatases (PtpA and PtpB) (Cole *et al*., 1998; Leonard *et al*., 1998). After the genome sequence came out, the prime emphasis was to test the activity of these proteins. These proteins have been implicated as potential candidates for switching the regulation of growth, development and pathogenesis. Presently, most of these kinases have been characterized; however, work on their natural substrates is scanty. Furthermore, the mechanism of regulation of these kinases has not yet been elucidated in detail. The following section will deal with the progress in characterization of mycobacterial Ser/Thr kinases, structures and mechanism of their activation and, identification of protein kinase substrates.

1.4.1 Eukaryotic-type Ser/Thr protein kinases in mycobacteria

The STPKs can broadly be categorized into two types, namely, kinases with sensor domains and those without sensor domains. The majority of the kinases have N-terminal kinase, a transmembrane and the C-terminal sensor domain, examples are; PknA, PknB, PknD, PknE, PknF, PknH, PknI, PknJ, PknL. Besides these, two of the kinases, PknG and PknK, do not have any sensor and transmembrane domains. Fig. 1.4 summarizes the knowledge of these kinases and the forthcoming section will provide a detail understanding.
**PknA**

The genome organisation of *M. tuberculosis* revealed the presence of the gene *pknA* in an operon with other Ser/Thr kinase *pknB* and genes encoding two morphogenic proteins (*php2A, rodA*), along with the only Ser/Thr phosphatase *mstP* (also known as *pstP*). The location of these genes adjacent to each other strongly suggested the involvement of these proteins in regulating cell morphology through phosphorylation/dephosphorylation cascades (Av-gay and Everett, 2000). Chaba *et al.* (2002) first reported the Mg$^{2+}$/Mn$^{2+}$ dependent autophosphorylating ability of PknA. Besides autophosphorylation, the protein was also found to phosphorylate the exogenous substrates. Interestingly, concomitant with the phylogenetic prediction of role of PknA in cell division, the constitutive expression of kinase in *E. coli* resulted
in elongated cell morphology. The finding was substantially progressed by the work of Kang et al. (2005) through demonstration of the involvement of the kinase in regulating cell morphology of mycobacteria. It has also been shown that PknA can phosphorylate PknB or vice versa. Further, using peptide library screening and proteomic methods, Wag31 and Rv1422 were identified as substrates of PknA as well as PknB. The identification of Wag31, a homolog of cell shape/cell division protein DivIVA of S. coelicolor, as a substrate of PknA strongly speculated the role of this kinase in cell division. However, detailed mechanism of action, physiological relevance etc. has not been worked out in detail. Furthermore, no structure-function analysis has been carried out for the kinase to aid in understanding the mechanism of its action.

PknB

Studies of PknB have yielded sizeable information regarding the structure of the kinase as well as its role in cell division. Av-Gay et al. (1999) for the first time showed that pknB encodes for a functional kinase which is constitutively transcribed in M. tuberculosis. After several reports on prediction of essentiality, only recently it has been demonstrated that the gene could be disrupted in the presence of a second functional copy in M. smegmatis as well as M. tuberculosis (Fernandez et al., 2006). The eukaryotic Ser/Thr protein kinase inhibitors, which inactivate PknB in vitro with a 50 % inhibitory concentration, were able to kill M. tuberculosis H37Rv, M. smegmatis mc²155, and M. aurum with MICs in the micro molar range, further demonstrating that PknB is essential for sustaining mycobacterial growth. Both pknA and pknB were predominantly transcribed in exponential phase and the over expression in mycobacteria led to major growth and morphological changes indicating a defect in cell wall synthesis and possibly in cell division (Kang et al., 2005).

PknB was the first mycobacterial Ser/Thr kinase for which the structure was solved (Young et al., 2003; Ortiz-Lombardia et al., 2003). Despite the diversity in sequence, the 3-D fold of the catalytic domain of PknB was found to be highly similar to eukaryotic cAMP-dependent protein kinase, PKA (Madhusudan et al., 2002). The fold comprised of an N-terminal sub domain of a curled β-sheet and a long α-helix, and a C-terminal lobe composed essentially of α-helices. The structure also revealed the presence of other kinase characteristic features, such as glycine rich motif acting as a flap, highly conserved active site, presence of P loop, P+1 loop and activation
loop (Ortiz-Lombardia et al., 2003). Concurrently, Young et al., (2003) reported the presence of a disordered activation loop and in turn supported the universal activation mechanism for PknB similar to most of the eukaryotic kinases. This mechanism was efficiently validated successively by Boitel et al. (2003) by identifying the regulation of the kinase activity through two threonines located within the activation loop of PknB. Recently, the structure of PknB in complex with mitoxantrone, an ATP-competitive inhibitor suggested a mode of protein kinase regulation in mycobacteria resembling that of the RNA-dependent protein kinase PKR (Wehehkel et al., 2006).

