SUMMARY

Cells usually receive or integrate signals and respond to changes happening in their immediate environment. These cellular events are usually followed by all organisms ranging from prokaryotes to eukaryotes for better adaptation to their surroundings. Among bacteria, *M. tuberculosis*, the causative agent of the disease tuberculosis, is one such successful pathogen that possesses the excellent ability to camouflage with its microenvironment and bears unique signal transduction mechanisms. To understand, how mycobacterial cells respond to the extracellular environment by relaying unique signal transduction within the interiors is a thrust area of research. The mechanisms that fortify signalling within *M. tuberculosis* are extremely complex and unravelling them requires great challenges. Therefore, the overriding goal of this dissertation is to elucidate the basic biology of *M. tuberculosis* in terms of the interplay between two independently existing signalling cascades like cAMP pathway and eukaryotic-type Ser/Thr kinase mediated phosphorylation.

cAMP is an essentially important signalling molecule of *M. tuberculosis* as it is known to regulate the expression of multitude of genes involved in the processes like stress response, adaptation, survival and pathogenesis. Compared to other bacteria, the levels of cAMP in *M. tuberculosis* are maintained high and interestingly its outburst is observed intra- and extracellularly at the time of infection. Catalytic bodies that are known to regulate such levels within the cells include adenylate cyclases responsible for synthesizing cAMP from ATP as well as phosphodiesterase that catalyses its hydrolysis to 5'-AMP. Therefore, maintaining equilibrium between synthesis and degradation of cAMP seems to be crucial for the microorganism to become a successful pathogen. *M. tuberculosis* genome possesses sixteen adenylate cyclases while in contrast there is a single phosphodiesterase (mPDE). Presence of a single phosphodiesterase against highly efficient cAMP synthesizing machinery along with its role in manipulating host signaling presumably hint towards the significance of mPDE as a critical regulatory molecule in *M. tuberculosis* for maintaining required cAMP pool within and outside the cells to support its survival especially during infection. Apart from this aspect, mPDE was also identified as a molecule that influences cell wall permeability.

Phosphorylation by Ser/Thr kinases plays a cardinal role in functional regulation of components involved in eukaryotic cAMP pathway. Such post-translational modification of either adenylate cyclase or phosphodiesterases, although known in eukaryotes, yet remains elusive in any prokaryote. In this scenario, the possibility of post-translational modification of mPDE by eukaryotic-type Ser/Thr kinases was of prime interest, as this forms the critical regulatory body for maintaining overall cAMP levels. Study was therefore initiated by performing *in vitro* kinase assays, utilizing mycobacterial Clade I eukaryotic-type Ser/Thr kinases (PknA, PknB, and PknL). Ni-NTA purified mPDE protein displayed phosphorylation on incubation with all three kinases, albeit with a varying degree. When mPDE was co-expressed one at a time with these kinases in *E. coli* BL21(DE3) cells, it was also identified by an anti-phosphothreonine antibody, thereby indicating its phosphorylating ability. Similarly, expression of mPDE in *M. smegmatis*, where all these eukaryotic-type Ser/Thr kinases are present, yielded a protein, which is recognized by anti-phosphothreonine antibody.

The phosphorylated mPDE protein obtained from *E. coli* strain BL21(DE3) following its co-expression with PknA, when used for mass spectrometric analysis identified Thr-309 as the phosphorylable residue. In concordance with this observation, anti-phosphothreonine antibody marginally recognized mPDE-T309A mutant protein, further validating this residue to be the predominantly phosphorylating site of mPDE. Interestingly, structural analysis revealed that Thr-309 is located within a short but de-structured stretch of C-terminal domain of mPDE. Reports from other groups have shown that mPDE is a cell wall localizing protein and this cellular recruitment is contributed by its C-terminal non-catalytic cap domain. This intrigued the necessity to identify if phosphorylation at Thr-309 is involved in the process. It was observed that wild-type mPDE protein localizes to the cell wall of *M. smegmatis* but in contrast, mPDE-T309A, the phosphoablative variant of mPDE, did not show such behaviour. On the other hand, phosphomimics of mPDE (T309D or T309E), exhibited similar cell wall anchorage as was observed with the wild-type. These results provide credence to the fact that eukaryotic-type Ser/Thr kinase mediated phosphorylation of mPDE renders negative charge to the protein, which in turn promotes its localization to cell wall. Furthermore, multiple sequence alignment revealed that Thr-309 is conserved among mPDE orthologs of *M. tuberculosis* complex, which presumably emphasizes evolutionary significance of such phosphorylation.

