Chapter 4
Biochemical and functional characterization of PknA, an eukaryotic-type serine/threonine kinase from *Mycobacterium tuberculosis*

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Accumulation of genome sequencing data in recent years has revealed the prevalence of putative genes encoding eukaryotic-type Ser/Thr kinases in many bacterial species (Zhang et al., 1992; Zhang, 1996; Hirakata et al., 1998; Shi et al., 1998; Zhang et al., 1998b). These sequences are usually annotated based on the presence of protein kinase ‘signature’, which constitutes eleven conserved subdomains (Hanks et al., 1988; Hanks and Hunter, 1995; Shi et al., 1998). Available reports have indicated that some of these kinases possess the ability to phosphorylate on serine and/or threonine residues as well as capable of phosphorylating basic substrates as has been observed with Ser/Thr kinases from eukaryotes (Zhang, 1996; Hanlon et al., 1997; Mukhopadhyay et al., 1999; Kamei et al., 2001; Yang et al., 2001; Neu et al., 2002). Involvement of such kinases in regulating growth and development has largely been documented in bacteria such as Myxococcus xanthus (Munoz-Dorado et al., 1991; Udo et al., 1996; Zhang et al., 1996; Hanlon et al., 1997), Anabaena (Zhang, 1993; Zhang and Libs, 1998; Zhang et al., 1998a), Streptomyces (Ueda et al., 1996; Nadvornik et al., 1999; Umeyama et al., 1999; Neu et al., 2002) and Bacillus subtilis (Gaidenko et al., 2002; Madec et al., 2002). In Yersinia pseudotuberculosis, YpkA has been identified as the first secretory prokaryotic Ser/Thr kinase involved in pathogenicity (Galyov et al., 1993). Besides these, eukaryotic-type Ser/Thr kinases have been implicated in virulence in opportunistic pathogens such as Pseudomonas aeruginosa (Wang et al., 1998). Thus a detailed study of these kinases, especially in pathogenic bacteria, could produce important insights into their contributions to signal transduction.

In M. tuberculosis eleven Ser/Thr kinases have been identified based on the presence of the consensus motifs (Cole et al., 1998). The cloning, expression and purification of one such homologue of mycobacterial Ser/Thr kinases, PknA has been described in Chapter 3. PknA was also shown to express constitutively in its natural host. In the present chapter, characterization of this protein will be carried out to elucidate whether PknA possesses intrinsic enzymatic activity.
4.1 Assessment of autophosphorylating ability of PknA

Ser/Thr kinases in both prokaryotes and eukaryotes have largely been shown to autophosphorylate (Cohen, 1992; Zhang, 1996). In vitro kinase assays were therefore performed with the recombinant fusion protein (MBP-PknA) to envisage the autophosphorylating ability of PknA. The autophosphorylating residues were identified by phosphoamino acid analysis. The amino acid sequence was analyzed to identify the domains of PknA followed by mutational studies to ascertain the region(s) responsible for autophosphorylation.

4.1.1 Autophosphorylation

The autophosphorylating ability of PknA was monitored by incubating the fusion protein with \( [\gamma^{-32}P] \)-ATP in the presence of 10 mM Mn\(^{2+} \) at room temperature for 20 min, followed by separation of reaction products by SDS-PAGE. Finally, the labelled protein was identified by autoradiography of the dried gel. As evident in Fig. 4.1, 6 \( \mu g \) of MBP-PknA exhibited significantly higher phosphorylation than 800 ng of the protein, hence revealing concentration dependence of enzymatic activity (compare lanes 2 and 3). As expected, MBP-\( \beta \)gal did not show any labelling (Fig. 4.1, lane 1), thereby confirming that the signal observed for MBP-PknA was due to the autophosphorylation of PknA. Further, the fusion protein was labelled with \( \gamma^{-32}P \) for different time periods (0 – 60 min). The results as depicted in Fig. 4.2 indicated the time dependent autophosphorylation of PknA.

4.1.1.1 Effect of divalent cations

Divalent cations are known to influence the autophosphorylating ability of bacterial Ser/Thr kinases (Peirs et al., 1997; Udo et al., 1997; Av-Gay et al., 1999; Petrickova et al., 2000). Therefore, to monitor their effect on the autophosphorylation of PknA, in vitro kinase assays were carried out in the presence or absence of \( Mg^{2+} / Mn^{2+} / Ca^{2+} \). As shown in Fig. 4.3A, phosphorylation is detectable only in the presence of either \( Mg^{2+} \) or \( Mn^{2+} \). Compared to the concentration of 1 mM, 10 and 100 mM \( Mg^{2+} \) exhibited \(~11 - 13\)-fold increase in the autophosphorylation of PknA.
Fig. 4.1  

**In vitro kinase assay with PknA.** The autophosphorylating ability of the fusion protein (MBP-PknA) was monitored by incubating with [γ-\(^{32}\)P]-ATP in the presence of Mn\(^{2+}\), followed by separation of reaction products by SDS-PAGE. The labelled protein was identified by autoradiography of the dried gel.  

*Lane 1*: 6 μg of MBP-βgal control;  
*lane 2*: 800 ng of MBP-PknA;  
*lane 3*: 6 μg of MBP-PknA. Position of molecular size standards is indicated.
**Fig. 4.2 Time course of PknA autophosphorylation.** The autophosphorylating ability of the fusion protein was monitored following incubation with [γ-³²P]-ATP in the presence of Mn²⁺ for indicated time periods. The reaction products were subjected to SDS-PAGE. (A) The labelled protein was identified by autoradiography of the dried gel. (B) The results were visualized by phosphoimager and the band intensities were quantitated using ‘Quantity one’ software.
Fig. 4.3  Effect of divalent cations on the autophosphorylation of PknA. *In vitro* kinase assays were carried out in the presence of indicated concentrations of Mg$^{2+}$ or Mn$^{2+}$. (A) The labelled product was visualized by autoradiography of the dried gel. Position of molecular size standards is indicated. (B) The dried gels were scanned in a phosphoimager and the band intensities were quantitated using 'Quantity one' software.
(Fig. 4.3A, upper panel and 4.3B). On the other hand, the autophosphorylating ability of PknA was augmented up to a concentration of 10 mM Mn$^{2+}$. However, it had an inhibitory effect on the enzyme activity at 100 mM concentration (Fig. 4.3A, lower panel and 4.3B). Interestingly, the magnitude of autophosphorylation of PknA in the presence of 10 mM of these divalent cations when considered, ~7-fold increase in the intensity of the phosphorylating protein band was observed with Mn$^{2+}$ compared to Mg$^{2+}$ (Fig. 4.3A and 4.3B). Ca$^{2+}$ alone did not influence the autophosphorylation of PknA. Furthermore, in vitro kinase assay when carried out in the presence of 10 mM Mn$^{2+}$, neither Ca$^{2+}$ nor Mg$^{2+}$ up to a concentration of 10 mM had any significant effect on the enzyme activity (Table 4.1).