The discovery of in vivo substrates of PknB added abundant knowledge to aid in understanding the role of this kinase in the signalling mechanisms of mycobacteria. A Forkhead associated (FHA) domain containing protein GarA (Rv1827) was credited to be the physiological substrate of PknB, using a global proteomic approach in whole cell extracts of M. tuberculosis as well as M. smegmatis. Binding studies strongly suggested that the activation loop of PknB and the C-terminal FHA domain of GarA contribute to docking interaction (Villarino et al., 2005). The FHA domains have been identified as protein-protein interaction modules in signalling which can bind to the phosphothreonine residues on the target protein. The identification of FHA domain containing proteins in the M. tuberculosis genome gave clues towards the role of these proteins in the signal transduction, possibly by acting as substrates of the Ser/Thr kinases and hence mediating interactions between different proteins in the cascade. In this track, two other FHA domain proteins, Rv0020c and the putative ABC transporter Rv1747 were also identified as the substrates of PknB in vitro, however, in vivo phosphorylation of these proteins by PknB has not yet been demonstrated (Grundner et al., 2005). Concurrently, apart from FHA domain containing proteins, Wag31 and Rv1422 were also identified to be the substrates of PknB.

The extra cellular domain of PknB was modelled as four linked PASTA (Pbp and Ser/Thr kinase attached) domains, structurally compatible with the proposed targeting domain of Streptococcus pneumoniae penicillin binding protein 2x (Pbp2x), a cell wall targeting enzyme (Wertz et al., 2007). These domains are functionally implicated in aspects of growth, cell division, and development. The presence of PASTA repeats in PknB together with its location in the operon comprising the candidate cell division gene pnb2A, raised the possibility of the regulation of penicillin binding protein PB2A by PknB. This hypothesis was validated by
detection of phosphorylation of PBP2A in presence of PknB, thus identifying it as one
of the substrates of the kinase (Dasgupta et al., 2006).

**PknD**

The first Ser/Thr protein kinase lying in the vicinity of phosphate specific
transporter *pstA*-1 was characterized by Piers et al. (1997) and named as Mbk
(mycobacterial protein kinase). However, with the advent of genome sequence in
1998, the kinase was later termed as PknD. The protein was confirmed to be
localized in the cell envelope in *M. tuberculosis*, however, the homologous protein in
*M. bovis* was found to be truncated to N-terminal kinase domain as a result of frame
shift, and thus presumably generated a free cytosolic protein (Piers et al., 2000).

The structure of the sensor domain of transmembrane receptor Ser/Thr kinase
PknD was solved by Good et al. in 2004. The extra cellular domain was shown to
form a rigid, six-bladed β-propeller with a flexible tether to the transmembrane
domain, thus mediating signalling presumably by directly or indirectly altering the
activity of the intracellular kinase domain. A recent study provided further insights
into the mechanism of signal activation by PknD through identification of a dimer
interface within the sequence and asserting that the dimer formation allosterically
activated unphosphorylated PknD and phosphorylation provided an allosteric
regulation mechanism (Greenstein et al., 2007).

The identification of substrates of PknD further deduced the contribution of
the kinase in mycobacterial signalling. As has been described in previous section, the
proteins with FHA domains serve as potential candidates for acting as substrates of
Ser/Thr kinases. Correspondingly, the FHA domain containing protein Rv1747,
identified as the substrate of PknB, was phosphorylated by PknD as well. Another
approach to identify the potential endogenous substrates of the kinase was undertaken
by Perez et al. (2006), wherein a mutant strain of *M. tuberculosis* with a deletion in
the kinase region was generated. The *in vitro* phosphorylation of cell-wall fractions in
the disruptant showed the absence of a family of phosphorylated peptides which was
identified by mass spectrometry as MmpL7, a transporter associated with the
deposition of components of the complex *M. tuberculosis* cell wall. Identification of
MmpL7 as a substrate of PknD perhaps suggested a critical role of this kinase in the
formation of the *M. tuberculosis* envelope, although the presence of the gene adjacent
to *pstS* suggested the involvement of the kinase in regulation of phosphate transport in
mycobacteria. Very recently, a putative anti-anti-sigma factor, Rv0516c was found to be phosphorylated by PknD in vitro as well as in vivo at threonine. As a consequence of phosphorylation, the binding of Rv0516c to another anti-anti-sigma factor Rv2638 was inhibited, thus supporting a model where signals transmitted through PknD alter the transcription program of *M. tuberculosis*. Apart from PknD, PknB and PknE have also been shown to phosphorylate sigma factor regulators (Greenstein *et al.*, 2007).

