SUMMARY AND CONCLUDING REMARKS

Survival of any organism depends upon its adaptability with the surrounding environment. One of the several strategies utilized by the organisms for better adaptation is recruitment of post-translational modifications (PTM) of macromolecules to coordinate diverse cellular processes. Unicellular organism such as bacteria, which could be pathogenic or non-pathogenic or free living, face diverse conditions and regulate several cellular activities such as cell division and growth, development, metabolism, virulence and stress etc. through modification of macromolecules post-translationally. Phosphorylation of protein is an established PTM event in bacteria and recent results of bacterial acetylome mapping highlighted the possibility of acetylation being another global regulator. In this context, the work embodied in this thesis is concentrated on *Mycobacterium tuberculosis*, a pathogen responsible for the dreadful disease, tuberculosis. Since *M. tuberculosis* leads complex lifestyle, role of PTM events in helping it to adapt to diverse and harsh environment within the host has been continuously under the scanner. Phosphoproteome and acetylome analyses established global influence of these PTMs over *M. tuberculosis* physiology. Acetylomes of *M. tuberculosis* H37Rv and *M. tuberculosis* H37Ra were mapped recently. Interestingly, *M. smegmatis*, which is often used as genetic model in carrying out experiments with *M. tuberculosis* was therefore, considered in this study to envisage commonality of the protein(s) associated with the process. Comparison of different bacterial acetylomes showed that acetylation of proteins in central carbon metabolism (CCM) is a conserved phenomenon. Acetylation of CCM enzymes indicated the possibility of the regulation of carbon flux by modulation of different enzymatic activities. Since, phosphorylation is also reported to regulate enzymes of CCM pathway, we sought to explore how overlapping PTMs could affect each other.

Carbon flux from TCA towards glyoxylate cycle in bacteria, particularly in intracellular pathogen like *M. tuberculosis* is an important step to save loss of carbon atom to promote mycobacterial survival in macrophage. In *E. coli* regulation of carbon flux through glyoxylate cycle is controlled by phosphorylation/dephosphorylation of Isocitrate dehydrogenase (ICD) by isocitrate dehydrogenase kinase-phosphatase (ICDH). Interestingly, homolog of ICDH is absent in *M. tuberculosis* genome. This observation prompted to explore how ICD2 is regulated in *M. tuberculosis*. In *E. coli* ICDH

phosphorylates ICD at serine residue. Although *M. tuberculosis* does not have this enzyme, it possesses eleven eukaryotic type Ser/Thr kinases. Therefore it was thought logical to speculate that these kinases could modulate the function of the ICD. Identification of ICD2 as a phosphorylated protein within *M. tuberculosis* phosphoproteome further supported this postulation. To have an insight on this aspect *in vitro* phosphorylation abilities of ICD1 or ICD2 or ICL were assessed using PknA, an essential mycobacterial kinase and also as a representative of mycobacterial eukaryotic-type Ser/Thr kinases. Interestingly it was observed that all these proteins were trans-phosphorylated by PknA. To confirm *in vitro* phosphorylation of these proteins in a cellular system, respective genes were cloned in pET-28c (one at a time) and co-transformed in *E. coli* strain BL21(DE3) along with PknA under regulation of a constitutive promoter. The expressed proteins were purified through Ni-NTA column and processed for western blotting. The purified ICD2 and ICL were recognised by anti-pThr antibody. However, similar experiment when performed with Ni-NTA purified protein from lysate co-expressing ICD2/ICL with a kinase dead mutant PknA-K42N or only His-tagged proteins (ICD2/ICL), they were not recognised by the anti-pThr antibody. In contrary to the *in vitro* findings of transphosphorylation ability of ICD1, the purified protein following co-expression with PknA was hardly recognised by the anti-pThr antibody. Since ICD2 and ICL were phosphorylated, their abilities to be acetylated were also assessed. Acetyl-phosphate mediated acetylation and NAD^+ dependent deacetylation were monitored with these proteins. Both phosphorylated and unphosphorylated forms of ICD2 and ICL were found to be acetylated and deacetylated by acetyl-phosphate and NAD^+ dependent deacetylase respectively. Interestingly, it seems deacetylation of the phosphorylated ICL was preferred over its unphosphorylated counterpart. Among ICD2 and ICL, the latter was deacetylated faster than ICD2. Activity of ICD2 and ICL reflected the carbon flux through TCA or Glyoxylate cycle. Since both these enzymes are prone to phosphorylation, their activities in phosphorylated and unphosphorylated forms were measured at physiological pH 7.5. No change in activity was observed upon phosphorylation of both ICL and ICD2. *M.tuberculosis* survives at acidic pH in macrophages and therefore activity of both ICD2 or p-ICD2 and ICL or p-ICL were measured at pH 5.6. ICD2/p-ICD2 exhibited decreased activity at pH 5.6 compared to that observed at pH 7.5. Interestingly, phosphorylated enzyme activity was significantly high compared to that of the unphosphorylated counterpart when compared between p-

