Conclusion and Future Work

6.1 Conclusion

The earliest case of tuberculosis dates back to approximately thousands of years ago. Tuberculosis is regarded as one of the oldest afflictions. There are several instances in the history of tuberculosis in which this contagious disease was taken care of and was almost eliminated. But the re-emergence of the disease even with more infectious repercussions has made it one of the top 10 leading causes of mortality resulting from the consequent post TB infection caused by the Human Immuno-Deficiency Virus (HIV). In spite of the continuous efforts to control TB, it still remains one of the major health hazards to mankind. In the past few decades although extensive research has been carried out to understand the pathogenicity of *Mycobacterium tuberculosis*, the causative agent of TB, the complete understanding of this pathogen is way behind. There are several questions that remain unanswered such as- (i) How did the bacterium evolve in such a way that led to acquisition of the attribute of resistance to the existing drugs? (ii) How is it possible for the bacteria to always find alternative pathways to overcome any encountered unfavorable condition? (iii) How do they bring about transformation of the fluidity of the cell wall at different environmental conditions thereby remaining in the two discernable latent and persistent phases? In view of the current status of the TB research, it is very interesting to understand the pathways and their components in the bacterium that contributes to its basic physiology.

The presence of the T7SS among all the species of *Mycobacterium* was considered unique to this genus until recently it was reported to be present in some of the Gram positive bacteria. The five clusters of T7SS designated as ESX1, ESX2, ESX3, ESX4, and ESX5 are present in the pathogenic strain, *M. tuberculosis* whereas only three out of the five ESX clusters are present in the non-pathogenic strain *M. smegmatis*. Of these three ESX clusters present in both the pathogenic and non-pathogenic strain, the ESX-3 secretion system is common to the two *Mycobacterium* species. So far, the ESX3 secretion system has been functionally characterized for the regulated uptake and acquisition of iron which in turn helps the bacterium within a host to adapt itself to stress conditions. But its presence and conserved nature in the non-pathogenic strain, *M. smegmatis* which have two dedicated pathways for iron sequestration - the exochelin pathway and the mycobactin pathway, is quite intriguing. Therefore to gain a better understanding of the role(s) of the conserved

nature of the ESX3 T7SS in the non-pathogenic strain, *M. smegmatis* an ESX3 deleted *M. smegmatis* mutant was characterized in our study.

In order to understand the role of the ESX3 secretion system in *M. smegmatis*, the complete copy of a gene known as the *eccD*3 gene was deleted from the genome of *M. smegmatis*. The ESX3 secretion system comprises of 11 genes of which the *eccD*3 gene is positioned at the 9th position. The ESX3 secretion system is a multimeric protein secretory apparatus in which each of the genes present in the ESX3 loci encodes a component that altogether forms the secretory apparatus. The *eccD*3 gene encodes for a transmembrane protein that traverses the outer mycomembrane of the mycobacterial cell wall and forms a central channel through which the secretory effector proteins are secreted. The complete copy of the *eccD*3 gene was deleted through efficient allelic exchange method. The deletion event achieved through homologous recombination aided to the generation of a *M. smegmatis* mutant in which there was absence of any chance in which the deletion event could be reverted back. Out of the 64 putative colonies, a single colony (Colony 56) with the desired deletion of the *eccD*3 gene was selected. In addition to the several screening procedures contributed by the selectable markers present in the pPR27 mycobacterial shuttle vector backbone, several sets of PCR primers were used for the identification of the putative colony with the desired deletion event. The colony 56 was screened against all the selectable markers and the amplification with the different sets of primers yielded the amplicon of expected size and was named as Ms0622. In order to confirm the phenotypes exhibited by the mutant strains was due to the deletion of the *eccD*3 gene, the complemented strain; Ms0622/pEccD3 was generated in which the wild-type copy of the gene was introduced in the mutant strain. The phenotypes exhibited by the mutant strain, Ms0622 could not be completely restored by the complemented strain, Ms0622/pEccD3. The reason for the failure of the complemented strain to restore the phenotypes completely can be attributed to the fact that the *eccD3* gene was cloned in an episomal vector and hence was not integrated into the *M. smegmatis* genome. Although the *eccD3* gene was expressed in the complemented strain, its expression was different from the wild type. Moreover, the *eccD3* gene encodes for a transmembrane protein, hence its expression in the complemented strain alone cannot confirm its correct positioning in the membrane. Furthermore, the expression of a gene with the help of an episomal vector

that is present in the 9th position of the ESX3 locus in *M. smegmatis* genome might not be adequate for the complete restoration of the wild-type phenotype.