4.1.1.2 Effect of oxyanions

Oxyanions imitate the trigonal-bipyramidal form of the phosphate group and thus bind to a large number of phosphotransferases and phosphohydrolases, thereby inhibiting the phosphoryl transfer reactions (Soman et al., 1983; Maruta et al., 1998). The effect of oxyanions (molybdate, tungstate and vanadate) on in vitro autophosphorylation of PknA was therefore assessed. The fusion protein was preincubated with oxyanions (0.5 - 2.5 mM) for 15 min at room temperature prior to the initiation of $\gamma^{32}$P labelling. Varying degree of inhibition was observed, molybdate being the most effective inhibitor and tungstate the least. At 2.5 mM concentration, both tungstate and vanadate showed ~40 - 50% inhibition of enzyme activity whereas molybdate at the same concentration completely abolished PknA autophosphorylation (Fig. 4.4).

4.1.1.3 Autophosphorylating residues

The autophosphorylating amino acids in PknA were identified by immunoblot analysis using antibodies against phosphoamino acids. ~800 ng of purified fusion protein was resolved in SDS-PAGE, electroblotted to nitrocellulose membrane and probed with anti-phosphoserine/anti-phosphothreonine/anti-phosphotyrosine antibodies. Following incubation with secondary antibodies, the signals were detected using chemiluminescent detection kit. Both anti-phosphoserine and anti-
Table 4.1  Effect of Ca\(^{2+}\) and Mg\(^{2+}\) on PknA autophosphorylation in the presence of Mn\(^{2+}\)

<table>
<thead>
<tr>
<th>Divalent ion (mM)</th>
<th>Activity retained (% in the presence of 10 mM Mn(^{2+}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{Ca}^{2+})</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>98.2 ± 9.9</td>
</tr>
<tr>
<td>10</td>
<td>75.2 ± 4.6</td>
</tr>
<tr>
<td>100</td>
<td>ND</td>
</tr>
<tr>
<td>(\text{Mg}^{2+})</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>96.0 ± 26.8</td>
</tr>
<tr>
<td>10</td>
<td>82.8 ± 14.5</td>
</tr>
<tr>
<td>100</td>
<td>13.6 ± 4.7</td>
</tr>
</tbody>
</table>

ND: Not detected
Fig. 4.4  Effect of oxyanions on the autophosphorylation of PknA. MBP-PknA fusion protein was preincubated for 15 min at room temperature with indicated concentrations of oxyanions and then assayed for phosphorylation activity. (A) The labelled protein was identified by autoradiography of the dried gel (upper panel). Coomassie stained gel as loading control (lower panel). (B) The dried gels were scanned in a phosphoimager and the band intensities were quantitated using ‘Quantity one’ software. Results were calculated as percentage of control.
phosphothreonine antibodies recognized MBP-PknA but not MBP-βgal (Fig. 4.5, compare lanes 2 and 4 as opposed to 1 and 3) suggesting that the phosphorylating residues are serine and threonine. However, these antibodies did not recognize PknA equally, as phosphorylation of threonine was more than that of serine (Fig. 4.5, compare lanes 2 and 4). This observation does not seem to be unusual as a number of bacterial Ser/Thr kinases have been reported to phosphorylate strongly at threonine (Urabe and Ogawara, 1995; Peirs et al., 1997; Vomastek et al., 1998; Mukhopadhyay et al., 1999; Neu et al., 2002). On the other hand, no specific signal was obtained in Western blots using anti-phosphotyrosine antibody. Thus, these lines of evidence argue that PknA is definitely a Ser/Thr kinase.

4.1.2 Identification of domain(s) involved in autophosphorylation of PknA

Ser/Thr kinases usually harbour two domains, conserved region designated as the catalytic domain and variable sequences termed as the regulatory domain (Taylor, 1989). In order to identify the domains of PknA, its amino acid sequence was analyzed followed by mutational studies to ascertain the region(s) responsible for autophosphorylation.

4.1.2.1 Bioinformatic analysis

Comparison of nucleotide derived predicted amino acid sequence of PknA with non-redundant database through PSI-BLAST programme showed homology with Ser/Thr kinases of both prokaryotic and eukaryotic origin. Catalytic domains of such kinases are reported to be conserved (Hanks et al., 1988; Hanks and Hunter, 1995). This region of PknA was therefore aligned (Fig. 4.6) with that of the selected (one representative from a genera exhibiting score value above 100) bacterial Ser/Thr kinases (PpkA of P. aeruginosa, score = 162, expect = 8e-39; Pkn1 of M. xanthus, score = 155, expect = 8e-37; Pkg2 of Streptomyces granaticolor, score = 126, expect = 4e-28) using Clustal W 1.74 programme (Thompson et al., 1994). The conserved subdomains I to XI, which are the characteristics of eukaryotic-type kinases (Hanks et al., 1988) are located towards the amino and central regions of PknA (amino acid residues 1-268). Like other Ser/Thr or Tyr kinases, the PknA sequence shows the
Fig. 4.5 Phosphoamino acid analysis of PknA. Purified fusion protein was subjected to SDS-PAGE and electroblotting. The blots were probed with anti-phosphothreonine (left panel) and anti-phosphoserine (right panel) antibodies. Lanes 1 and 3: MBP-β gal; lanes 2 and 4: MBP-PknA. Position of molecular weight standards is indicated.
Fig. 4.6  Alignment of amino acid sequence of *M. tuberculosis* PknA with other bacterial Ser/Thr kinases. Nucleotide derived amino acid sequence of different bacterial Ser/Thr kinases were aligned using Clustal W 1.74 programme. The conserved regions are shown by shading the sequence. Roman numerals (I - XI) indicate Hanks's type conserved subdomains of Ser/Thr kinases. Abbreviations used: PpkA.Pa - PpkA from *P. aeruginosa*, Pkn1.Mx - Pkn1 from *M. xanthus*, Pkg2.Sg - Pkg2 from *S. granaticolor*. 
ATP binding motif GXGXXG (alanine is present instead of first glycine and X is a variable amino acid) in subdomain I and an invariant lysine residue involved in phosphotransfer reaction presumably by mediating proton transfer in subdomain II. Strikingly, PknA possesses typical DXKPXN and GTXXYXAPE sequences of Hank's subdomain VI and VIII respectively, which distinguishes Ser/Thr from Tyr kinases (Hanks et al., 1988; Taylor, 1989). However, an alanine residue instead of a proline (underlined) is present in the typical triads of amino acids, RDL[X]nDFG[X]nGTPXXX[A/S]PE (Matsumoto et al., 1994) in subdomains VI - VIII of PknA (Fig. 4.6 and 4.7).