ICD2 and ICD2 at pH 5.6. ICL/p-ICL showed enhanced activity, however, the activity of phosphorylated and unphosphorylated forms of this enzyme was same. Thus it seems apparent from these results the preference in flow of carbon through glyoxylate cycle and its possible regulation by phosphorylation as well as deacetylation to adapt with changes in the surroundings (eg. alteration of pH).

In eukaryotes sirtuins or NAD^+ dependent deacetylases are reported to be regulated by phosphorylation, and its homolog in *M. tuberculosis* seems to play a major role in controlling levels of protein acetylation. To examine this aspect, the only NAD+ dependent deacetylase from *M. tuberculosis* (mDAC) was cloned, expressed and the His-tagged recombinant protein was purified. mDAC was indeed phosphorylated by eukaryotic-type Ser/Thr kinases of *M. tuberculosis* such as PknA and PknB. Among them it was observed that mDAC was preferentially phosphorylated by PknA and this event was reversed by cognate phosphatase, PPP. Effect of phosphorylation over activity of mDAC was monitored in a deacetylation reaction using acetylated peptide with varying NAD^+ (0-16 mM) concentrations. The phosphorylated mDAC was a deficient enzyme which had low K_{cat} compared to that of the unphosphorylated mDAC; however, K_m value was unaltered. Mass spectrometric analysis of the phosphorylated mDAC identified seven sites (Ser-38, Thr-39, Ser-179, Thr-197, Ser-212, Thr-214 and Ser-222). Mutational analysis of mDAC, replacing these residues one at a time with alanine, followed by kinase assay of these variants revealed that Thr-214 is the predominant site of phosphorylation.

To elucidate the relevance of mDAC phosphorylation, the wild type or T214A was cloned in pVV-vector and transformed individually in *M. smegmatis*. Both wild type and T214A proteins were expressed and purified using Ni-NTA affinity chromatography followed by assessment of phosphorylation status in western blotting using anti-pThr as primary antibody. Although both mDAC and T214A were recognised by anti-pThr antibody, the magnitude of band intensity was significantly low with the mutant protein. Further, to get insight towards role of phosphorylation of mDAC in bacterial physiology, *E. coli* knock out of CobB (homologue of mDAC) was used. CobB knock out of *E. coli* was compromised in its growth profile in nutrient deprived condition (acetate medium). This growth profile, however, was reversed upon mDAC expression. Interestingly, expression of PknA in the mDAC complemented *E. coli* cells exhibited growth inhibition in acetate

medium, indicating phosphorylation mediated regulation of mycobacterial sirtuin. Furthermore, expression of a phosphomimic, T214E also exhibited growth compromised behaviour compared to that of the wild-type. Thus, phosphorylation mediated control of deacetylase activity of a *M. tuberculosis* sirtuin provides evidence for cross-talk between two distinct post-translational modifications.

Acetylation of proteins influences its metabolic process and it is usually regulated by NAD^+ dependent deacetylase. The work presented here also established that deacetylase like mDAC was regulated by phosphorylation/dephosphorylation reactions. Thus it is certain that phosphorylation along with acetylation maintains tight control over *M. tuberculosis* metabolism. One of the key pathways upon which metabolic status depends is nucleotide biosynthesis pathway. Thus the final part of the study deals with exploring the possibility of regulation of mycobacterial nucleotide biosynthesis by PTM events, such as, phosphorylation mediated regulation of enzyme(s) in this pathway.

Previous studies in the lab identified several partners of PknA by utilizing bacterial two hybrid system. Strikingly, one of the interacting partners was guanylate kinase (GMK), an essential enzyme involved in biosynthesis of GDP by transferring phosphate to GMP. The *M. tuberculosis* GMK (mGMK) was phosphorylated by PknA *in vitro* or upon co-expression in *E. coli* system as well as its over expression in *M. smegmatis*. Phosphorylation of mGMK was reversed by PPP, the only Ser/Thr phosphatase present in the *M. tuberculosis* genome. Other enzymes in this pathway, ADK and NDK, involved in conversion of NMP to NDP and NDP to NTP respectively were also examined for PknA mediated phosphorylation. ADK was not phosphorylated while NDK reflected weak transphosphorylation by PknA in *in vitro* kinase assays. These findings established that in purine nucleotide biosynthesis pathway involving NMP to NTP synthesis, only mGMK is preffered for regulation by PknA mediated phosphorylation. Kinetic analysis of phosphorylated and unphosphorylated mGMK revealed p-mGMK was a deficient enzyme, as its turnover rate is affected. To identify the major phosphosites within mGMK, phosphorylated mGMK protein was subjected to mass spectrometry, which identified Thr-101 and Thr-169 as phosphorylating residues. These residues were individually mutated to alanine and a double mutant T101-T169A was also generated. The effect of phosphorylation on these mutant proteins by PknA was monitored by performing *in vitro* kinase assay. The magnitude of *in vitro* phosphorylation of T101A or