**PknE**

The transmembrane eukaryotic-type Ser/Thr protein kinase PknE, was first characterized by Molle *et al.* (2003a). Although detailed characterization of the protein was lacking, the membrane topology of the protein was demonstrated and using MALDI-TOF mass spectrometry several phosphorylation sites were also determined within the catalytic domain of the protein. The *pknE* gene was not found to be essential for bacterial growth in vitro or in mice (Sassetti and Rubin, 2003; Sassetti *et al.*, 2003). The crystal structure of the ligand free, activated kinase domain of PknE suggested the formation of dimer similar to PknB, despite only 37% sequence conservation, putting forward a functional role of dimerization in both the kinases and possibly other *M. tuberculosis* receptor STPKs. The crystal structure of apo-PknE revealed a catalytic site similar to PknB, however, there were substantial changes in the nucleotide complexes and the structure of the catalytic loop (Gay *et al.*, 2006). Although the specific substrates have not yet been identified, the kinase domain was found to phosphorylate several proteins reported to be phosphorylated by other kinases in vitro, such as the FHA domain containing protein Rv1747 and sigma factor regulators Rv0516c, Rv1365c, Rv1904 and Rv3221A (Grundner *et al.*, 2005).

**PknF**

The location of the *pknF* in operon with an ATP-binding cassette (ABC) transporter gene (Rv1747) and close proximity to *pknE* (three ORFs apart) suggested a possible role of the kinase in transport. Although the presence of the gene was confirmed by southern hybridization in pathogenic *M. tuberculosis* H37Ra and *M. bovis* BCG, the homolog could not be detected in avirulent strain *M. smegmatis*, suggesting its possible involvement in the processes specific to pathogenic mycobacteria (Koul *et al.*, 2001). The enhanced growth and deformed cell morphology in the antisense strain of *M. tuberculosis* expressing low level of PknF, implicated the role of the kinase in cell division. Further, the strain also showed a
significant increase in the specific uptake of D-glucose and thus substantiated the involvement of the kinase in glucose transport (Deol et al., 2005).

Although no structural information is available for the kinase as yet, the FHA domain containing protein has been demonstrated to be the substrate of this kinase. It has been shown that PknF was able to phosphorylate Rv1747, a newly described ABC transporter located in the same operon, and the recruitment as well as phosphorylation was mediated through the two non-redundant FHA domains present in PknF. The interaction was found to be phospho-dependent, since any mutation that compromised the phosphorylation ability of the kinase, abrogated the interaction between PknF and Rv1747 (Molle et al., 2004). The role of Rv1747 in survival of M. tuberculosis in mice has recently been demonstrated. A deletion mutant of Rv1747 although did not affect growth in vitro, exhibited a significantly impaired growth in mouse bone marrow-derived macrophages and dendritic cells (Curry et al., 2005). Taken together, these observations insinuate that Rv1747 not only functions as a transporter with potential importance in virulence, but also serves as a part of the complex signalling system involving a eukaryote-like Ser/Thr protein kinase pathway.

_PknG_

The predominantly cytosolic enzyme PknG, comprising of a kinase domain flanked by a large N- and C- terminal domain unlike other receptor Ser/Thr kinases was initially proposed to be involved in pathogenesis due to its explicit absence from non-pathogenic mycobacteria. The pathogenic nature was elegantly demonstrated by Walburger et al. (2004), where they found that the kinase from pathogenic bacteria was secreted within the macrophage phagosome and inhibited phagosome-lysosome fusion, thus mediating the intracellular survival of mycobacteria. Further, inactivation of the kinase in mycobacteria resulted in lysosomal localization followed by mycobacterial cell death in infected macrophages. Apart from its crucial role in pathogenesis, the location of _pknG_ in an operon with _glnH_ gene encoding for an extra cellular glutamine binding protein suggested its role in glutamine uptake. However, experimental evidences excluded any defect in glutamate uptake or alteration in intracellular concentration as a result of depletion of PknG, thus eliminating the involvement of the kinase in glutamine metabolism (Nguyen et al., 2005; Cowley et al., 2004).

