Chapter II

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2.1 INTRODUCTION

Kinases mediate their effects on the cell by phosphorylating various cellular proteins. One of the important mechanisms underlying the regulation and functioning of protein kinase is through interaction with other cellular proteins called substrates. Thus, identifying the direct substrates of kinases is an area of rigorous investigation. It has been difficult to uncover bona-fide kinase substrates because kinases often display overlapping target specificity. In-vivo substrates of kinases have been identified using gene knock-out studies, immuno-precipitations and kinase inhibitors. Various methods have been employed by different groups to identify kinase substrates in *M. tuberculosis*. Substrates of PknA and PknB have been identified using a combination of 2D gel electrophoresis and immunoblot analysis with a phosphor-(S/T)Q antibody (Kang et al., 2005). GarA, a substrate of PknB was identified in an approach involving an in vitro kinase reaction with the kinase and mycobacterium whole cell lysate, followed by resolution of the reaction products on 2D gels, and identification of substrates by mass spectrometry (Villarino et al., 2005). Recently, substrates of PknD have been identified by carrying out transcription profiling of wild type vs PknD overexpressing cells with the help of microarrays (Greenstein et al., 2007). Novel substrates of PknA (Dasgupta et al., 2006), PknB, PknH (Molle et al., 2003) and PknF (Molle et al., 2004) have also been identified by carrying out in-vitro kinase assays with the kinase and tested the proteins encoded by neighboring genes as substrates. In this study, we have identified substrates of PknK.

SUBSTRATE IDENTIFICATION – Approach 1: Neighbouring Genes of PknK

*M. tuberculosis* faces various stress conditions inside the host and responds to them through a coordinated regulation of gene expression. *virS* gene of *M. tuberculosis* (Rv3082c), known to be involved in virulence, belongs to the ArsC family of transcriptional regulators. VirS exhibits homology with the VirF protein of *Shigella*, the VirFy protein of *Yersinia* and the Cfad, Rns and FapR proteins from various enterotoxigenic *Escherichia coli* strains. *virS* was shown to be present exclusively in the species belonging to the *M. tuberculosis* complex as revealed by Southern blot hybridization and PCR analysis (Gupta et al., 1999). VirS is similar with the regulatory proteins involved in invasion, survival and spread of infection. Analysis of
VirS protein sequence revealed the presence of two putative helix-turn-helix (HTH) motifs in its C-terminal region from amino acids (254-275) and (301-324). Sequence alignment of VirS protein with those of the other regulatory proteins shows a conserved helix-turn-helix motif. The sequence and structural similarities between VirS and its homologs from bacterial systems, where they regulate the transcription of genes required for establishment of disease suggested that VirS could carry out similar functions in *M. tuberculosis*. Sequence homology search revealed that PknK may be involved in transcription regulation. Since PknK is located in vicinity of virS and the serine/threonine protein kinases have been shown to be involved in transcription regulation, we investigated the possibility of VirS being a substrate of PknK.

The *mymA* operon, located divergently from the *virS* gene, is named after the first gene in the operon, *mym* (Mycobacterial monoxygenase), which is important for maintaining the integrity of the *M. tuberculosis* cell wall (Singh et al., 2003). Transcription of the *mymA* operon was shown to be dependent on the presence of VirS and disruption of *virS* or *mym* results in altered cell wall structure (Singh et al., 2003). When exposed to acidic pH, the transcription of *mymA* operon in the wild type *M. tuberculosis* increased by four-fold, suggesting a possible role of these proteins in the severely acidic conditions of activated macrophages (Sassetti et al., 2003). MymA operon encoded proteins are involved in the modification of fatty acids required for cell envelope of mycobacteria.

The *MymA* operon consists of seven open reading frames namely, Rv3083- Rv3089 in same orientation in the *M. tuberculosis* genome, with a maximum distance of 38 bp between any two neighboring genes (Figure 2.1).
Figure 2.1
Schematic diagram showing arrangement of seven MymA operon genes while VirS is arranged divergently from mymA operon.

