The phosphate specific transporter (Pst) is a multi-subunit system known to be operative during phosphate limiting conditions in prokaryotes. In *Mycobacterium tuberculosis* several copies of this transporter are present. PstB of *M. tuberculosis* is the nucleotide binding subunit of Pst. Like other ABC proteins, being the energy transducing unit, PstB in *M. tuberculosis* has a vital role in the ATP-consuming import of phosphate. Besides this, PstB in *M. tuberculosis* gains additional importance since several copies of all the components of the operon are present in the genome but there is single copy of *pstB* gene. Furthermore, earlier reports from our laboratory indicated that *pstB* is overexpressed as well as amplified in a fluoroquinolone resistant colony of *M. smegmatis* where drug efflux plays a pivotal role in conferring resistance. These consequences together with the fact that ABC proteins are involved in diverse biological processes, led us to study the PstB subunit from *M. tuberculosis*.

The work embodied in this thesis, therefore, deals with stepwise characterization of *M. tuberculosis* PstB. The initial focus was to isolate the *pstB* gene from *M. tuberculosis* genome. Since the genome sequence was known therefore polymerase chain reaction was employed to amplify the *pstB* gene from the genomic DNA of *M. tuberculosis* strain H37Rv using suitably designed primers. The amplified gene was cloned in vector pET23a, expressed in *E. coli* and finally purified as a recombinant protein. Overexpression of PstB in *E. coli* resulted in its accumulation as aggregates and it was found in the pellet fraction (inclusion bodies) following centrifugation of sonicated cultures. The renaturation of solubilized protein in urea or guanidine hydrochloride proved to be a difficult task since it yielded completely denatured protein which could not be refolded following conventional methods. Hence, the protein was solubilized using a mild ionic detergent N-lauroyl sarcosine. Analyses of the size of the native (obtained through gel filtration chromatography) and denatured (as in SDS-PAGE gels as well as in Western blots) proteins revealed that the PstB presumably existed as a dimer. ABC proteins in

bacteria have already been shown to be active as dimer therefore, this did not seem to be a unique feature for mycobacterial protein. The details in this regard have been presented in chapter 3.

In chapter 4, a detailed biochemical analysis of the purified PstB protein was carried out. ABC proteins are known to have nucleotide binding as well as ATP hydrolysing ability, which is very important for the functionality of the transporter. The ATP-binding ability of PstB was monitored using a non hydrolyzable analogue of ATP, FSBA. Binding of FSBA not only confirmed the ATP-binding ability of PstB but also ensured correct folding of the protein. The ATP hydrolysing ability of mycobacterial PstB exhibited many properties characteristic to typical ABC proteins, however, subtle differences made it distinct from others, like MalK or HisP of *Salmonella typhimurium*. Among different nucleotides, ATP has been found to be a preferred substrate for the recombinant PstB. Similarly, divalent cations (Mg^{2+} and Ca^{2+}) which have been reported to be stimulatory for the enzymatic activity had no effect on the mycobacterial PstB-ATPase. Like membrane bound thermostable ATPase from *Sulfolobus acidocaldarus*, the enzymatic activity of mycobacterial PstB was found to be resistant to known inhibitors, such as, sodium orthovanadate and sodium azide. Although this observation has also been found to be true for HisP or MalK, N-ethylmaleimide which had a strong inhibitory effect on their activity, was unable to affect the mycobacterial enzyme. The sensitivity of these proteins to N-ethylmaleimide has often been correlated to the presence of a cysteine residue at or near the highly conserved Walker A motif. Interestingly, no such cysteine residue was present in *M. tuberculosis* PstB. The ATPase activity of *M. tuberculosis* could however be inhibited if incubated with non hydrolyzable ATP analogue, FSBA. This suggests a possible competition of the non-hydrolysing ATP-analogue with ATP for substrate binding site.

An interesting aspect of the PstB-ATPase is its stability at high temperature.