The catalytic domain is followed by a sequence of 70 amino acid residues (269-338) containing 7 serine, 6 threonine, 15 alanine, 7 glycine and 11 proline. These residues account for 66% of this sequence. Downstream to the Ala/Pro-rich region, there is a hydrophobic stretch of 23 amino acid residues (339 to 361), which is flanked by glutamine-arginine at the amino terminal side and aspartate-asparagine at the carboxy terminal side (Fig. 4.7). The charge distribution around the hydrophobic region fits well with that for the transmembrane domain (Gennity and Inouye, 1991; Pugsley, 1993; Gafvelin and von Heijne, 1994; Kim et al., 1994). Ala/Pro-rich region and transmembrane domain have been identified in a number of bacterial Ser/Thr kinases (Udo et al., 1995; Zhang et al., 1996; Vomastek et al., 1998; Yang et al., 2001). However, the amino acid residues 362-431 (Fig. 4.7) share negligible homology with other eukaryotic-type Ser/Thr kinases and is thus unique to PknA. This stretch of amino acids presumably represents the regulatory domain of PknA.

4.1.2.2 Deletion analysis

In order to have an insight into the minimal region(s) responsible for exhibiting the autophosphorylating ability of PknA, a series of deletion mutants were created. The deletion scheme has been outlined in Fig. 4.8. The mutants, Δ23-33, Δ17-130 and Δ143-200 representing deletions in the catalytic subdomains were created by digesting the wild type construct pMAL-PknA with unique restriction enzyme sites in the gene i.e., Agel, Psti and AatII respectively, followed by religating the backbone. On the other hand, the remaining three deletion mutants (1-338, 1-268
Fig. 4.7 Different domains of PknA. (A) Schematic representation showing different domains of PknA. Numbers indicate the span of amino acids in a particular domain. Abbreviations used: Catalytic – Catalytic domain; Ala/Pro – Ala/Pro-rich region; Tm – Transmembrane domain; Regulatory – Regulatory domain. (B) Predicted amino acid composition of each domain. Colour codes in A and B are identical.
<table>
<thead>
<tr>
<th>Mutant Code</th>
<th>Amino acids deleted</th>
<th>Domains deleted</th>
<th>Catalytic</th>
<th>Ala/Pro</th>
<th>Tm Regulatory</th>
</tr>
</thead>
<tbody>
<tr>
<td>PknA</td>
<td>NONE</td>
<td>NONE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-338</td>
<td>339-431</td>
<td>Transmembrane and Regulatory</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-268</td>
<td>269-431</td>
<td>Ala-Pro rich, Transmembrane and Regulatory</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Δ 23-33</td>
<td>23-33</td>
<td>Part of Subdomain I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Δ 17-130</td>
<td>17-130</td>
<td>Subdomains I-V</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Δ 143-200</td>
<td>143-200</td>
<td>Part of Subdomain VI, whole VII, VIII, Part of Subdomain IX</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>253-431</td>
<td>1-252</td>
<td>Subdomains I-X</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 4.8 Schematic diagram of PknA and its deletion mutants.** Numbers indicate the span of amino acids in the mutants. Δ followed by numbers indicate the deleted regions. Abbreviations used: Catalytic – Catalytic domain; Ala/Pro – Ala/Pro-rich region; Tm – Transmembrane domain; Regulatory – Regulatory domain.
and 253-431) were constructed by PCR cloning using specific primers and pMAL-PknA as template. Primer pairs CC58/CC69, CC58/CC68 and CC13/CC14 were used for amplifying fragments for 1-338, 1-268 and 253-431 deletion mutants respectively. Primer sequences are provided in Chapter 2, Table 2.2. The forward primer CC58 for 1-338 and 1-268 deletion mutants was designed from the first nucleotide of \( pknA \). ‘CAT’ in this primer did not correspond to the genome sequence but was introduced to incorporate a \( NdeI \) site at the 5’ end of the PCR amplified fragment. The primers CC68 and CC69 were designed based on the \( pknA \) sequence. However, in these primers stop codons have been introduced after the catalytic domain (CC68) and Ala/Pro-rich region (CC69). The fragments obtained by PCR amplification with these primers were digested with \( \text{EcoRI/HindIII} \) (sites incorporated in the forward and reverse primers respectively). This was followed by their ligation with pMAL-c2 digested with the same restriction enzymes. For 253-431 mutant, the forward primer CC13 was designed to start after the subdomain X and had an \( \text{EcoRI} \) site incorporated at the 5’ end. The reverse primer CC14 designed from the last nucleotide of \( pknA \) had a \( \text{SalI} \) site at its 5’ end. The fragment amplified with these primers was digested with \( \text{EcoRI/SalI} \) and cloned at the corresponding sites in pMAL-c2. All the mutants were confirmed by restriction analysis and sequencing.