The genes Rv3083- Rv3089 are transcribed from a single promoter. Computational analysis revealed that besides \textit{M. tuberculosis}, this operon is also present in \textit{M. bovis}. While homologs of genes of this operon are also present in \textit{M. leprae} and \textit{M. smegmatis}, they are however not organised in form of operon.

Bioinformatic analysis was performed for mymA operon genes and the operon was examined for the presence of any conserved motifs. The possible roles of gene products encoded by mymA operon is given in table 2.1. Inspection of sequences of proteins encoded by these genes revealed that mym gene codes for a monooxygenase showing 58% homology with dimethylaniline monooxygenase of humans. Mym has NADPH and FAD binding domains. Monooxygenases can hydroxylate long chain fatty acids. BLAST search revealed the presence of HHxxxDG motif in Rv3087 and Rv3088 which is a common feature of acyl-transferases and condensation domains of Non Ribosomal Peptide Synthetases (NRPSs). These two proteins may be involved in transferring acetylated product to Rv3089. Such a functional association is known in several instances such as phospholipid synthesis in \textit{E. coli}, mycocerosic acid synthesis in mycobacteria and wax ester synthesis in \textit{Acinetobacter calcoaceticus} ADP1. Rv3089 (\textit{FadD 13}) contains three domains which are highly conserved among members of acyl-CoA synthases. It has a consensus sequence SGXXGXPKG where glycine residues form the phosphate binding loop (p-loop), found in the ATP and GTP binding proteins.
Table 2.1: Hypothetical proteins encoded by genes of MymA operon

<table>
<thead>
<tr>
<th>ORF number</th>
<th>Gene name</th>
<th>Gene product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rv3083</td>
<td>mymA</td>
<td>Mycobacterial monooxygenase</td>
</tr>
<tr>
<td>Rv3084</td>
<td>lipR</td>
<td>Probable acetyl hydrolase/esterase</td>
</tr>
<tr>
<td>Rv3085</td>
<td>sadh</td>
<td>Short chain alcohol dehydrogenase</td>
</tr>
<tr>
<td>Rv3086</td>
<td>adhD</td>
<td>Zinc-containing alcohol dehydrogenase</td>
</tr>
<tr>
<td>Rv3087</td>
<td>chp</td>
<td>Conserved hypothetical protein</td>
</tr>
<tr>
<td>Rv3088</td>
<td>chp</td>
<td>Conserved hypothetical protein</td>
</tr>
<tr>
<td>Rv3089</td>
<td>fadD 13</td>
<td>Acyl-CoA synthase</td>
</tr>
</tbody>
</table>

The functional link between two acyl transferases (Rv3087 and Rv3088) and acyl-CoA synthase (Rv3089) is indicated by a translational fusion between these three proteins. Mycobacteria might use this operon for modification, activation and transfer of fatty acids to the appropriate acceptor(s) in its cell wall.

It is possible that mymA, a monooxygenase encoded by Rv3083, could potentially oxygenate mycobacterial fatty acids (Singh et al., 2003). The oxygenated fatty acids could be further modified by the acetyl hydrolase/esterase (Rv3084), short chain alcohol dehydrogenase (Rv3085) and zinc containing alcohol dehydrogenase (Rv3086). Finally, the acyl-CoA synthase homolog (Rv3089) could activate the fatty acids (modified by the products of genes Rv3083-Rv3086), which could subsequently be transferred to an acceptor in the cell wall of mycobacteria by acyl transferases (Rv3087 and Rv3088). Mycolic acid synthesis pathway is depicted in Figure 2.2.

Since MymA operon is located in the vicinity of pknK, and nearby genes have previously been shown to be interacting partners of other serine/threonine protein kinases of mycobacterium, we have examined the possibility of the mymA operon proteins as direct targets of PknK mediated phosphorylation.
In the present work, we have identified and characterized the DNA-binding domain of VirS. Region of DNA-binding motif (20 amino-acids) at C-terminus was deleted which abolished DNA binding activity of VirS. The results indicate the importance of C-terminus of VirS in DNA binding activity.

