SUMMARY & CONCLUDING REMARKS

Mycobacterium tuberculosis is the causative agent of tuberculosis, a major health problem worldwide that is responsible for the deaths of over two million people every year. A better understanding of the physiopathology of the tubercle bacillus aiming towards identification of new targets is imperative for improving the treatment of the disease. Emerging body of evidences suggest that signalling elements, particularly those involved in Ser/Thr protein phosphorylation, may represent a new class of promising therapeutic intervention molecules. Recent studies have unveiled the importance of Ser/Thr protein kinases (STPKs) and phosphatases in mycobacterial physiology and virulence. The present study, therefore, targeted towards investigating the role of one such kinase PknA in mycobacterial signalling through identification of natural substrates of the kinase and the mechanism of autophosphorylation.

In an earlier study from the lab, PknA was cloned and characterized from *M. tuberculosis*. The location of the transmembrane Ser/Thr protein kinase PknA within the genome in a cluster with the morphogenic proteins (Pbp2A and RodA) suggested its involvement in regulating cell morphology. The regulatory role of PknA in cell division has been experimentally demonstrated by expression in *E. coli* devoid of such kinases. Interestingly, the presence of PknA in *E. coli* remarkably elongated the cells and thus validated the proposed function of the kinase. Later, the studies from other investigators with over expression of the protein in mycobacteria suggested the involvement of the kinase in cell division. However, the precise mechanism underlying the regulatory role of the kinase is poorly understood. This opened a new horizon to investigate the mechanism of regulation of PknA including its autophosphorylation and transphosphorylation, and ultimately regulation through the phosphatase.

Bioinformatic analysis revealed that PknA has an N-terminal catalytic domain which is linked to the transmembrane region through a variable length o juxtamembrane linker. The C-terminal domain outside the cell presumably bind signalling ligands and is attached to the transmembrane sequence. It was therefor

intriguing to identify the contribution of these domains in imparting functionality to the kinases. The first approach was to fish out the minimum domain of PknA that could be capable of auto as well as substrate phosphorylation. For this, several deletion mutants of PknA were constructed based upon the prediction from the primary sequence and secondary as well as tertiary structures. Surprizingly, unlike other Ser/Thr kinases, the catalytic domain of PknA itself was not sufficient for exhibiting phosphorylating ability. However, juxtamembrane region together with the kinase domain was found to be necessary for the enzymatic activity of the protein. This construct comprising of catalytic and juxtamembrane regions was capable for auto as well as *trans* phosphorylation in a manner similar to full length protein, and was referred as core. This finding highlighted a unique feature of the kinase distinct from few other mycobacterial Ser/Thr kinases where catalytic domain alone can exhibit autophosphorylation or substrate phosphorylation abilities.

The core was utilized to demonstrate whether autophosphorylation occurs in a *cis*- or *trans*- acting manner. *In vitro* phosphorylation assays utilizing the kinase active and inactive versions of full length and PknA core respectively, demonstrated that the autophosphorylation occurs in *trans*. Further, *in vivo* phosphorylation of the full length kinase inactive mutant by the co-expressed kinase active core, emphasized the prevalence of intermolecular autophosphorylation of the PknA. Thus, the results ostensibly established that like the majority of histidine kinases in bacteria and most of the eukaryotic receptors, the mycobacterial Ser/Thr kinase PknA exhibits bimolecular autophosphorylation. Further, by mutation of the conserved threonines to alanine in the predicted activation loop of the protein established that the kinase activates through a universal activation mechanism like the majority of eukaryotic kinases. These studies laid insights into the mechanism of autophosphorylation of the kinase and the importance of catalytic and juxtamembrane regions. It was now interesting to elucidate the role of other domains in the functionality of the protein.

Earlier studies from the lab have established that the constitutive expression of PknA in *E. coli* elongates the cells. However, on transforming the core in *E. coli*, no elongation could be seen. This was a surprising observation since the core mimicked the wild type protein in phosphorylation ability and further gave a clue for the importance of the other domains towards the functionality of the protein. To analyze

this, the core was co-transformed with the transmembrane and the extracellular domain individually as well as in concert. Interestingly, co-transformation with either the transmembrane or the extra cellular domain alone did not lead to any change in phenotype of the cells. However, on transforming the construct harboring both the transmembrane and extra cellular region with the core, the elongation phenotype as manifested by the full length protein was restored. These findings, therefore, argued that the transmembrane and extra-cellular domains of PknA, although dispensable for phosphorylation activities, are crucial in responding to signals and absence of a single domain compromises the functionality of the protein. Thus, the results presented here for the first time established the significance of different domains in a bacterial eukaryotic-type Ser/Thr kinase for reconstitution of its functionality.