T169A or T101A-T169A compared to wild type mGMK was decreased. The T101A-T169A showed maximum decrease in transphosphorylation ability followed by T169A and T101A. To evaluate the possible cause of decreased phosphorylation of these proteins, positions of these residues in already reported crystal structure of mGMK were visualized utilizing PyMOL software. Thr-101 was found to exist in close proximity (3.86 Å) to substrate binding site which suggests that phosphorylation at this position might affect activity of the protein. Although the reason for decreased phosphorylation of the mGMK protein due to mutation at Thr-169 could not be predicted from the structure, results of biochemical experiments indicated the reality. Nonetheless, it seems logical to presume that phosphorylation could bring changes in the protein leading to altered interactions thereby affecting enzyme activity. In fact, sequence analysis of different mycobacterial GMKs revealed that both these residues are conserved. Available literature from eukaryotes indicated that a kinase may have number of substrates and thus regulate several processes depending on the signal. The work presented in this thesis, thus claims its distinction by providing biochemical evidence with eukaryotic-type Ser/Thr kinases in *M. tuberculosis*, particularly considering PknA as a representative.

CONCLUDING REMARKS

Interplay of different PTMs in fine tuning regulation of different cellular processes is well known in eukaryotes. However, reports regarding controlling array of activities through cross-talk between PTMs in prokaryotes is scanty. In this context, the work presented in this dissertation deals with *M. tuberculosis* protein(s) associated with bacterial cell growth/metabolism and regulated through PTMs, such as phosphorylation /dephosphorylation and/or acetylation/deacetylation. The prime focus in this direction was initiated through central carbon metabolic enzymes like ICD and ICL that governs flow of carbon through glyoxylate shunt instead of TCA cycle in pathogens like *M. tuberculosis* for saving at least 'two carbon atoms'. The results of acetylome mapping identified acetylation/deacetylation mediated control of these (ICD and ICL) mycobacterial (*M. tuberculosis* strains H37Ra, H37Rv and *M. smegmatis*) proteins. Utilizing PknA as a representative of mycobacterial eukaryotic-type Ser/Thr kinase, this study further established the trans-phosphorylating abilities of these proteins. Thus it is apparent that at least two PTMs might regulate them. Apart from PTMs, pH was observed to play a key role in rerouting carbon flux through glyoxylate cycle. This

finding fits quite well with earlier observation wherein ICL knock out mycobacterium was observed to get killed during macrophage infection as its carbon flux through glyoxylate cycle was inhibited. The present study thus indicated the possible means by which the carbon flux in mycobacterium could be regulated.

Eukaryotic-type Ser/Thr kinases in *M. tuberculosis* regulate a number of metabolic processes through reversible phosphorylation. In this direction, this study also established the phosphorylation mediated control of NAD^+ dependent deacetylase activity of the only sirtuin from *M. tuberculosis*. Such an observation ostensibly rendered evidence for cross-talk between two distinct post-translational modifications. In addition to exploring relationship between PTM events, identification of novel substrates of PknA was also focused. Utilization of bacterial two hybrid revealed guanylate kinase (GMK) to be one of the interacting partners of PknA and confirmed as a substrate of PknA by utilising different assays and mutagenesis experiments. Nucleotide biosynthesis is important for generation of energy metabolites like ATP, GTP in addition to co-factors including NAD^+. As it is well established that ATP, GTP and cofactors regulate enzymatic functions, regulation of a pathway by PTM event could affect several processes simultaneously without directly targeting the participating enzymes.

All these lines of evidence presented in this thesis indicated existence of several levels of regulation that affect functionality of a cell. Future study in the direction, particularly on interplay between different PTMs in regulating functionality of proteins belonging to different pathways would lead to identification of drug targets. This would open up new vistas to increase our understanding of *M. tuberculosis* biology and also how to combat this pathogen, which remains one of the biggest killers of human even today.