**SUBSTRATE IDENTIFICATION – Approach 2: FHA-Domain Containing Proteins**

Several protein harboring FHA domains have been identified to be the endogenous substrates of some of kinases (Ponting et al., 1999; Krupa and Srinivasan, 2005). FHA domain was initially found in transcription factors and has been identified as
protein-protein interaction module known to bind to pThr residues on target protein. The critical motifs identified in eukaryotic FHA domains are conserved in the bacterial FHA domains as well. In *M. tuberculosis*, of the seven FHA domains in six proteins (Rv0019c, Rv0020c, Rv1267, two in Rv1747, Rv1827 and Rv3360), five have been characterized (Rv0020c, Rv1267, two in Rv1747 and Rv1827). The FHA domain present in EmbR is a phosphoprotein recognition domain.

EmbR belongs to SARP family (*Streptocolor* antibiotic regulatory gene), known to regulate genes involved in biosynthesis of secondary metabolites in *Streptocolor* through binding to specific DNA sequences. SARP is a family of transcription factors that bind through HTH motifs. Central HTH motif presents site of interaction with RNA polymerase. Phosphorylation of the mycobacterial transcriptional activator, *embR*, is essential for transcriptional regulation of the *embCAB* operon that encodes arabinosyltransferases. EmbR, as a transcriptional regulator, interacts with RNA polymerase and possesses a phosphorylation-dependent ATPase activity that might play a role in forming an open complex between EmbR and RNA polymerase. Recently, FHA domain was found to play a crucial role in interaction and phosphorylation of EmbR by PknH, a mycobacterial Ser/Thr protein kinase (STPK) (Molle et al., 2003). A mutation in EmbR FHA domain was shown to be associated with ethambutol (EMB) resistance (Ramaswamy et al., 2000), suggesting that alteration in this “kinase interacting domain” of EmbR can result in an ethambutol resistance phenotype. This observation provided an indirect evidence for existence of some functional relationship between PknH, EmbR, *embCAB* operon and ethambutol resistance. EmbR-mediated transcriptional control of EMB arabinosyltransferases was shown to be phosphorylation dependent (Sharma et al., 2006).

Cross-genomic comparisons of bacterial protein kinases were done to identify homologs of kinases with known substrates. Putative homologs of AfsK, a well characterized Ser/Thr kinase of *S. coelicolor* have been identified in *Deinococcus radiodurans* and *Streptomyces avermitilis*. In *S. coelicolor*, AfsK phosphorylates a global regulator(AfsR) of secondary metabolism that controls the production of Actinorhodin. AfsK has significant homology with PknK at C-terminus. In this chapter, we investigated the possibility of EmbR being a PknK substrate and probability of EmbR being phosphorylated at different sites by multiple kinases.
SUBSTRATE IDENTIFICATION – Approach 3
Adenylyl Cyclases

Nucleotide cyclases, the enzymes that convert nucleotide triphosphates (NTP’s) into the respective 3’-5’-cyclic nucleoside monophosphates, are a large group of proteins in which the function of synthesizing these secondary messengers has evolved through convergent evolution. In recent years, the adenyl cyclases present in cyanobacteria and mycobacteria, have been well studied (Shenoy et al., 2006). Rv0386 was the first biochemically characterized representative of the family of putative DNA-binding domain-containing cyclases in mycobacteria. Interestingly, Rv0386 shows both adenylyl and a guanylyl cyclase activity. Domain organization of this protein appears to be very similar to one of serine/threonine kinase of Mycobacterium tuberculosis, PknK (Figure 2.3). Previously adenylyl cyclases have been reported to be activated by kinases (Comer et al., 2006). It was therefore interesting to investigate the possibility of Rv0386 being phosphorylated by PknK.