Studies regarding thermal aggregation profile of the recombinant protein through Raleigh's scattering and subsequently monitoring of enzymatic activities indeed showed that PstB-ATPase from *M. tuberculosis* is thermostable compared to *E. coli* counterpart. Several thermostable ATPases have been characterized, but they all are present in thermophilic bacteria. Therefore, what made the finding significant is not only the fact that the recombinant PstB being an ABC-ATPase is thermostable but presence of such an enzyme in a mesophilic bacteria like *M. tuberculosis*.

In Chapter 5, structure function analysis of this protein was carried out, as it was essential for the better understanding of its unique characteristics. Although with the advancement in molecular methods, expression and purification of recombinant proteins are almost a routine work now a days, obtaining their three-dimensional structures are not always easily achievable. Therefore, as an alternative strategy, homology modeling for protein structure predictions are often adopted. Using this technique a comparative model of PstB was generated based on the solved structure of *S. typhimurium* HisP, the ATP-binding subunit of the histidine transporter. The structure displayed all the conserved motifs of an ABC-ATPase and formed the basis for mutational analysis.

Mutations were carried out in all the conserved regions of ABC proteins, such as Walker A, Walker B, Q-loop and signature motif of mycobacterial PstB . Two mutants were created in the Walker A motif of the *M. tuberculosis* PstB. Among three glycine residues in this region, two of them (amino acid residues 57 and 59) are conserved throughout the ABC proteins. The equivalent residue of glycine 54, however, in *M. smegmatis* PstB has a serine. The glycines at codons 54 and 57 were substituted with aspartic acid, resulting in mutants G54D and G57D. Like other ABC proteins, G57D did not show any ATP binding as well as ATPase activity. G54D mutant, on the other hand, showed both nucleotide binding and ATP hydrolyzing ability quite comparable to that of the wildtype . Thus, the results with G54D made *M. tuberculosis* PstB distinct from other ABC-ATPases considering *in vitro* nucleotide binding as well as hydrolysis. A complete

loss of activity was observed for mutants D188K and E189D where mutations were in the Walker B region. This was similar to results observed with other ABC-ATPases. Mutation in the conserved glutamate of Q loop in the *M. tuberculosis* PstB resulted in a significant loss in ATPase activity though the nucleotide binding function was unaffected. In MalK, mutation in this residue does not cause a significant loss in ATPase activity, however in HisP this loop has been found to be important for ATP hydrolysis.

Signature motif is well conserved in all ABC-ATPase and it has been implicated to perform different functions. One group holds the view that it plays a role in ATP-binding and hydrolysis by acting as a sensor for γ-phosphate in the opposing molecule in the dimer. The other school of thought is that the motif has a role in interaction with membrane spanning domains rather than ATP-hydrolysis function. This view is also supported by the crystal structures of HisP and MalK. The experimental evidence provided in this study with mycobacterial PstB also indicated that alteration in these conserved residues of the signature motif did not result in any loss of *in vitro* nucleotide binding or ATP hydrolysis. Thus the mutational analysis of the conserved motifs of the mycobacterial PstB revealed that although it confirmed most of the structural properties typical of other ABC proteins, it also exhibited subtle variations.

The temperature at which organisms survive does not exactly reflect the thermostability status of their proteins. PstB-ATPase from *M. tuberculosis* is an example. Understanding the mechanisms of thermo tolerance is a daunting task since a large number of factors have been implicated to play a role. However, increased charge and decreased polarity have been proposed to make the most significant contribution. Since one of the most consistent factors implicated to contribute towards thermostability is increased proportion of charged residues this aspect was emphasized in this study. Analysis of the homology model of PstB revealed a completely charged solvent exposed α-helix. This helix consisted of several charged residues which were mutated into uncharged ones. It

was assumed that a decrease in net charge of the protein by as many as five residues would drastically decrease the thermostability of the protein. However, mutational studies revealed that thermostability of mycobacterial PstB-ATPase was not affected by these mutations except for ~45% loss in overall enzyme activity. In fact, the multiple mutant (combined mutant) having all five point mutations together, displayed a similar aggregation profile as has been observed with the wildtype. Thus implication of surface charge being an essential factor for protein thermostability did not seem to be true for *M. tuberculosis* PstB-ATPase.