The deletion mutants were expressed as MBP fusion protein and purified on an amylose column. The expression was confirmed by Western blot analysis with the rabbit polyclonal antisera against MBP-PknA (Fig. 4.9, upper panel). The autophosphorylating ability of the deletion mutants was further monitored in an in vitro kinase assay (Fig. 4.9, lower panel). Wildtype PknA and its deletion mutant, 1-338 (lacking transmembrane and regulatory domains) exhibited autophosphorylating ability (Fig. 4.9, lanes 1 and 2). However, deletion of the Ala/Pro-rich region in addition to the transmembrane and regulatory domains as in 1-268 mutant completely abolished autophosphorylation (Fig. 4.9, lane 3). These results thereby indicated the essentiality of Ala/Pro-rich region in displaying the autophosphorylating ability of PknA. The mutants of PknA deleted in the various regions of its putative catalytic domain, \( \Delta23-33, \Delta17-130 \) and \( \Delta143-200 \) did not autophosphorylate (Fig. 4.9, lanes 4, 5 and 6) suggesting that these conserved subdomains are elementary to the
Fig. 4.9  Autophosphorylating ability of the deletion mutants of PknA. Western blot analysis with rabbit polyclonal anti-MBP-PknA sera (upper panel) and $\gamma^{-32}$P labelling (lower panel) of different deletion mutants of PknA. Lane 1: MBP-PknA; lane 2: MBP 1-338; lane 3: MBP 1-268; lane 4: MBP $\Delta$23-33; lane 5: MBP $\Delta$17-130; lane 6: MBP $\Delta$143-200; lane 7: MBP 253-431; lane 8: MBP-βgal. Position of molecular weight markers is indicated.
manifestation of autophosphorylating ability. Absence of signal for 253-431 mutant further emphasized the necessity of the catalytic domain and non-essentiality of the regulatory domain for the autophosphorylation of kinase (Fig. 4.9, lane 7). The results of the deletion analysis have been summarized in Table 4.2.

4.1.2.3 Effect of point mutation on PknA autophosphorylation

Among the eleven conserved subdomains of eukaryotic-type kinases, an invariant lysine in subdomain II is the best-characterized residue. This lysine is known to be directly involved in the phosphotransfer reaction (Snyder et al., 1985; Kamps and Sefton, 1986; Weinmaster et al., 1986; Carrera et al., 1993). It has been reported that all substitutions of this residue, including arginine, in the bacterial eukaryotic-type kinases result in the loss of enzymatic activity (Hanks et al., 1988; Vomastek et al., 1998; Motley and Lory, 1999; Nadvomik et al., 1999). Similar studies were carried out with PknA. The lysine (K) at amino acid position 42 in subdomain II of PknA was mutated to Asparagine (N). Two forward (CC58 and CC62) and two reverse (CC61 and CC63) primers were used for generating the mutant. Sequences of the primers have been provided in Chapter 2, Table 2.2. Base mismatches for the desired mutations were incorporated in primers CC62 and CC63. To generate the mutant, two sets of primary and one set of secondary PCR reactions were carried out by overlap extension method (Ho et al., 1989) as illustrated in Chapter 2, Fig. 2.1. Primary reactions were carried out with primers CC58/CC63 and CC61/CC62 using pMAL-PknA as template. The mixture of primary PCR products for individual mutant served as template for carrying out secondary reactions with primers CC58 and CC61. PCR conditions were same as used for amplification of wildtype pknA from M. tuberculosis genomic DNA (Chapter 3, Section 3.1). Thus, the mutation was contained within the amplified ~460 bp fragment of pknA, which had a unique XhoI site in addition to EcoRI and NdeI sites incorporated in the primer CC58 (Fig. 4.10). To express the K42N mutant in fusion with MBP, the ~460 bp fragment of mutated pknA was digested with EcoRI/XhoI and substituted for the corresponding wildtype fragment in the pMAL-PknA backbone (Fig. 4.10). The resulting construct, pMAL-K42N was sequenced to confirm the mutation.
Table 4.2  Deletion analysis of PknA

<table>
<thead>
<tr>
<th>Mutant code</th>
<th>Amino acids deleted</th>
<th>Domains deleted</th>
<th>Autophosphorylating ability</th>
</tr>
</thead>
<tbody>
<tr>
<td>PknA</td>
<td>NONE</td>
<td>NONE</td>
<td>+</td>
</tr>
<tr>
<td>1-338</td>
<td>339-431</td>
<td>Transmembrane and Regulatory</td>
<td>+</td>
</tr>
<tr>
<td>1-268</td>
<td>269-431</td>
<td>Ala-Pro rich, Transmembrane and Regulatory</td>
<td>-</td>
</tr>
<tr>
<td>Δ 23-33</td>
<td>23-33</td>
<td>Part of Subdomain I</td>
<td>-</td>
</tr>
<tr>
<td>Δ 17-130</td>
<td>17-130</td>
<td>Subdomains I-V</td>
<td>-</td>
</tr>
<tr>
<td>Δ 143-200</td>
<td>143-200</td>
<td>Part of Subdomain VI, whole VII, VIII, Part of Subdomain IX</td>
<td>-</td>
</tr>
<tr>
<td>253-431</td>
<td>1-252</td>
<td>Subdomains I-X</td>
<td>-</td>
</tr>
</tbody>
</table>

‘+’ indicates autophosphorylating ability
‘-’ denotes inability to autophosphorylate
Fig. 4.10 Construction of plasmid pMAL-K42N. The ~375 bp EcoRI/XhoI fragment was excised out from plasmid pMAL-PknA. The mutagenized EcoRI/XhoI fragment was substituted for the analogous wildtype region to obtain pMAL-K42N. Relevant restriction enzyme sites have been mentioned. WT represents wildtype pknA. K42N indicates that lysine (K) residue at amino acid position 42 has been mutated to Asparagine (N).
The K42N mutant was expressed and purified as MBP fusion protein (MBP-K42N). The purity of MBP-K42N was monitored by running the samples on SDS-PAGE followed by Coomassie brilliant blue staining (Fig. 4.11A) and was further confirmed by Western blot analysis with the rabbit polyclonal antisera against MBP-PknA (Fig. 4.11B). Interestingly, the mutant showed faster migration than the wild-type protein (Fig. 4.11A and 4.11B). The molecular mass of $89.34 \pm 6.79 \text{kDa}$ (Mean $\pm$ SD, $n = 6$) for the mutant was comparable to that predicted from the sequence (88.7 kDa) for the wildtype protein but significantly lower than the latter’s observed molecular weight ($97.10 \pm 1.29 \text{kDa}$; Mean $\pm$ SD, $n = 4$). Furthermore, the phosphorylating ability of the mutant when monitored in an in vitro kinase assay, MBP-K42N did not show any labelling (Fig. 4.11C, lanes 4 and 5). These results clearly indicate that the lysine of subdomain II is critical for catalyzing the phosphorylation reaction.

### 4.2 Assessment of substrate phosphorylating ability of PknA

In order to mediate its effect, Ser/Thr kinases are known to phosphorylate substrate proteins present downstream in the regulatory cascades (Cohen, 1992). The ability of PknA to phosphorylate known exogenous substrates as well as cellular proteins was therefore examined.