Figure 2.3
Domain organization of one of mycobacterial adenylyl cyclase is very similar to serine/threonine protein kinase PknK.

SUBSTRATE IDENTIFICATION – Approach 4
Elongation Factor-Tu

Purification of proteins in native conditions often results in co-purification of biologically important interacting partners. Purification of PknK-KD (290aminoacids) from E. coli under native conditions persistently resulted in co-purification of E. coli proteins corresponding to ~50kDa. Therefore, it was tempting to speculate that the contaminant was actually specific interacting protein. The unknown protein corresponding to ~50kDa was then identified by MALDI-MS as EF-Tu. Post-translational phosphorylation of EF-Tu has been shown to prevent its binding to amino-acylated transfer RNA as well as to kirromycin, an antibiotic known to inhibit EF-Tu function (Cristel. et al., 2005). Subsequently, M.
tuberculosis homolog of elongation factor (Rv0685) was cloned, purified and used in-vitro kinase assay to investigate the possibility of Elongation factor-Tu, being a PknK substrate.

2.2 RESULTS AND DISCUSSION

2.2.1 Expression and Purification of Substrate Proteins

The genes encoding, virS (Rv3082), mym (Rv3083), lipR (Rv3084), fadD13 (Rv3089) Rv3085, Rv3086, Rv3087 and Rv3088 (figure 2.4), as well as genes Rv3079 (ODR), Rv3081 (PBGR), eF-Tu and adenylate cyclase (figure 2.5) were cloned by amplification from M. tuberculosis H37Rv genomic DNA, using the corresponding forward and reverse primers (materials and methods, table 2) and Phusion DNA polymerase, followed by cloning of the amplicons into pENTR/Directional-TOPO vector. The genes encoding VirS, Mym, LipR, Rv3085, Rv3086, Rv3088 and Rv3089 (FadD13) were cloned into the vector with GST tag which had caspase cleavage sites designated as PC6 while eF-Tu, adenylate cyclase, were cloned into pQE11 vector. The gene encoding Rv3087 was cloned into pMAL-c2X.

The genes encoding Mycobacterial EF-Tu, Adenylate cyclase, were subcloned into pQE11 vector with His tag. Positive clones were identified by restriction digestion (Figure 2.5).
Expression constructs were transformed into competent cells of BL-21 DE3 codon plus strain. Proteins were expressed and purified by affinity chromatography. Purified VirS and seven of MymA operon proteins purified as putative substrates are shown in Figure 2.6.

Figure 2.5
Screening of clones of genes by restriction digestion of Elongation factor-Tu (Rv0685), Rv3079 (Oxidoreductase), Rv3081 (PBGR) and adenylate cyclase (Rv0386).

Figure 2.6: Purification of proteins encoded by mymA operon.
Lane 1: GST-VirS, Lane 2: GST-Mym, Lane 3: GST-LipR, Lane 4: GST-Rv3085, Lane 5: GST-Rv3086, Lane 6: MBP Rv3087, Lane 7: GST-Rv3088, Lane 8: GST FadD13 and maltose binding protein tagged -Rv3087 were expressed in E. coli and purified. The eluted protein were run on 10% SDS PAGE and visualized by CBB staining.
Since proteins were purified with recombinant GST tag and GST tag itself is known to be phosphorylated by kinases, we carried out cleavage of GST tag to release the native protein prior to phosphorylation reaction. The vector PC6 carries a sequence coding for caspase cleavage site between sequence coding GST and cloned protein. The caspase 6 cleavage reaction on various substrate GST-tagged proteins was carried out in the kinase buffer for 1 h (at 4°C) prior to performing \textit{in vitro} kinase reactions. Figure 2.7 shows five proteins encoded by genes of MymA operon after removal of tag by caspase cleavage.