The deletion of the *eccD3* gene from the *M. smegmatis* genome resulted in some drastically different phenotypes than the parental strain. One of such phenotypes was the aberrant colony morphology of the *eccD3* deleted mutant, Ms0622. The abnormal colony morphology of the deleted strain was also associated with the inability of the *eccD3* deleted strain to spread in motility agar plates and form pellicles at the air-liquid interface. The inability of the mutant strain to form biofilm in the biofilm media and even in the Chelated Sautons minimal media supplemented with increasing concentration of iron hinted at the fact that the precise architecture of the cell wall must have been disturbed. The decrease in the integrity of the cell wall was further confirmed by the decrease in the uptake of both the representative hydrophobic and hydrophilic dyes by the *eccD3* deleted mutant. The fact that the *eccD3* gene might have a functional role in maintaining the precise architecture of the cell wall was evident from the hyper sensitivity of the *eccD3* deleted strains to the β-lactam drug, ampicillin and also from the gain in the resistance to the four first line anti-TB drugs by the *eccD3* deleted strain as compared to the wild type. Based on these results it was concluded that the ESX3 associated *eccD3* gene conserved among all the *Mycobacterium* species was related to the maintenance of the precise architecture and integrity of the cell wall. Although the functional role of the ESX3 associated *eccD3* gene in maintaining the integrity of the cell wall was deciphered, the mechanism by which the *eccD3* gene helps in the maintenance of the cell wall was studied in the preceding chapter.

During the last decade, several significant advances have been made in tuberculosis research. Meanwhile, a number of potent enzymes and pathways essential for the survival of the *Mycobacteria* has been discovered against which effective anti TB drugs can be designed and discovered. However, *Mycobacteria* always succeed in using an alternative pathway that bypasses the essential enzymes or pathways that ultimately leads to the desired product. In such a scenario of acquiring evolved pathways and resistance to the applied anti TB drugs, the cell wall of the bacterium has been the most interesting target for the development of anti TB drugs. The unique architecture of the mycobacterial cell wall which comprises of the outer layer, the mycomembrane and the plasma membrane that acts as a tough barrier between the extracellular environment and the interior of the bacterial cell. It is very

interesting to understand as how mycobacteria maintain the less fluidic nature of the cell wall that aids to the lower permeability of the cell wall.

The mycomembrane of the mycobacterial cell wall is known for its less fluidic nature which contributes to the lower permeability of the cell wall of mycobacteria. The mycolic acid present in the mycomembrane comprises of fatty acid with very long carbon atom chains ranging from C_{60}- C_{82}. In addition to the long carbon chain, the presence of the desaturated carbon atoms contributes to the lower fluidity of the mycobacterial cell wall. Together with the maintenance of lower permeability of the mycobacterial cell, the cell wall also contributes to the shape of the bacterium. It is interesting to note that different bacterial species assume different shapes so as to- firstly adapt to various environmental niches and secondly to maintain a uniformity to be able to take the advantage of the particular shape. The morphology of a bacterium is an important trait which can be mapped in an evolutionary scale with the rod shaped bacteria as the most primitive form. The species belonging to *Mycobacterium* are rod shaped cells and the cell wall of the bacteria plays an important role in the maintenance of the cell shape. The *eccD3* deleted mutant of *M. smegmatis* was observed to be transformed to spherical shaped cells. The change in the cell shape in the *eccD3* deleted strain was a clear indication of the fact that architecture of the cell wall has been damaged. When the mycolic acid from the mycomembrane of both the wild-type and mutant strains were separated on a TLC plate, two (α′ and epoxy) out of the three (α, α′ and epoxy) mycolic acid components were absent in the mutant strain. Further, when the α subspecies of mycolic acid from both the wild type and mutant were subjected to GC-MS analyses, the α subspecies of mycolic acid from the mutant strain, Ms06622 consisted of less number of carbon atoms. In addition, the ^{1}H NMR analyses of the α and epoxy subspecies of mycolic acid from the *eccD3* deleted mutant demonstrated the transformation of the unsaturated fatty acids present in the parental strain to saturated fatty acid. The lack of the presence of the characteristic *cis* double bond in the α subspecies of mycolic acid and the absence of the characteristic double bond and epoxy group from the epoxy subspecies of mycolic acid of the *eccD3* deleted strain resulted in the formation of a saturated mycomembrane contributing to the much less fluidity and permeability of the cell wall in the *eccD3* deleted mutants as compared to the wild type.

In summary, our findings suggest that the ESX3 associated *eccD3* gene is essential for the maintenance of the mycobacterial cell shape and precise architecture of the cell wall thereby contributing to the normal physiology of the bacterium.

6.2 Future Works

In continuation of the present study, it is proposed that investigations relating to the following aspects in future may lead to interesting findings.

- Study the effect of overexpression of the *eccD3* gene in *M. smegmatis*.
- Understanding the molecular mechanism by which the effectors of the ESX3 secretion system interact with the cell wall synthesizing enzymes.
- Study the role of ESX3 secretion system in the coordinated synthesis of cell wall and cell division.
- Study the role of ESX3 secretion system in the oxidative stress response pathway.