Despite the demonstration of its involvement in pathogenesis, the role of PknG in mycobacterial signalling is less implicated, as no substrate of the kinase has been
identified so far. However, the most recent crystal structure of PknG in complex with the highly selective low molecular-weight inhibitor AX20017 revealed an affluence of information. The unique multidomain topology of PknG revealed the presence of N-terminal rubredoxin and C-terminal tetratrico-peptide repeat domains edging the central kinase domain. The structure divulged that the inhibitor was buried deep within the adenosine-binding site and targeted an active conformation of the kinase domain. Remarkably, the inhibitor binding pocket was shaped by a unique set of amino acid side chains not present in any human kinase. These results explained the specific mode of action of AX20017, and provided insight into specificity for blocking the kinase and hence proliferation of mycobacteria without compromising homologous kinases in eukaryotes (Scherr et al., 2007).

**PknH**

The autokinase activity of PknH was first demonstrated by Molle et al. (2003b). Differential expression of PknH during stress conditions, transmembrane localization and absence in non-pathogenic strains suggested a possible role of the kinase in processes unique to pathogenic strains (Sharma et al., 2004). Interestingly, evidence was presented for the ability of PknH to phosphorylate EmbR, a FHA domain containing protein assumed to modulate the level of arabinogalactan. The interaction was essentially FHA domain dependent, as mutation of some critical residues of the FHA domain abolished the PknH mediated transphosphorylation of EmbR. Progressively, the crystal structure of EmbR (Alderwick et al., 2006) presented a unique domain architecture: the N-terminal winged-helix DNA-binding domain forming an extensive interface with the all-helical central bacterial transcription activation domain positioned adjacent to the regulatory C-terminal FHA domain, which mediates binding to a Thr-phosphorylated site in PknH. The key feature for PknH autophosphorylation was involvement of Thr170 within the activation loop, a hallmark for the activation of a majority of eukaryotic kinases. However, the crystallographic and peptide-binding data suggested that the activation loop of the PknH did not constitute a docking site for the substrate EmbR.

In contrast to attenuated growth of *pknG* knockout strain, Av-Gay and co-workers showed that the disruption of PknH resulted in a hyper virulent phenotype in BALB/c mice (Papavinasasundaram et al., 2005). Additionally, they demonstrated that the *emb* and *ini* operons, encoding proteins associated with mycobacterial cell-wall, belonged to PknH signalling cascades, since a down regulated expression of
these genes was observed in kinase deleted mutant strain. The PknH mediated increase in transcription of embAB genes significantly altering resistance to ethambutol, together with the *in vivo* upregulation of PknH inside the host macrophages and the dephosphorylation of EmbR by the Ser/Thr phosphatase MstP, suggested a functionally relevant signalling mechanism involving PknH-EmbR-embCAB and phosphatase system (Sharma *et al.*, 2006a; Sharma *et al.*, 2006b). Furthermore, the recent identification of two novel *in vitro* substrates of PknH, Rv0681c and Dacb1, has opened the possibility of participation of PknH in novel signalling pathways in mycobacteria (Zheng *et al.*, 2007).

**PknI**

The Ser/Thr protein kinase PknI was first characterized for its autophosphorylation ability by Gopalaswamy *et al.* (2004). The closest homology of the kinase with Stkl from *S. coelicolor*, predicted a role in virulence and cell-segregation, however, no functional studies have till date been carried out. Recently, immunoblot analysis of sub-cellular fractions of *M tuberculosis*, using anti-PknI antibodies raised in rabbits, showed that PknI was localized to the bacterial cytosol. Furthermore, the relative expression of protein, as measured by combining molecular beacons and RT-PCR, decreased during infection of THP-1 human macrophages (Singh *et al.*, 2006). PknI was thus distinct from PknA in being largely cytosolic despite the presence of a transmembrane helix, and down regulated upon infection unlike the later. However, detailed studies regarding the essentiality of the kinase, mechanism of action and identification of *in vivo* substrates warrant attention.