![Silver Stained gel](image)

**Figure 2.7**
SDS PAGE gel showing proteins of mymA operon after caspase cleavage. Cleaved GST-tag is indicated by arrows. GST tag from the GST-VirS, GST-Mym, GST-LipR GST-Rv3085 and GST-Rv3088 proteins were cleaved by incubating these proteins with purified caspase 6 for 1 hour at 4°C in kinase reaction buffer. samples were resolved on 10% SDS-PAGE.

**Purification of Oxidoreductase (ODR Rv3079) and Phosphate Binding Glycine Residue (PBGR, Rv3081)**

The genomic organization of Rv3079 shows close proximity with PknK. Sequence analysis done using ClustalW alignment revealed homology of Rv3079c with Rv0953c which is a known oxidoreductase. It showed 41% identity in 288 amino acids overlap (Figure 2.8).

Sequence analysis of PBGR shows glycine radical signature and possible helix-turn-helix motif between 53-74 amino-acids. A number of proteins containing helix-turn-helix motifs are known to be phosphorylated by eukaryotic kinases. Both of these
proteins were purified as GST fusion protein (Figure 2.9) and assayed for phosphorylation by PknK.

**Figure 2.8:** Amino acid sequence alignment (ClustalW) of the Rv3079c and *M. tuberculosis* Oxidoreductase Rv0953c. Alignment was generated using amino acid sequences of Rv3079c and its homolog Rv0953c. Sequence alignment shows 41% identity in 288aa overlap. The conserved residues are marked by asterix.

**Figure 2.9:** GST-ODR(Rv3079c) and GST-PBGR(Rv3081) were expressed in *E. coli* and purified. The eluted proteins were run on 10% SDS PAGE and visualized by CBB staining.

**Purification of Elongation Factor (EF-Tu) and Adenylate Cyclase (Rv0386)**

**Elongation Factor (EF-Tu)**

Purification of proteins in native conditions often results in co-purification of biologically relevant interacting partners. EF-Tu was identified by MALDI-MS as
a co-purified protein with PknK-KD (290aa). Subsequently, its mycobacterial homolog was cloned into pQE11 vector, expressed and purified as His tag EF-Tu ~50kDa. (Figure 2.10 A).

To investigate the possibility of adenylate cyclase being a PknK substrate, Adenylate cyclase from *Mycobacterium tuberculosis* was cloned under His-tag expressed and purified to homogeneity as 130kDa protein (Figure 2.10B).

### 2.2.2 Identification of Substrates

**MymA Operon Proteins**

Purified recombinant proteins encoded by *mymA* operon in presence of \([\gamma^{32}P]\) ATP in kinase buffer were incubated with PknK. The proteins carried GST tag, therefore GST alone was used as control. GST alone displayed very low levels of phosphorylation by PknK (Figure 2.11). However phosphorylation of VirS (lane 4), Mym (lane 6), LipR (lane 8), Rv3085 (lane 10) and Rv3088 (lane 14) was 3-5 fold above background levels compared to GST (lane 2), Rv3086 (lane 12) and FadD13 (lane 16).
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Figure 2.11: Phosphorylation of mymA operon proteins by PknK.

Similar reactions were carried out with maltose binding protein-tagged Rv3087 and revealed that Rv3087 is not phosphorylated by PknK. Results of the kinase assays with these substrates clearly demonstrated that proteins VirS, Mym, LipR, Rv3085 and Rv3088 are indeed substrates of PknK.

In another kinase assay, PknK phosphorylated the caspase 6 cleaved untagged VirS and mymA operon proteins. Bands corresponding to the proteins without GST tag and those phosphorylated by PknK are shown in figure 2.12.
Figure 2.12: *In-vitro* kinase assay of PknK with proteins without tag.

