General Introduction and Review of Literature

1.1 Tuberculosis - An Ancient Affliction

Tuberculosis (TB) is one of the ancient diseases that have been distressing mankind for ages. The first evidence of tuberculosis (TB) infection was noted in the fossil of an extinct bison dating back to about 17,000 years ago and also in the human remains of the Neolithic era about 9000 years ago [1,2]. With the help of the modern molecular DNA sequencing techniques, presence of the *Mycobacterium tuberculosis* complex was proved in the remains of the mummies which indicated the pervasiveness of this deadly disease in ancient times. Gutierrez *et al*., 2005 reported that the early progenitor of *Mycobacterium tuberculosis* originated in East Africa as early as 3 million years ago and the organisms belonging to the *M. tuberculosis* complex had a common African ancestor about 35,000-15,000 years ago [3]. The modern strains of *M. tuberculosis* complex seem to be an outcome of an evolutionary process that possibly originated about 20,000–15,000 years ago [4]. In 2006, Gagneux et al. demonstrated that *M. tuberculosis* has six phylogeographical lineages, each of which is associated with specific sympatric human population [5].

The presence of the tubercle bacilli and the skeletal deformities found in the Egyptian mummies depicted that the East Africa was the original home of the tubercle bacilli and the human hosts [6,7]. About 1.7 million years ago, people from Africa started to move out to different places and in due course of time the places were inhabited by the migrants from different parts of the world. Apparently people migrating from Africa carried the tubercle bacilli along with them [8]. In India, tuberculosis was known as *Yaksma* as mentioned in the *Rigveda* and dates back to about 3300 years ago. In the classical Chinese medical texts also tuberculosis is referred to as a weak consumptive disease which dates back to about 2300 years ago [9]. In ancient Greece, tuberculosis was known as *Pthisis* or consumption. Hippocrates, the '*Father of Medicine'* had a clear understanding of the symptoms of the disease and recommended good food, milk and physical exercise for its treatment and believed it to be hereditary in nature [10]. It was Aristotle (384-322 BC) who disagreed with Hippocrates and believed that the disease is infectious and contagious. During the middle ages in Europe, tuberculosis emerged as a widespread disease claiming thousands of lives [11]. The first declaration came in 1790 from Benjamin Marten who claimed that tuberculosis was infectious in nature [12]. It was in 1865 when a French military surgeon Jean-Antoine Villemin conclusively demonstrated the contagious nature of tuberculosis.

Table 1.1: Milestones in the history of Tuberculosis

Time	Event/Discovery	References
3 million years ago	Evolution of *M. tuberculosis*	Gutierrez et al., 2005 [3]
460-370 BC	Hippocrates described the clinical signs of TB	Coar, 1982 [10]
384-322 BC	Aristotle described TB as infectious	Garrison, 1913 [13]
1781-1826	Concept of pulmonary or extrapulmonary TB	Laennec, 1962 [14]
1834	Schonlein coined the term 'tuberculosis'	Ferlinz, 1995 [15]
1881	Discovery of *M. tuberculosis* and tuberculin (Robert Kotch)	Koch, 1932 [16]
1907-1926	Tuberculin skin test (Clemens Freiherr von Pirquet, Charles Mantoux, Florence Seibert)	von Pirquet, 1907, Lebedeva, 1977, Seibert, 1926 [17,18]
1920s	The Vaccine 'BCG' (Albert Calmette and Camille Guerin) was developed	Calmette and Guérin, 1924, Calmette, 1928 [19,20]
1950	Discovery of Streptomycin and other antibiotics	Schatz et al., 1944, Jones et al., 1944 [21,22]
1990	MDR-and XDR-TB pandemic	Pablos-Mendez et al., 1998 [23]