**PknK**

Although no characterization of PknK has been carried out, it has been predicted to regulate the production of secondary metabolite(s) in *M tuberculosis*, on the basis of its homology to the enzymes involved in production of secondary metabolites.

**PknJ and PknL**

The characterization of these transmembrane Ser/Thr kinases has not yet been carried out.

1.4.2 *Eukaryotic-type protein phosphatases in mycobacteria*

The presence of two different types of protein phosphatases (Ser/Thr and Tyr) have been documented in *M. tuberculosis* genome. Available reports indicated them
to be active proteins (Boitel et al., 2003; Chopra et al., 2003). However, detailed mechanism of their action has not worked out. In vitro studies have revealed that protein phosphatases interact with kinases, however, the physiological relevance has not yet been unravelled.

Ser/Thr phosphatases: The Ser/Thr protein phosphatase PPP (also referred as PstP or MstP, will be referred as MstP in this thesis), is located in an operon with the Ser/Thr kinases pknA and pknB and genes encoding morphogenic proteins pbp2A and rodA. Its activity was found to be Mn\(^{2+}\) dependent and has been grafted under PP2C family of Ser/Thr protein phosphatases (Chopra et al., 2003; Boitel et al., 2003). The crystal structure of this phosphatase has recently been solved (Pullen et al., 2004; Alzari 2004). The fold within the catalytic domain and two-metal centre revealed in the high resolution crystal structure of MstP shared homology with the human PP2Ca, however, it showed the binding of a third Mn\(^{2+}\) in a site created by a large shift in a previously unrecognized flap sub domain adjacent to the active site (Pullen et al., 2004; Alzari 2004). Thus, in contrast to the human enzyme, MstP can bind third Mn\(^{2+}\) thus reasoning the metal dependency of the enzyme and the alternate conformation of the enzyme may help in affording versatility in binding different STPKs. Recently, the atomic resolution structure of a soluble PPM phosphatase from the saprophyte M. smegmatis in complex with different ligands has supported a reaction mechanism different from the currently accepted model, thus reinforcing the hypothesis of convergent evolution in phosphatases (Bellinzoni et al., 2007).

Tyr phosphatases: The presence of two tyrosine phosphatases (PtpA and PtpB) within the genome of M. tuberculosis is definitely surprising in the absence of endogenous tyrosine kinases. Both PtpA and PtpB exhibit protein tyrosine phosphatase activity (Cowley et al., 2002), however sequence comparison suggested that they belong to different PTP families. PtpA has been classified as a low-molecular weight (LMW) phosphatase, whereas, the PtpB sequence falls into the conventional PTP or dual-specificity phosphatase (DSP) class. The crystal structure of PtpA (Madhurantakam et al., 2005) revealed a classic LMW PTP fold structurally homologous to eukaryotes. The structure of PtpB revealed a single domain comprising of a central typical four-stranded parallel \(\beta\)-sheet surrounded by \(\alpha\)-helices
with the overall fold reminiscent of dual-specificity phosphatases. The structure also highlighted an unprecedented lid associated with protection from reactive oxygen species \textit{in vitro}, as exposure to H$_2$O$_2$ inactivated PtpB to a considerably lower extent in comparison to PtpA which has an open active site.

There have been evidences for the importance of \textit{ptpA} and \textit{ptpB} in persistence of mycobacterial infection. The expression of PtpA in \textit{M. bovis} BCG increased in stationary phase \textit{in vitro} or upon infection of human monocytes (Bach \textit{et al.}, 2006). Furthermore, the transient expression of LMW PtpA led to reduced phagocytosis in \textit{M. tuberculosis} and \textit{M. smegmatis} cells and increased actin polymerization in phagosomes, and hence proposed to inhibit phagosome-lysosome fusion. The deletion of \textit{ptpB} did not show any growth phenotype in culture, however, the defects were apparent in activated macrophages and guinea pig model (Grundner \textit{et al.}, 2007; Grundner \textit{et al.}, 2005). Recently, Beresford \textit{et al.} (2007) have provided experimental evidence for the dual-specificity protein phosphatase activity and phosphoinositide phosphatase activity of PtpB, thereby suggesting a potential novel role of the phosphatase in PtdIns metabolism in the host and hence mycobacteria pathogenicity.