The role of phosphorylation mediated regulation of mPDE enzyme activity was also evaluated. Reports suggest that few of the eukaryotic phosphodiesterases undergo activity modulation upon phosphorylation mediated post-translational modification, thus influencing feed-back inhibition mechanism. Such a regulation was not reported for any prokaryotic phosphodiesterase. In this study, it was observed that phosphorylation of mPDE by PknA exhibited a decrease in its enzyme turnover rate. To elucidate the role of mPDE phosphorylation on its functionality, we utilized a phosphodiesterase knock-out *E. coli* strain JW3000-1, where interference of endogenous phosphodiesterase and mycobacterial kinases could be excluded. Interestingly, the mPDE complemented JW3000-1 strain showed enhanced cAMP levels in the presence of PknA, while this effect was antagonized by PknA-K42N, a kinase-dead variant. Structural analysis of mPDE revealed that four of its Ser/Thr residues (Ser-20/Thr-22/Thr-182/Thr-240) are close to the active site, indicating their possible role in phosphorylation mediated alteration in enzymatic activity. In fact, mutation of these residues one at a time to alanine or combination of all four (mPDE-4A) affected the mPDE activity. In addition, mPDE-4A protein in kinase assay exhibited reduction in phosphorylation compared to wild-type mPDE. Furthermore, mPDE-S20A/-T240A proteins yielded on co-expression with PknA in *E. coli* BL21(DE3) cells, were marginally recognized by anti-phosphoserine/phosphothreonine antibodies in immunoblotting. Such an observation delineates the role of Ser-20 and Thr-240 in phosphorylation mediated modulation of mPDE enzyme activity. Interestingly, phosphorylation at C-terminal Thr-309 of mPDE leads to cell wall localization in *M. smegmatis* without affecting its enzyme activity. On the other hand, phosphorylation of mPDE at Ser-20 and Thr-240 modulates enzyme activity, but did not affect the recruitment of protein to cell wall. Taken together, these findings established mutually exclusive dual functionality of mPDE upon PknA mediated phosphorylation.

Phosphoproteome mapping in *M. tuberculosis* indicated that Rv0891 is the only adenylate cyclase as a phosphoprotein. To evaluate further on this aspect, PknA was utilized as a representative of eukaryotic-type Ser/Thr kinase to trans-phosphorylate Rv0891. *In vitro* kinase assay revealed that the phospho-signal generated in the presence of PknA was diminished on treatment with either kinase dead mutant (PknA-K42N) or Ser/Thr phosphatase (PPP). Co-expression of Rv0891 with PknA in *E. coli* BL21(DE3), also led to the purification of a

phosphorylated histidine-tagged protein. In order to ensure that the phosphorylation of Rv0891 is not an experimental artefact, *in vitro* kinase assay was performed with another well characterized mycobacterial adenylate cyclase, Rv1647, which did not show such behaviour. Finally, through sub-cellular fractionation studies it was demonstrated that Rv0891 is a cell wall localizing protein and undergoes phosphorylation when expressed in *M. smegmatis*. All these lines of evidence, therefore established that Rv0891 is a substrate of eukaryotic-type Ser/Thr kinases, like PknA.

Concluding Remarks and future perspectives

Cyclic-AMP is an essentially important signalling molecule in *M. tuberculosis*. The enzymes involved in its synthesis and degradation requires tight regulation. Phosphorylation by Ser/Thr protein kinases is an important means of regulation for eukaryotic adenylate cyclases and phosphodiesterases, but such phenomenon was not evident in any prokaryote. In this scenario, the work presented in this dissertation established that phosphorylation of mPDE influences its cell wall localization as well as enzyme activity in a mutually exclusive manner. Further, the study helped in identifying the post-translational modification by eukaryotic-type Ser/Thr kinases over one of the adenylate cyclases, Rv0891 of *M. tuberculosis*. This study, therefore, provided new perspectives in the field of mycobacterial cAMP signaling, albeit it needs further elucidation. It would be interesting to determine the significance of mPDE localization to cell wall upon phosphorylation and whether such recruitment influences cAMP levels intra- and extracellularly. Also, it demands further interpretation on the outcome of phosphorylation of adenylate cyclase and to study the importance of this phenomena happening on two antagonizing events; one involving cAMP synthesis and other causing its hydrolysis. Finally, identification of the exact kinase involved in regulating cAMP signaling pathway under physiological conditions also remains a challenge. This would be an interesting arena to elucidate as it would provide hints towards the development of drug interventional strategies. Nonetheless, this study identified for the first time that phosphorylation of adenylate cyclase and phosphodiesterase is not only a eukaryotic feature, but occurs in bacteria as well. It is indeed the beginning, but definitely opens up new vista for the understanding of the biology of *M. tuberculosis*. Unravelling such exceptionally unique signalling cascade(s) involving eukaryotic-type Ser/Thr

kinase mediated regulation of small molecule trafficking machinery in *M. tuberculosis*, would definitely offer promising perspectives for rationalizing the development of new anti-mycobacterials in the years to come.