On 24th March 1882, in Berlin, a famous lecture entitled '*Die Ätiologie der Tuberkulose*' was delivered by Robert Koch which subsequently changed the history of tuberculosis. He demonstrated the causative agent of tuberculosis and named it *Mycobacterium tuberculosis*. He used microscopic techniques and identified the causative organisms as rod shaped bacilli and also proved the Henle-Koch postulate regarding the infectious organism [24]. Subsequently in 1890, Robert Koch described a glycerin derivative of the tubercle bacilli and named it tuberculin. He described that upon administration of the tuberculin, a violet colour appeared in the skin with a rise in the body temperature. Later, he described the use of tuberculin for the diagnosis of

the patients with tuberculosis. In 1907, Clemens Freiherr, a well-known pediatrician reported that tuberculin could be used to diagnose latent TB (a term he introduced) in children. Charles Mantoux carried forward Clemens work and introduced the use of a cannulated needle and syringe to inject tuberculin intracutaneously in 1908. With the immense contribution of understanding the infection, diagnosis and treatment by the pioneer scientists, practitioners were able to contain the epidemic of tuberculosis [25,26]. Although the incidence of tuberculosis was declining, after the World War-1, the resurgence of the disease was seen. With the discovery of para amino salicylic acid (PAS) by Jorgen Lehmann in 1943 followed by thiosemicarbazone by Gerhard Domagk, the first therapeutic agents for the treatment of TB was popularized. Soon after in 1944, Albert Schatz, Elizabeth Bugie, and Selman Waksman demonstrated the isolation of the first antibiotic and bactericidal agent effective against *M. tuberculosis* and named it streptomycin [21,27].

1.2 The Genus *Mycobacterium*

The genus *Mycobacterium* belonging to the family Mycobacteriaceae, order Actinomycetales comprises of over 190 species of pathogenic and non-pathogenic bacteria [28]. The genus shares a circular genome rich in G+C content (about 65.6%) and consists about 4,411,529bp that codes for around 4006 proteins and 50 stable RNAs. Out of the total genome, 52% of the genes have been annotated while 48% of it is still unknown [29, 30]. The species belonging to the *Mycobacterium* are obligate, non-sporulating, non-motile (except *Mycobacterium marinum*), rod shaped (0.2-0.6 µm wide and between 1-10 µm long) and aerobic. The phylogenetic tree of *Mycobacterium* can be divided into fast growing mycobacteria, slow growing mycobacteria and unculturable mycobacteria [31]. *Mycobacterium sp*. possess the characteristics of both Gram positive and Gram negative bacteria and exhibit the characteristic acid fast staining (Ziehl–Neelsen acid stain stain) due to the presence of a lipid rich thick cell wall [32]. The presence of the thick lipid layer provide the colonies the waxy appearance and the cells have a tendency to cluster together. Based on the recently reported phylogenetic analysis of the mycobacterial core genomes, *Mycobacteria* can be classified into several groups. The fast growing *Mycobacteria* includes mostly the non-pathogenic strains such as *M. smegmatis, M. chelonae, M. fortuitum, M. aurum, M. agri, M.chitae, M. aichiense, M. phlei and M. thermoresistible*. The colonies of these bacteria appear in less than 7days in selective

medium. The slow growing mycobacteria are mostly pathogenic and require several weeks to appear in the selective medium. This includes the *Mycobacterium tuberculosis* complex that comprises of *M. microti, M. leprae,* the causal organism of Hansen's disease or leprosy and *M. bovis*, *M. africanum*, M. *tuberculosis*, the causative agent of tuberculosis [33].

1.3 *Mycobacterium tuberculosis*

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB) in human is one of the most successful obligate pathogens which currently infects one-third of the world's population in its latent form and claimed about 1.3 million lives in 2016 making it among the top 10 leading cause of death from an infectious disease worldwide, ranking above the Human Immunodeficiency Virus (HIV) (Figure 1.1). About 10.4 million people of the total population were infected with TB in 2016 out of which, 90% were adults and 56% of these cases were reported from India, Indonesia, China, the Philippines and Pakistan. With the emerging drug resistant strains there was a report of 600,000 new cases of drug resistant TB in 2016. Out of these, almost 47% cases were in India, China and the Russian Federation [34].