\subsection*{1.4.3 Interplay between protein kinases and phosphatases}

The presence of single Ser/Thr phosphatase for eleven STPKs reinforced the hypothesis that MstP acts as antagonist for all the kinases. MstP has been found to dephosphorylate PknA, PknB, PknD, PknE, PknF (Duran \textit{et al.}, 2005) as well as exogenous substrates phosphorylated by these kinases. In addition, the natural substrates such as phosphorylated EmbR, have also been shown to be dephosphorylated by the MstP (Sharma \textit{et al.}, 2006a). In a systemic approach, Molle \textit{et al.} (2006) identified the phosphorylation of FAS-II components by eleven STPKs. While PknA, PknB, PknE, PknF, PknH efficiently phosphorylated $\beta$-ketoacyl-ACP synthases (KasA and KasB), phosphorylation by other kinases could not be detected. In addition, both the proteins could also be dephosphorylated by MstP. Taken together, the dephosphorylation of Ser/Thr kinases as well as their substrates by MstP suggests the prevalence of phosphorylation dephosphorylation cascades co-ordinately regulating the signalling in mycobacteria.
SCOPE OF THE PRESENT STUDY

The diversity in the mechanism of phosphorylation of eukaryotic protein kinases is widely reported (Hunter, 2000). However, the mechanistic aspects of such event in bacterial eukaryotic-type Ser/Thr kinases in bacteria are inadequately comprehended. Although majority of these kinases have been shown to catalyze autophosphorylation, the mechanism of their regulation is still unclear. The work embodied in this thesis, therefore, concentrates on one such kinase PknA from *M. tuberculosis*, which is thought to be involved in the process of cell division (Chaba *et al.*, 2002; Kang *et al.*, 2005)

*M. tuberculosis* genome sequence reveals that PknA is localized in an operon with the other Ser/Thr kinase PknB, morphogenic proteins like PBP2A and RodA, and the only Ser/Thr protein phosphatase MstP. Earlier studies from our lab have reported cloning and characterization of *pknA* from *M. tuberculosis*. PknA has been shown to autophosphorylate as well as phosphorylate exogenous substrates like histone and myelin basic protein (Chaba *et al.*, 2002). The PknA has also been shown to be a substrate of PknB and vice-versa (Kang *et al.*, 2005). Interestingly, its constitutive expression in *E. coli* cells resulted in morphological changes associated with cell-division (Chaba *et al.*, 2002). Although the precise mechanism is still obscure, the ability of PknA to phosphorylate Wag31 (homologous to DivIIV of *S. coelicolor*) in vitro, led to the speculation of its involvement in the process of cell division (Kang *et al.*, 2005). In vitro studies established that MstP has the ability to dephosphorylate specifically the autophosphorylated PknA as well as the exogenous substrates phosphorylated by this kinase (Boitel *et al.*, 2003, Chopra *et al.*, 2003). Since reversible phosphorylation is an important regulatory event and MstP is a member of the operon, it would really be interesting to know about the functional coupling of these two enzymes.

The crystal structure of mycobacterial Ser/Thr kinase PknB, has highlighted the universal activation mechanism, which is a hallmark of eukaryotic protein kinases (Ortiz-Lombardia *et al.*, 2003). The structures of apo-PknE and the sensor domain of PknD have also put forward insights into mechanisms of these kinases. However, the molecular mechanisms underlying the regulation of phosphorylation activity as such towards the functionality of PknA have not yet been established. Studies are still
warranted that could aid in deducing the mechanism of phosphorylation of the kinase and further identify other functionally relevant substrates to decipher the role of this kinase in cell division.

Underscoring the importance of PknA, the present work has been focussed on the mechanism of phosphorylation of the kinase by the identification of certain critical residues/regions involved in the process. PknA has been postulated to comprise of catalytic, transmembrane, juxtamembrane and extracellular domains. However, role of these domains in signalling is still unknown. Therefore, efforts will be made to unravel the collaboration between various domains of PknA in order to understand the mechanism of regulation of this kinase. In addition, attempt towards identification of natural substrates, if any, together with the role of the phosphatase in the dephosphorylation process would presumably help in gaining insight into the PknA mediated signalling pathway. Since PknA has been postulated to be an essential gene in mycobacteria (Kang et al., 2005), elaborative knowledge on these aspects will aid in considering PknA for framing new/novel drug intervention strategies (Noble et al., 2004). This is the crying need at present considering the fact that drug resistance in M. tuberculosis is one of the prime causes for rapid resurgence of tuberculosis throughout the world (Bloom and Murray, 1992; Ginsberg and Spigelman, 2007).