GST tag from the GST-VirS, GST-Mym, GST-LipR and GST-Rv3085 and GST Rv 3088 proteins were cleaved by incubating these proteins with purified caspase 6 for 1 h at 4°C in kinase reaction buffer. Subsequent to the cleavage, *in vitro* kinase reactions were performed with or without PknK for 15 min at 30°C. Samples were resolved on 10% SDS-PAGE, silver-stained, dried and exposed for autoradiography. Bands corresponding to autophosphorylated PknK and phosphorylated substrates are indicated with arrows in the autoradiogram. Lane 1: GST-VirS, Lane 2: PknK + GST-VirS, Lane 3: GST-Mym, Lane 4: GST-Mym + PknK, Lane 5: GST-LipR, Lane 6: GST-LipR+PknK, Lane 7: GST-Rv3085, Lane 8 GST-Rv3085+PknK, Lane 9: GST-Rv3088, Lane 10: GST-Rv3088 + PknK.

- ODR, PBGR, EF-Tu, and adenylate cyclase

*In vitro* kinase assays with ODR, PBGR, EF-Tu and adenylate cyclase ruled out the possibility of these proteins being substrate of PknK.

EmbR

Considering the homology of AfsR with EmbR as well as the significant sequence similarity observed between AfsK and PknK, it seemed of interest to study EmbR as a possible substrate of PknK.
EmbR had earlier been shown to be phosphorylated by the cognate mycobacterial serine/threonine (Ser/Thr) kinase, PknH. EmbR was cloned as His tag fusion protein. In vitro kinase assay was performed to check if EmbR was substrate of PknK.

EmbR proteins were incubated with PknK, PknB and PknA in the presence of [γ-32P] ATP in *in vitro* kinase assay and found to be phosphorylated by all the three kinases. Positions of PknK, PknA and PknB in autoradiogram is shown by arrows. Thus EmbR proved to be the substrate of PknK (figure 2.13).

### 2.2.3 Identification of Phosphorylated Residues

Phospho-peptide map of EmbR phosphorylated by PknB, PknH, PknA and PknK suggested differential phosphorylation. While PknA, PknB and PknH phosphorylated EmbR at single site, PknK had at least phosphorylated EmbR on at least three different residues (Figure 2.14).

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*Figure 2.13: Phosphorylation of EmbR by PknK, PknB and PknA.*

Peptide maps of EmbR phosphorylated by PknB, PknH and PknK.

Figure 2.14
Comparison of phosphopeptide map EmbR phosphorylated by different kinases.

Phospho-peptide analysis of Mym phosphorylated by PknK suggests a single major site of phosphorylation (Figure 2.15).

Figure 2.15
Phosphopeptide map of protein encoded by mym phosphorylated by PknK. Phosphorylated residue is indicated by arrow.

Phospho- amino acid analysis of Mym phosphorylated by PknK suggests threonine as major site of phosphorylation with minor phosphorylation on serine residues (Figure 2.16).
Phospho-amino acid analysis and phosphopeptide analysis of EmbR when phosphorylated by PknK, PknA, and PknB was also done. Threonine residue was identified as the primary amino acid phosphorylated on EmbR by PknK and PknB (Figure 2.17).
2.2.4 Role of Substrate Phosphorylation

The primary sequence analysis of VirS shows the presence of nine putative protein kinaseC phosphorylation motifs [ST]-x-[RK](Singh et al., tuberculosis 2003). The expression of VirS was up-regulated under acidic conditions. The transcription of mymA operon is controlled by VirS. The acidic pH may lead to post-translational modification of VirS such as phosphorylation that could induce conformation of VirS which is suitable for regulation of the promoter region of mymA operon.

The interaction of proteins with DNA is central to transcription regulation. The technique that is used to study protein and DNA interactions is the electrophoretic mobility shift assay (EMSA). EMSA is based on the observation that protein-DNA complexes migrate more slowly than free DNA molecules when subjected to non-denaturing polyacrylamide electrophoresis. Because the rate of DNA migration is
shifted or retarded upon protein binding, the assay is also referred to as a gel shift or gel retardation assay.