#### 4.2.1 Phosphorylation of exogenous substrates

Purified MBP-PknA fusion protein was added to the reaction mixtures containing [$\gamma$-$^{32}$P]-ATP and either histone or myelin basic protein. The reaction products were subjected to SDS-PAGE, gels were dried and labelled proteins were identified by autoradiography. As shown in Fig. 4.12, in addition to an autophosphorylating band of MBP-PknA at $97 \text{kDa}$, substrate phosphorylation was also observed (Fig. 4.12, lanes 4, 5, 8 and 9). In contrast, exogenous substrates alone showed negligible phosphorylation (Fig. 4.12, lanes 2 and 6). Even in the presence of boiled fusion protein, phosphorylation of histone/myelin basic protein could not be seen (Fig. 4.12, lanes 3 and 7).
Fig. 4.11 Western blot analysis and \textit{in vitro} kinase assay of the K42N mutant of PknA. (A) Purified fusion proteins were subjected to SDS-PAGE and the gel was stained with Coomassie brilliant blue. \textit{Lane} 1: Molecular weight marker; \textit{lane} 2: 6 µg of MBP-βgal; \textit{lane} 3: 800 ng of MBP-PknA; \textit{lane} 4: 6 µg of MBP-PknA; \textit{lane} 5: 800 ng of MBP-K42N; \textit{lane} 6: 6 µg of MBP-K42N. (B) Purified MBP-PknA (\textit{lane} 1) and MBP-K42N (\textit{lane} 2) following Western blot with rabbit polyclonal anti-MBP-PknA sera. (C) Autophosphorylating ability of the mutant protein was monitored in an \textit{in vitro} kinase assay. 6 µg of MBP-βgal control (\textit{lane} 1), 800 ng (\textit{lane} 2) and 6 µg (\textit{lane} 3) of MBP-PknA, 800 ng (\textit{lane} 4) and 6 µg (\textit{lane} 5) of MBP-K42N mutant. Position of molecular weight markers is indicated.
Fig. 4.12  **Substrate phosphorylation by PknA.** Phosphorylation of exogenous substrates was monitored by incubating these with PknA in the presence of [γ-32P]-ATP. The reaction products were separated on 12.5% SDS-PAGE and the labelled proteins were identified by autoradiography of the dried gel. *Lane 1*: MBP-PknA; *lane 2*: Histone (50 μg); *lane 3*: Histone (50 μg) with boiled MBP-PknA; *lane 4*: Histone (1 μg) with MBP-PknA; *lane 5*: Histone (50 μg) with MBP-PknA; *lane 6*: Myelin basic protein (50 μg); *lane 7*: Myelin basic protein (50 μg) with boiled MBP-PknA; *lane 8*: Myelin basic protein (1 μg) with MBP-PknA; *lane 9*: Myelin basic protein (50 μg) with MBP-PknA. Arrows denote the position of phosphorylated histone/myelin basic protein. Size of molecular weight markers is indicated.
4.2.2 Interaction with unknown protein

To elucidate the possibility of interaction of PknA with unknown protein(s), soluble fraction of cell lysates from *Escherichia coli* strain DH5α was incubated for 10 h at 4°C with MBP-PknA fusion protein that was immobilized on amylose resin. *In vitro* kinase assays with aliquots of the resin after thorough washing indicated the phosphorylation of a 56.36 ± 0.83 kDa (Mean ± SD, n = 3) protein in addition to ~97 kDa autophosphorylating MBP-PknA (Fig. 4.13, lane 7). The MBP-PknA immobilized amylose resin when incubated with or without boiled lysate showed the phosphorylation of only ~97 kDa fusion protein (Fig. 4.13, lanes 4 and 6). This ~56 kDa band did not seem to be an experimental artifact, since it was absent in the controls (resin only, resin with either lysate or MBP-βgal and lysate) used in the assay. Furthermore, immobilization of the boiled MBP-PknA on amylose resin followed by incubation with the lysate neither showed autophosphorylation of the fusion protein nor highlighted the phosphorylation of the ~56 kDa band (Fig. 4.13, lane 5). Thus, these results indicate that at least a ~56 kDa soluble protein of *E. coli* interacts with PknA.

4.3 Effect of constitutive expression of PknA on the morphology of *E. coli* cells

In a preliminary study, pMAL-PknA transformed cells of *E. coli* strain TB1 grown for 2 - 10 h upon IPTG induction were observed to exhibit unusual elongation pattern compared to the cells harbouring pMAL-c2 plasmid. To elucidate the contribution of PknA in this process, morphological studies were carried out following the constitutive expression of the protein in *E. coli* strain DH5α.

4.3.1 Constitutive expression of PknA in *E. coli*

In order to examine the involvement of PknA in the process of cell elongation, we sought to express the protein constitutively in the *E. coli* host strain DH5α using a
**Fig. 4.13** Phosphorylation of soluble protein of *E. coli* by PknA. MBP-βgal or MBP-PknA (100 μg) was immobilized on amylose resin and incubated with crude soluble protein extracts of *E. coli* strain DH5α (250 μg) for 10 h at 4°C. *In vitro* phosphorylation was carried out at room temperature for 30 min with aliquots (12 μl) of washed amylose beads suspended in buffer. *Lane 1*: resin only; *lane 2*: resin incubated with crude soluble protein extracts of *E. coli*; *lane 3*: resin incubated with MBP-βgal and crude soluble protein extracts of *E. coli*; *lane 4*: resin incubated with MBP-PknA; *lane 5*: resin incubated with boiled MBP-PknA and crude soluble protein extracts of *E. coli*; *lane 6*: resin incubated with MBP-PknA and boiled crude soluble protein extracts of *E. coli*; *lane 7*: resin incubated with MBP-PknA and crude soluble protein extracts of *E. coli*. The position of the ~56 kDa band is denoted by an arrow. Position of molecular weight standards is indicated.
low copy vector. However, expression of mycobacterial protein in *E. coli* is known to be difficult, especially under the control of a heterologous promoter (Matsuo et al., 1990; Garbe et al., 1993). We therefore used a *Mycobacterium-E. coli* shuttle vector p19Kpro, derived from p16R1 (Garbe et al., 1994) containing a mycobacterial 19 kDa antigen promoter. These series of vectors are known to elicit a low level of mycobacterial gene expression in *E. coli* (Garbe et al., 1993).