After deducing the mechanism of auto regulation of the kinase, it was intriguing to know the involvement of the kinase in regulating the other cellular proteins. In earlier reports, the involvement of PknA, in the process of cell division has been reported. However, the precise regulation of activities by this kinase has not yet been elucidated. Therefore, in order to understand the mechanism of regulation of cell division by PknA, the elongation phenotype generated by the expression of PknA in *E. coli* was utilized. The presence of multiple nucleoids in the filamentous *E. coli* cells expressing PknA with no signs of cellular constriction, as visualized by DAPI staining, argued that the defect was not in the DNA replication or segregation; indeed the morphology seemed to be a consequence of aberrant septum formation. The key molecule involved in the formation of septal Z-ring in bacteria is FtsZ. Therefore, to have an insight into the regulation of septum formation by PknA, its crosstalk with FtsZ was assessed. *ftsZ* was amplified from the genome of *E. coli* K12 and expressed as GST fusion protein (GST-eFtsZ). Interestingly, co-expression of GST-eFtsZ with PknA in *E. coli* led to its phosphorylation as identified by immunoblotting with anti-phosphothreonine (anti-pThr) antibody. The phosphorylation of eFtsZ by mycobacterial PknA, together with the presence of homologous protein in mycobacteria further opened way to explore the association between mycobacterial FtsZ (mFtsZ) and PknA. In this consequence, *ftsZ* was amplified from the genome of *M. tuberculosis* H37Ra. Sequencing revealed 100 % identity at the nucleotide level

between the non-pathogenic (H37Ra) and the pathogenic (H37Rv) strains. mFtsZ was purified as GST fusion protein (GST-mFtsZ) and used for further experiments. *In vitro* kinase assays established that PknA was capable of transphosphorylating GST-mFtsZ protein. The phosphorylation of mFtsZ by PknA was further monitored *in vivo* in *E. coli* using co-expression strategy. Interestingly, in concordance with earlier observations, the results further revealed that mFtsZ was phosphorylated on co-expression with PknA in *E. coli* cells. These results were provocative to monitor the effect of phosphorylation on the functionality of the protein. To test this, the GST-mFtsZ protein overexpressed from *E. coli* in the presence of PknA was purified by affinity chromatography. The detection of protein in western blotting with anti-pThr antibody confirmed that the protein purified was in a phosphorylated state unlike the one purified in the absence of PknA. Both unphosphorylated as well the phosphorylated proteins were tested for the GTPase activity. Interestingly, the phosphorylated protein purified as GST fusion protein showed a markedly reduced GTPase activity as compared to the unphosphorylated protein. Further, as it is well known that FtsZ possesses GTP dependent polymerization ability, therefore, it was intriguing to know if the polymerization ability of the protein has any effect as a result of phsphorylation. To assess the polymerization ability *in vitro,* light scattering and high speed sedimentation was carried out. The results revealed that phosphorylation of the FtsZ led to a decrease in the polymerization tendency, since no difference in partitioning of the protein or the light scattering could be seen in presence or absence of GTP. Thus, these results for the first time established the association of PknA, in regulating functionality of FtsZ, the protein involved in the process of cytokinesis throughout the bacterial lineage.

Although alteration in cell morphology and septum formation in bacteria is known to be mutually exclusive processes, there exists a co-ordination between them. After establishing the role of PknA in septum formation, it was tempting to see its involvement, if any, in the process of cell wall synthesis. Although unraveling the complex signalling network of PknA is an ardous task, as an initial step in this direction bacterial two hybrid screening system was employed to identify the

interacting proteins and substrates of PknA. For this, *pknA* was cloned as bait and used for whole genome two hybrid screening. The earlier studies revealed that expression of PknA resulted in elongation of *E. coli* cells. This alteration in phenotype suggested that PknA is likely to affect some cell division or cell wall biosynthesis machinery. Therefore, to screen for the protein(s) involved in the process, genomic DNA library of *E. coli* (constructed by partial digestion of *E. coli* strain K12 genomic DNA with *Sau*3A1) was taken as target in the two-hybrid screening. Recombinant bait and target plasmids were co-transformed in the reporter strain and clones were selected on His drop out media and further confirmed on streptomycin. Among the putative open reading frames (ORFs) identified through BLAST search, a gene encoding for the cytoplamic enzyme, UDP-N-acetylmuramyl-L-alanine:D-glutamate ligase (MurD), has been reported to be involved in peptidoglycan biosynthesis by catalyzing the addition of D-glutamate to the nucleotide precursor UDP-N-acetylmuramoyl-L-alanine. Since MurD does not show similarity to any of the host (human) proteins, it has the potential to be considered as a drug target. As a consequence, the MurD from M. *tuberculosis* was explored for its ability to act as substrate of PknA.

