Chapter 5

Summary and Conclusions
Preponderance of P-type ATPases reflects importance of ion homeostasis in *M. tuberculosis*. Twelve P-type ATPases in *M. tuberculosis* may indicate that they possess different substrate specificities, which allow *M. tuberculosis* to acquire different essential ions under nutrient depriving condition in the host. Some of them responsible for their virulence too (Botella *et al.*, 2011; Ward *et al.*, 2010). Out of twelve P-type ATPases, six of them have homologue in *M. smegmatis* mc²155 and provide an opportunity to unravel the unknown function of P-type ATPases prevailing in mycobacteria.

In this study, CtpE (P-type ATPase) of *M. smegmatis* mc²155 was functionally characterized. This study confirmed that CtpE is a member of a new family of P-type ATPases, which is functionally uncharacterized and is present only in bacteria. CtpE belongs to FUPA 23 (Functionally Uncharacterized P-Type ATPase) family according to Chan *et al.* classification system given in 2010. Genomic organization confirmed that it presents in most of mycobacterial sp.

To characterize CtpE, deletion mutant was made for *ctpE* in *M. smegmatis* using allelic exchange mutagenesis with the help of suicidal vector. Further this mutant referred as MHK1. *ctpE* is not an essential gene for *M. tuberculosis* (Sassetti *et al.*, 2003; Griffin *et al.*, 2011). MHK1 was sensitive towards calcium deprivation and this effect was reverted by adding extracellular calcium in the medium, but not with other cations. These results indicate that CtpE would be an uptake pump for calcium. Further radioactive calcium accumulation assays confirmed that CtpE is a Ca²⁺ uptake ATPase. Till now, Ca²⁺ ATPases were characterized in bacteria as efflux pump, no ATPases were known as calcium uptake pump in bacterial system before. This is the major finding of this study. Complementation study with wild type *M. smegmatis* mc²155 confirmed that destructive phenotype of MHK1 was due to disruption of *ctpE* only. CtpE homologues from *M. tuberculosis* H₃⁷Rv Rv0908 also complement the MHK1 function, indicates that *M. tuberculosis* H₃⁷Rv Rv0908 has same function as *M. smegmatis* mc²155. CtpE did not complement the yeast PMR1 (SERCA like Ca²⁺ ATPases) mutants (AA542 and K616) (Rudolph *et al.*, 1989) and also not sensitive towards manganese, which make CtpE different from PMR1.
Inhibition of radioactive calcium accumulation assay proved that CtpE exhibits specificity for calcium and calcium regulated expression. Accumulation of calcium in wild type *M. smegmatis* is affected by extracellular calcium, culture grown in medium supplemented with EGTA showed approximately four times more calcium accumulation than control culture grown in medium without any supplements. This might be due to strong signal to the cell for low calcium in presence of EGTA, which enhance the transcription or expression of *ctpE* and result in increase calcium accumulation. Culture grown in medium supplemented with extra calcium has lower calcium accumulation than control culture due to down regulation in signal for low calcium and result in low expression and less calcium accumulation. But in complemented strain, calcium accumulation was not affected in same condition, due to constitutive expression of CtpE from *hsp60* promoter. Reverse transcription analysis of *ctpE* reveals that *ctpE* is transcribed as a polycistronic operon and its transcription was also regulated by calcium. Cultures grown in presence of EGTA or extra calcium have 35% more or 30% less transcription of *ctpE* operon respectively than control.

Membrane topology study of CtpE reveals that it has ten trans-membrane helices. P-type ATPases with ten trans-membrane helices came in topological type II family of P-type ATPases. Type II ATPase subgroups contains the family of Na⁺, K⁺-ATPases, Na⁺ or K⁺ or Ca²⁺-ATPases of animals, Ca²⁺-ATPases, proton ATPases of fungi and lower plants, Mg²⁺-ATPases, aminophospholipid translocase, FUPA 23 ATPases, and FUPA 24 ATPases (Thever and Saier, 2009).

A report by Clarke *et al.* (1989) reveals that six amino acid residues were responsible for binding of calcium in two calcium binding sites. Glu-771 in TMH5, Glu-908 in TMH8 make calcium binding site I and Glu-309 in TMH4, Asn-796, Thr-798, and Asp-799 in TMH6 makes calcium binding site II (Clarke *et al.*, 1989). But in *S. cerevisiae*, out of six, only three residues are conserved in TMH4 and TMH6 at calcium binding site II and transporting calcium. CtpE has only single conserved residues in TMH4 other residues are not conserved and still transport calcium, this indicates that other highly conserved motifs of CtpE might be involved in calcium transport. However CtpE transporting Ca²⁺, but it has no other conserved residues.
except one (Glu) for Ca$^{2+}$ binding, didn’t grouped with other characterized Ca$^{2+}$ transporting P-type ATPases and has very less similarity with characterized Ca$^{2+}$ ATPases. Thus these results also confirmed that CtpE is a new family of calcium transporting P-type ATPases.

ctpE lies in an operon with \textit{php4} (Penicillin binding protein 4), \textit{ml} (Metallo β-lactamase), \textit{echA} (Enoyl-CoA hydratase), \textit{1.6-g} (Amylo-α-1,6-glucosidase) and \textit{gt} (Glycosyl transferase). PBP4 involves in the synthesis of peptidoglycan, which is the major component of bacterial cell wall. PBP4 catalyze the last step of murine biosynthesis. ML provides resistance against β-lactam ring containing antibiotics. EchA used in β-oxidation of unsaturated fatty acid and GT are enzymes act as a catalyst for the transfer of a monosaccharide unit from an activated sugar to a glycosyl acceptor. 1.6-G also exists as operon and involved in glycogen metabolism, but no glycogen metabolism occurs in \textit{M. smegmatis}. All genes seem to be involved in synthesis of cell wall and have some role in maintaining integrity of cell wall. The \textit{Aspergillus fumigates} PmrA (Golgi apparatus Ca$^{2+}$ P-type ATPase) mutant strain exhibit hypersensitivity to cell wall inhibitors (Pinchai \textit{et al.}, 2010).

To confirm whether MHK1 has any sensitivity towards cell wall or cell membrane disrupting agents, survival assays were done for lysozyme and SDS. Several cell wall attacking antibiotics were also checked and MHK1 was found sensitive toward polymyxin B, eight fold more than wild type. Polymyxin B alter bacterial membrane permeability by binding to a negatively charged site in lipopolysaccharide layer, which has an electrostatic attraction for the positively charged amino groups in the cyclic peptide portion (this site is normally binding site for calcium and magnesium counter ions), and dissolves fatty acid portion in hydrophobic region of cytoplasmic membrane and thus disrupts membrane integrity. Addition of CaCl$_2$ in the medium reverses the effect of polymyxin B.

This indicates that CtpE might have some role in cytoplasmic membrane integrity. Considering all these points into account, role in cell wall or cell membrane integrity can be proposed for \textit{ctpE} operon, but assignment of exact function for this operon requires further studies on mutant MHK1.