In order to clone *pknA* in p19Kpro, pPknA (Chapter 3, Fig. 3.3) and p19Kpro were digested with *NdeI* and *BamHI* respectively, followed by treatment with Klenow to obtain blunt-ended fragments. Both these fragments were further restriction digested with *HindIII* and ligated. The resulting construct was named as p19Kpro-PknA (Fig. 4.14). The K42N mutant of *pknA* was also cloned in p19Kpro (p19Kpro-K42N). The strategy adopted was same as for the construction of p19Kpro-PknA, but here instead of pPknA, pMAL-K42N (Fig. 4.10) was used as the template for obtaining K42N insert by restriction digestion. Further, to clone *pknA* in an antisense orientation, pPknA was initially digested with *NdeI* and treated with Klenow to obtain a blunt-ended fragment. After restriction digestion with *BamHI* this fragment was subsequently ligated to p19Kpro that had already been digested with *BamHI* and *EcoRV*. The antisense construct of *pknA* was designated as p19Kpro-aPknA (Fig. 4.14). All three constructs, p19Kpro-PknA, p19Kpro-K42N and p19Kpro-aPknA were transformed in *E. coli* strain DH5α. Clones carrying the gene of interest were confirmed at all steps by restriction analysis.

The constitutive expression of PknA was monitored at the mRNA as well as protein levels. Total RNA was isolated from cultures harbouring p19Kpro or p19Kpro-PknA plasmid by hot phenol extraction method as described in Chapter 2, Section 2.2.3. For Northern analysis, RNA samples were electrophoresed on 1.2% agarose gel containing formaldehyde, transferred onto a nylon membrane and hybridized with [α-32P]-CTP labelled *pknA* as the probe. As shown in Fig. 4.15 (upper panel), hybridization signal was observed with RNA extracted from cells transformed with p19Kpro-PknA, thereby confirming the expression of the kinase at the mRNA level. Further, to monitor the expression of PknA at the protein level, overnight cultures (~15 h at 37°C) of p19Kpro and p19Kpro-PknA were reinoculated
Fig. 4.14 Strategy for cloning *pknA* in p19Kpro. pPknA was digested with appropriate restriction enzymes in order to subclone *pknA* in p19Kpro in both sense (p19Kpro-PknA) and antisense (p19Kpro-aPknA) orientations.
Fig. 4.15  Northern analysis following constitutive expression of *pknA* in *E. coli*. Total RNA was isolated from *E. coli* DH5α cells transformed either with p19Kpro (*lane 1*) or p19Kpro-PknA (*lane 2*), electrophoresed on 1.2% agarose gel containing formaldehyde, transferred onto a nylon membrane and processed for hybridization. **Upper panel:** the blot following hybridization using [α-32P]-CTP labelled *pknA* as probe. **Lower panel:** the same blot following methylene blue staining served as a loading control.
and grown till an OD$_{600}$ of 0.5. Cells were harvested after 3 h, lysates were prepared and expression was monitored by running the samples on SDS-PAGE followed by Coomassie brilliant blue staining. The constitutive expression of PknA was evident from the induced band (Fig. 4.16A, compare lanes 2 and 3) and was further confirmed by Western blot analysis with anti-MBP-PknA sera (Fig. 4.16B, lane 2). The protein was found to be in the soluble fraction. Furthermore, _in vitro_ kinase assay with crude cell extracts indicated autophosphorylating ability of the expressed protein (Fig. 4.16C, compare lanes 1 and 2).

### 4.3.2 Phenotypic effect of PknA on _E. coli_

The effect of constitutive expression of PknA on the phenotype of _E. coli_ cells was evaluated by scanning electron microscopy. As shown in Fig. 4.17, _E. coli_ alone (panel ‘a’) or that transformed with p19Kpro (panel ‘b’) were normal rods of size 1 - 2 μm. On the other hand, _E. coli_ cells transformed with p19Kpro-PknA (panel ‘c’) showed remarkable elongation (more than 95% of the cells were in the range of 60 – 70 μm). Interestingly, _E. coli_ transformed with either the kinase-dead variant, p19Kpro-K42N (panel ‘d’) or the antisense construct, p19Kpro-aPknA (panel ‘e’) did not show such phenotypic alteration. Taken together, these findings convincingly established that the constitutive expression of PknA resulted in the elongation of _E. coli_ cells.

### 4.4 Placement of PknA among bacterial Ser/Thr kinases

Bioinformatic analyses revealed that most of the mycobacterial Ser/Thr kinases including PknA exhibit highest sequence homology with other known and putative kinases from _Mycobacterium leprae_ or _Streptomyces coelicolor_ (Peirs _et al._, 1997; Av-Gay and Everett, 2000). In order to relate PknA to other bacterial Ser/Thr kinases, for which functions had already been assigned, we carried out sequence comparisons with non-redundant database in the BLAST and PSI-BLAST programmes using the mail server at NIH. Nine different bacterial Ser/Thr kinase sequences were retrieved through these searches where homology score varied from
Fig. 4.16  Constitutive expression of PknA in \textit{E. coli} and its kinase activity. (A) Soluble fractions of crude lysates of \textit{E. coli} DH5\(\alpha\) cells transformed either with p19Kpro vector or p19Kpro-PknA were subjected to SDS-PAGE and Coomassie brilliant blue staining. \textit{Lane 1}: Molecular weight marker; \textit{lane 2}: p19Kpro; \textit{lane 3}: p19Kpro-PknA. (B) Constitutive expression of PknA in \textit{E. coli} was confirmed by Western blot analysis with rabbit polyclonal anti-MBP-PknA sera. \textit{Lane 1}: crude lysate of \textit{E. coli} DH5\(\alpha\) cells transformed with p19Kpro; \textit{lane 2}: crude lysate of \textit{E. coli} DH5\(\alpha\) cells transformed with p19Kpro-PknA. (C) Kinase activity of the constitutively expressed PknA. \textit{In vitro} kinase assay was carried out with soluble fractions of crude lysates of \textit{E. coli} DH5\(\alpha\) cells transformed either with p19Kpro vector (\textit{lane 1}) or p19Kpro-PknA (\textit{lane 2}). Position of molecular weight markers is indicated. Arrow in all the panels denotes the position of constitutively expressed PknA.
Fig. 4.17 Effect of constitutive expression of PknA on the morphology of *E. coli* cells. Morphology of the cells was determined by scanning electron microscopy. Panel a: *E. coli* DH5α cells; Panels b – e: *E. coli* DH5α cells transformed with p19Kpro (b) or p19Kpro-PknA (c) or p19Kpro-K42N (d) or p19Kpro-aPknA (e). Bar in each panel indicates magnification.
80 to 162 with expect values between $e^{-15}$ and $e^{-39}$. In contrast, YpkA, a Ser/Thr
kinase of *Y. pseudotuberculosis* known to be associated with virulence (Galyov *et al.*, 1993),
showed insignificant homology (score = 39.9, expect value = 0.054). The multiple sequence alignment of PknA with bacterial Ser/Thr kinases was carried out
using the CLUSTAL W 1.74 programme (Thompson *et al.*, 1994) after truncating the
highly variable N- and C- termini of the selected sequences. The phylogenetic tree
was constructed after 100 cycles of bootstrapping using PROTDIST, UPGMA and
CONSENSE programmes available at the PHYLIP site (Felsenstein, 1993) and was
drawn with TREEVIEW (Page, 1996). The phylogenetic placement revealed PknA to
be very close to Pkn1 and Pkn9 of *M. xanthus* (Fig. 4.18). Since these kinases in *M.
* xanthus have been implicated in sporulation or cell division/differentiation (Munoz-
Dorado *et al.*, 1991; Hanlon *et al.*, 1997), these results, thereby suggested the
involvement of PknA in similar functions. The phylogenetic data thus corroborated
with the findings of the constitutive expression of PknA in *E. coli*.