On analyzing the genome of *M. tuberculosis*, *murD* gene (Rv2155c) was found to be located in a cluster near the other cell division genes. *murD* was therefore, PCR amplified from the genome of *M. tuberculosis* strain H37Ra. Interestingly, *murD* gene was identical between pathogenic and non-pathogenic strains of *M. tuberculosis*. The amplified fragment (m-*murD*) through an intermediate subcloning in pUC19 (pUC-mMurD) was finally cloned in pGEX-KG (GST-mMurD) for expression in *E. coli* strain BL21 (DE3) as a GST fusion protein. The overexpressed protein from the supernatant fraction was purified on glutathione sepharose column. To ensure interaction between PknA and MurD from *M. tuberculosis* 'pull-down' assay was performed and the results revealed that PknA could interact with mMurD. To know the affinity of interaction between the two proteins, solid phase binding assay was carried out. The enzyme activity increased with increasing concentrations of biotinylated PknA with the half maximal binding of 0.62 ±.07 μg/ml and a dissociation constant of 1.12 ± 0.35 μM. Compared to

different kinase-substrate reactions in eukaryotes, the affinity of PknA-mMurD interaction seems to be within physiologically relevant range. The specificity of the interaction was determined by displacing the biotin labelled PknA with the non-biotinylated proteins. Increasing concentration of non-biotinylated mMurD as well as PknA displaced the binding of biotinylated protein. All these results, therefore, pointed towards interaction between PknA and mMurD.

The next aim was to evaluate the phosphorylation status of mMurD expressed in mycobacteria. Comparison of the genome sequences revealed a high order of homology between MurD from *M. bovis*, *M. leprae*, *M. smegmatis* and *M. tuberculosis*. Therefore, to examine intracellular phosphorylation status of mMurD within mycobacteria, it was overexpressed in *M. smegmatis* using histidine tagged *Mycobacterium-E. coli* shuttle vector pVV. The overexpression of the protein was seen on induction with H_2O_2 and the purification was performed using Ni-NTA resin. The phosphorylation status of the purified protein was monitored. Interestingly, Western blotting of purified mMurD using anti-pThr antibody revealed it to be a phosphorylated protein, while its identity was confirmed by immunoblotting with anti-His antibody. This observation validated the *in vitro* phosphorylation of the protein *in vivo*. Although the possibility of MurD phosphorylation by other Ser/Thr kinases present in mycobacteria cannot be ruled out, the propensity of its phosphorylation by PknA is quite high as the *in vitro* observations strongly support the PknA mediated transphosphorylation of MurD as well as the association between the two. In fact, mMurD can be apprehended as a substrate of PknA and hence the kinase might be involved in regulation of switch between peptidoglycan biosynthetic pathway as well as cell division in mycobacteria.

After unraveling the mechanism of auto regulation and regulation of other proteins by PknA, it was enticing to explore another key mode of regulation of kinase which is mediated through protein phosphatases. Reversible protein phosphorylation is a major regulatory mechanism of fundamental biological processes, not only in eukaryotes but also in bacteria. Opposing activities of protein phosphatases have been shown to regulate the cognate kinases as well as the substrates of these kinases. The *M. tuberculosis* genome also includes three genes (*pstP, ptpA, ptpB*) encoding eukaryotic-like protein phosphatases in addition to protein kinases. While *ptpA* and

ptpB encode for tyrosine phosphatases, only one gene *pstP* encodes for Ser/Thr protein phosphatase. Therefore, the presence of single Ser/Thr protein phosphatase in the organism expressing eleven Ser/Thr kinases makes it an important molecule to study. The location of the phosphatase in the operon with the kinase PknA, further supported its role in the regulation of the kinase. Earlier reports from the lab as well as other groups have established the role of MstP in dephposhorylating PknA as well as the artificial substrates phosphorylated by the kinase. Further studies were carried out to assess the role of phosphatase on PknA mediated phosphorylation of the natural substrates and identify the regulatory role *in vivo*. As expected, MstP could dephosphorylate the core. Furthermore, the dephosphorylation of both the natural substrates FtsZ as well as MurD could be seen on incubation with MstP. The dephosphorylation was specific since the phosphatase mutant G117D did not dephosphorylate the kinase or its natural substrates identified.

The *in vitro* dephosphorylation studies strongly suggested the regulatory role of MstP towards PknA. However, the opposing action of the kinase and the phosphatase remained to be validated *in vivo*. As mentioned earlier, expression of PknA in a *Mycobacterium-E. coli* shuttle vector p19Kpro led to remarkable elongation of *E. coli* cells. Therefore, to assess the role of phosphatase in such a situation, pMAL-MstP was co-transformed with p19Kpro-PknA in *E. coli* cells and the morphology of the cells was examined as a result of co-expression of the kinase and the phosphatase. Interestingly, co-expression of MstP reversed the elongation phenotype manifested by the kinase. The specificity of the association was further confirmed by co-transfornation of the phosphatase dead mutant with the kinase, which showed majority of elongated cells indicating that the mutant could not revert the elongation phenotype of the kinase. Besides, the co-transformation of kinase dead mutant together with the phosphatase also showed normal morphology. Thus, all these lines of evidences argued that PknA and MstP form a functional pair *in vivo*.