An inverted repeat is a sequence of nucleotides that is the reversed complement of another sequence further downstream (figure 2.18). For example, 5’GACTGC....GCAGTC—3’. Inverted repeats also indicate the regions capable of self-complementary base pairing (regions within a single sequence which can base pair with each other). MymA promoter region (~156bp) has indirect repeats of 24 base pair. We synthesized a probe that has 63bp on either side of the 24bp inverted repeats. The probe thus synthesized was used in mobility shift assay using VirS protein.

The region of VirS from (254-275) and (301-324) aminoacids has helix-turn-helix motif known to be involved in DNA-binding. Therefore, *M. tuberculosis* VirS was subjected to Electrophoretic mobility shift assay (EMSA) to elucidate the role of PknK in transcription regulation and overall signaling pathway.

For protein–DNA binding assay, γ-32P labeled probe DNA was prepared by end labeling using Polynucleotide kinase. Labeled PCR products were incubated with various amounts of VirS at 37°C for 30 min. After incubation, complexes and free DNA were resolved on 5% non-denaturing polyacrylamide gels. Gels were then subjected to autoradiography. Altered electrophoretic mobility, indicative of VirS binding to DNA was detected with a minimum of 20ng of VirS used (Figure 2.19). The binding was found to be specific, as VirS deletion mutant (C-terminal deletion)
lacking the DNA binding domain, was unable to bind to upstream region of the \textit{virS} gene itself up to the concentration of 200 ng.

**Figure 2.19:** Detection of \textit{virS} DNA complex by EMSA. 

[\gamma^{32}P] labeled promoter region of \textit{Myx} gene promoter region (156bp) was incubated with increasing amounts of VirS Protein. Gel shift detected in form of DNA-VirS protein complex is indicated by arrows. 

Lane 1: Free probe, Lane 2: Free probe incubated with VirS deletion mutant, Lane 3, 4, 5 have probe incubated with 50 ng, 100 ng and 200 ng of VirS respectively.

In view of the fact that VirS is phosphorylated by PknK, the effect of such phosphorylation on DNA-binding activity of VirS was examined. The phosphorylated VirS was incubated with labelled promoter region.
A secondary shift was observed with phosphorylated VirS which was not prominent with unphosphorylated VirS (Figure 2.20). This increased secondary shift thus observed could be due to phosphorylation induced multimerization of VirS. PknK phosphorylated VirS showed enhanced DNA binding activity after multimerization. EMSA analysis performed with 156bp probe and increasing amounts of phosphorylated VirS to the binding reaction affected VirS-DNA binding. It is possible that phosphorylation causes conformational change in VirS, resulting in an altered DNA-binding ability and increased multimerization properties. Phosphorylation of VirS increases the strength of secondary binding. Consequently, this may enable initiation of transcription of the mynA operon genes.

These results indicate that MymA operon is regulated by PknK mediated phosphorylation of VirS.
2.3 CONCLUSION

The present study reveals VirS protein to be a substrate of PknK. PknK-mediated phosphorylation may play a role in activating this transcription factor, and thus in regulating the activation of the *mymA* operon. In addition, several of the *mymA* operon proteins – Mym, LipR, Rv3085 and Rv3088 - are targets of direct phosphorylation by PknK (Figure 2.21), suggesting the possible modulation of the activities of these target proteins by phosphorylation.

*MymA* operon which is involved in synthesis of fatty acids and various polyketides as secondary metabolite, being regulated by a kinase, will open new roads in unraveling pathway for secondary metabolite production. Present study marks the beginning by identification of novel substrates of PknK and opens up new area for identifying PknK mediated signaling pathway in mycobacterium.

![Proposed model of regulation of *mymA* operon by PknK](image)

**Figure 2.21: Hypothesis Figure**
Proposed model of regulation of MymA operon by PknK
A schematic figure showing regulation by phosphorylation of *mymA* operon by PknK/VirS system in *M. tuberculosis*. PknK phosphorylates VirS (block arrow) and four of MymA operon proteins as indicated by arrows.