The other factor found to make a positive contribution towards thermostability is decreased content of polar amino acids. However, verification of the influence of polarity on *M. tuberculosis* PstB through mutational analysis was difficult, since a large number of amino acids would have to be mutated into polar residues in all possible combinations, so that, there is a net increase in polarity of the protein sufficient to cause a change. A theoretical approach was therefore adopted to get an insight into this feature. Since the thermophilic and mesophilic counterparts of a given protein are composed of the same twenty essential amino acids, therefore, it is apparent that the mystery of its thermo tolerance should be encoded within the sequence itself. This prompted us to get an insight into the placement of this thermostable ATPase among other bacterial PstB proteins.

BLAST search using *M. tuberculosis* protein as a search probe in non-redundant protein sequence databases exhibited significant homology with different PstB and ABC-ATPases. Among them the first thirty-nine hits were different bacterial PstB of either mesophilic or thermophilic origin. A phylogenetic tree obtained from multiple sequence alignment of PstB from different bacteria, confirmed the mesophilic origin of *M. tuberculosis* protein. It showed a clustering with *M. intracellulare*, *Mesorhizobium loti* and *Xylella fastidiosa*. Interestingly, the protein from the thermophiles did not show any distinct pattern, rather they were randomly distributed throughout the phylogram.

Conversely, based on simple statistical tests with stabilizing factors (eg. protein size, number of residues involved in hydrogen bonding, β-strand content, helix stabilization through ion pairs, relative amount of hydrophobic β-branched amino acids etc.) and secondary structure analysis, the *M. tuberculosis* PstB protein confirms many of the properties characteristic to thermostable proteins.

To resolve this ambiguity, the amino acid composition which is known to be one of the important parameters in determining protein thermostability was analyzed. Thermostable proteins contain increased proportion of charged residues at the expense of uncharged polar amino acids conferring them rigidity as well as stability by minimizing deamidation and backbone cleavages. The contribution of charge and polarity on all the PstB sequences including that of the *M. tuberculosis* was therefore determined using OC software. The data was plotted as the function of percentage of either charged or polar amino acid residues. On the basis of percentage of charged amino acid residues in each PstB, two major clustering were observed and *M. tuberculosis* PstB was grouped with mesophiles only. On the other hand, on the basis of polarity of different PstBs, thermophiles were found to be distinct from mesophiles. PstB from *M. tuberculosis* protein was placed with thermophiles in conformity with the experimental evidence. Such an observation was not only *M. tuberculosis* specific since other pathogenic mycobacteria (*M. intracellulare, M. leprae*) showed a higher propensity towards thermophilic characteristics compared to the nonpathogen (*M. smegmatis*). The genome analysis data also predicted that PstB from *Pseudomonas aeruginosa* and *Halobacterium sp.* are thermostable. However, the rationale behind such thermo tolerance of this ABC protein in some mesophiles remains to be elucidated.

Finally, our results revealed that PstB from *M. tuberculosis* is an atypical ABC-ATPase. Although it exhibits many characteristics of known ABC-ATPases, its thermostable nature is the most interesting and unique feature. The work presented in this thesis has attempted to gain an insight into some of the factors underlying this

thermostability. However, an in depth understanding of the factors making a specific contribution towards the thermostable nature of PstB will only be unravelled once the crystal structure is solved and a complete analysis can be carried out. Also the detailed study on the thermostability of other ABC proteins is not available yet. Therefore, such a property brings them in sharp focus and indicates that they might be playing a more pivotal role in bacteria than a cursory glance reveals.