**Discussion**

Signal transduction pathways in both prokaryotes and eukaryotes often utilize
protein phosphorylation catalyzed by kinases as a molecular switch in regulating
different cellular activities. In prokaryotes, phosphorylation event is controlled by
two proteins, His kinase and response regulator thereby forming two-component
signal transduction pathways (Fabret *et al.*, 1999; Robinson *et al.*, 2000; West and
Stock, 2001). Such signalling in eukaryotes is mediated by Ser/Thr or Tyr kinases
(Hanks *et al.*, 1988; Hanks and Hunter, 1995). Interestingly, in recent years, genome
sequence data revealed the presence of eukaryotic-type Ser/Thr kinases in a number
of bacterial species (Zhang *et al.*, 1992; Zhang, 1996; Hirakata *et al.*, 1998; Shi *et al*.,
1998; Zhang *et al.*, 1998b). *E. coli* genome search also revealed the presence of
sequences exhibiting homology with eukaryotic-type Ser/Thr kinases, however, they
have neither been characterized biochemically nor functionally (Shi *et al.*, 1998).
Eleven such Ser/Thr kinases have been identified in the genome of *M. tuberculosis*
(Cole *et al.*, 1998). Among these, PknB, PknD, PknF and PknG have been
Fig. 4.18  Phylogenetic placement of PknA from *M. tuberculosis* with respect to other bacterial Ser/Thr kinases with known function. Multiple sequence alignment was carried out by Clustal W 1.74 programme and the tree was constructed using PROTDIST, UPGMA and CONSENSE programmes available at the PHYLIP site. Abbreviations used: PknA.mtb - PknA from *M. tuberculosis*; Pkn1.mx - Pkn1, Pkn2.mx - Pkn2, Pkn5.mx - Pkn5, Pkn6.mx - Pkn6 and Pkn9.mx - Pkn9 from *M. xanthus*; AfsK.sc - AfsK from *S. coelicolor*; Pkg2.sg - Pkg2 from *S. granaticolor*; PpkA.pa - PpkA from *P. aeruginosa*; PknA.ana - PknA from *Anabaena*; YpkA.yp - YpkA from *Y. pseudotuberculosis*.
biochemically characterized (Peirs et al., 1997; Av-Gay et al., 1999; Koul et al., 2001) but their biological functions are still unknown. In this chapter, we have described the detailed characterization of another mycobacterial Ser/Thr kinase, PknA, which is located adjacent to genes encoding bacterial morphogenic proteins. Thus mycobacterial PknA demands special attention due to its location within the genome, which seems to be unique among all prokaryotes (Av-Gay and Everett, 2000).

PknA was expressed and purified in fusion with maltose binding protein (MBP). Biochemical studies with the fusion protein convincingly established PknA to be a Ser/Thr protein kinase capable of autophosphorylation on serine and threonine residues (Figs. 4.1 and 4.5) as well as phosphorylating model substrates, histone and myelin basic protein (Fig. 4.12). These findings are in agreement with the previously characterized Ser/Thr kinases, PknB and PknD of *M. tuberculosis* (Peirs et al., 1997; Av-Gay et al., 1999). Presence of aspartate residue in Hank’s subdomains VI and VII of Ser/Thr kinases have been implicated in their interactions with the phosphate group of ATP through Mg$^{2+}$ salt bridges (Hanks et al., 1988; Matsumoto et al., 1994). Thus the observation regarding the effect of Mg$^{2+}$ on the enzyme activity of PknA (Fig. 4.3) was in agreement with such hypothesis. It seems that PknA is distinct from PknD of *M. tuberculosis* where Mg$^{2+}$ did not influence the enzyme activity (Peirs et al., 1997). Interestingly, PknA autophosphorylation was stimulated significantly in the presence of Mn$^{2+}$ compared to Mg$^{2+}$ (Fig. 4.3). Only a few bacterial Ser/Thr kinases have been reported to prefer Mn$^{2+}$ compared to Mg$^{2+}$ (Peirs et al., 1997; Udo et al., 1997; Petrickova et al., 2000). Even in eukaryotic protein kinases known to utilize Mn$^{2+}$, enzyme activities in the presence of either Mn$^{2+}$ or Mg$^{2+}$ have been found to be similar (Udo et al., 1997). Moreover, divalent cations like Ca$^{2+}$ in the presence of Mn$^{2+}$ did not strongly inhibit the autophosphorylation of PknA (Table 4.1), which is in contrast to PknD of *M. tuberculosis* (Peirs et al., 1997).

Ser/Thr and Tyr kinases share eleven characteristic sequence motifs, termed as Hank’s subdomains (Hanks et al., 1988; Hanks and Hunter, 1995). Analysis of nucleotide derived amino acid sequence of PknA indicated the presence of these conserved subdomains (Fig. 4.6). Like other Ser/Thr and Tyr kinases, the PknA
sequence showed the ATP binding motif in subdomain I and an invariant lysine residue involved in phosphotransfer reaction in subdomain II. The essentiality of this lysine for exhibiting the kinase activity of PknA was highlighted by the inability of K42N mutant to autophosphorylate (Fig. 4.11C). The faster migration of the K42N mutant compared to the wildtype protein on SDS-PAGE also indicated the lack of post-translational modification of the mutant protein (Fig. 4.11A and 4.11B). Furthermore, typical DXKPXN and GTXXYXAPE sequences of Hanks's subdomain VI and VIII respectively (Hanks et al., 1988; Taylor, 1989) argued the inclusion of PknA as a member of eukaryotic-type Ser/Thr protein kinases (Fig. 4.6).

The catalytic domain of PknA is followed immediately by an Ala/Pro-rich region (Fig. 4.7). Similar amino acid stretch containing in addition many serine and threonine residues has been described for Pkn2 from *M. xanthus*. Deletion of this region resulted in the inability of Pkn2 to phosphorylate its substrate, β-lactamase. It has been proposed that the phosphorylation of this domain may be involved in the regulation of Pkn2 function (Udo et al., 1995). In PknA, the Ala/Pro-rich sequence also has 7 serine and 6 threonine residues (Fig. 4.7). Thus, it is logical to presume that this region might be associated with similar activity. Interestingly, the deletion of Ala/Pro-rich region in PknA despite the presence of the catalytic domain resulted in the loss of autophosphorylating ability (Fig. 4.9). Further, Pro-rich regions are known to have diverse binding functions regulated by phosphorylation (Williamson, 1994). In certain eukaryotic signalling molecules, Pro-rich sequences are involved in protein-protein interaction as targets of fixation for the SH3 domains found in many signalling proteins (Koch et al., 1991; Williamson, 1994; Alexandropoulos et al., 1995).

In prokaryotes, transmembrane histidine kinases function as sensors for various external signals (Stock et al., 1990). However recently, it has been shown that *Myxobacteria* and *Streptomyces* contain several transmembrane Ser/Thr kinases that may serve as receptors for outer signals (Udo et al., 1995; Zhang et al., 1996; Hanlon et al., 1997; Nadvornik et al., 1999). The charge distribution around the hydrophobic stretch (Fig. 4.7) suggests PknA to be a transmembrane sensory kinase acting as a signalling molecule between the cell exterior and interior. This
observation is consistent with the 'N-in, C-out' topology of PknA predicted using the programme HMMTOP (Av-Gay and Everett, 2000).

_E. coli_ strain TB1 transformed with pMAL-PknA exhibited remarkable elongation upon induction with IPTG. This observation led towards monitoring the effect of constitutive expression of mycobacterial PknA on _E. coli_. Apparently, expression of PknA (Figs. 4.15 and 4.16) resulted in dramatic alteration in the phenotype of _E. coli_ cells (Fig. 4.17). The elongation of cells was about 20 to 30 fold compared to the normal rods of _E. coli_ (Fig. 4.17, compare panels ‘a’ and ‘c’). This observation was not an experimental artifact or the effect of plasmid load since _E. coli_ transformed with the vector (p19Kpro) as well as the antisense construct (p19Kpro-aPknA) did not show such phenotypic alteration (Fig. 4.17, panels ‘b’ and ‘e’). The elongation of cells did not seem to result in any toxicity from ‘out of context’ expression of the mycobacterial gene since growth curves between experimental and controls were very similar. There are, in fact, examples of mycobacterial gene expression using _E. coli_ as a host (Okino _et al._, 1999; Koul _et al._, 2000). Further, the fact that the kinase dead mutant, p19Kpro-K42N did not result in elongation of _E. coli_ cells (Fig. 4.17, panel ‘d’), convincingly established that the kinase activity of mycobacterial PknA is involved in regulating these morphological changes.

Genome analysis of _M. tuberculosis_ revealed that _pknA_ is located adjacent to _pbpA_ and _rodA_ genes encoding putative morphogenic proteins belonging to SEDS (shape, elongation, division and sporulation) family (Henriques _et al._, 1998). These SEDS family of proteins have been said to be present in all eubacteria where a constituent of cell envelope is the peptidoglycan. These proteins are known to be involved in control of cell shape and peptidoglycan synthesis in bacteria like _E. coli_ (Begg and Donachie, 1985; Signoretto _et al._, 1996) and _B. subtilis_ (Henriques _et al._, 1998; Daniel _et al._, 2000). Thus the presence of _pknA_ at this location in the genome together with the altered morphology of _E. coli_ cells observed upon its expression suggests the potential role of this kinase in the process of cell division/differentiation.

A bioinformatic approach was adopted to further envisage the functional aspect of PknA. Sequence database comparison was carried out to place PknA among other bacterial Ser/Thr kinases where functions had already been assigned. Nine
different bacterial Ser/Thr kinase sequences were retrieved through searches. A phylogenetic tree when generated following multiple sequence alignment with these kinases, PknA was found to be very close to Pkn1 and Pkn9 of *M. xanthus* (Fig. 4.18). These Ser/Thr kinases of *M. xanthus* are involved in the process of cell differentiation, mainly in sporulation (Munoz-Dorado *et al.*, 1991; Hanlon *et al.*, 1997). Thus, placement of PknA along with Pkn1 and Pkn9 of *M. xanthus* argued in favour of its role in similar functions. In contrast to myxobacteria, mycobacteria lack a complex developmental cycle. However, it has been proposed that in a dormant condition associated with prolonged latent infection, *M. tuberculosis* enters a spore-like state with no cell division or metabolic function (DeMaio *et al.*, 1996; Parrish *et al.*, 1998). Interestingly, Western blot analysis with the cell lysates from *in vitro* culture of *M. tuberculosis* using anti-PknA antibody showed significant reduction in PknA expression in stationary compared to exponential growth phases (Chapter 3, Fig. 3.11). Thus, *pknA* is downregulated when the organism enters a state of limited metabolism with little or no cell turnover.

Collectively, the evidence presented in this chapter established that the mycobacterial Ser/Thr kinase, *pknA* encodes an active enzyme capable of protein phosphorylation. However, such protein modifications governed by kinases needs to be regulated in order to maintain a state of homeostasis. Interestingly, a putative and the only Ser/Thr phosphatase, *ppp* in *M. tuberculosis* is present in a cluster with *pknA* (Cole *et al.*, 1998). In a recent study it has been postulated that *pknA* and *ppp* are genetically linked, suggesting the regulation of phosphorylation/dephosphorylation events (Treuner-Lange *et al.*, 2001). Therefore, it would be interesting to gain insight into the biochemical aspects of this phosphatase and elucidate whether it affects the phosphorylating ability of PknA.