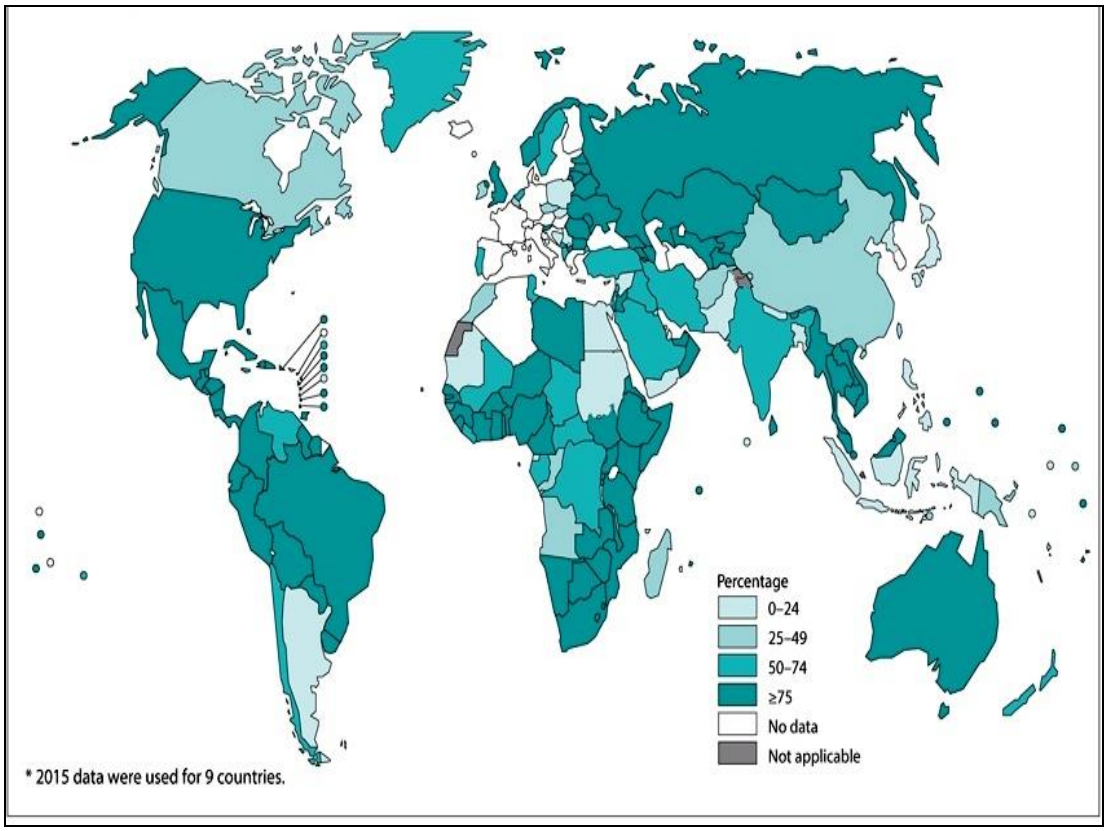

Figure 1.1: Percentage of new and relapse TB cases in 2016. (Courtesy: http://www.who.int/tb/publications/global_report/gtbr2016).

M. tuberculosis is an airborne pathogen and is spread by the tiny air droplets (droplet infection) containing the bacterium expelled by an infectious person. Consequently, the bacteria travel to the terminal bronchioles and alveoli where they are phagocytized by the alveolar macrophages [35]. The phagocytized bacilli fuse with the lysosome where the bacterium is capable of efficiently inhibiting the phagosome-lysosome fusion. The bacilli grow within the phagolysosome and are released after sometime. Eventually the inactivated macrophages continue to phagocytize the tubercle bacilli and in the due course of time the bacteria lyse the macrophages and get dispersed in the host tissue [36]. In immunocompetent individuals, the presence of the bacteria elicits robust immunological response resulting in activation of the Th1 signaling pathway involving INF-γ, Il-2, and Il-12. The activated macrophages are recruited at the periphery of the infection lesion. The lysed bacteria and cellular debris form a caseous granuloma and in this stage the disease is halted [37]. The bacteria stay in the body of the host but it does not show any characteristic symptoms of TB. This phase is known as the phase of latent TB infection. When the host immune system weakens or in the immunologically compromised individual, the bacteria continue to multiply and lyse the inadequately activated macrophages [38]. The toxic substances produced by the recruited T cells lyse the host tissue and gradually the granuloma increases in size. Eventually, cavity formation starts followed by the liquefaction of the granuloma. This phase is known as the phase of active TB [39].

Tuberculosis can be classified in two types based on the site of occurrence and appearance of the characteristic symptoms. The pulmonary TB refers to the bacterial infection in the lungs whereas infection by the bacteria in parts other than the lungs is known as extra-pulmonary TB, with the former being most prevalent [40]. Despite the recent advances made in the diagnosis and treatment of tuberculosis it still remains the major cause of mortality in the developing and underdeveloped countries fueled by poverty, ignorance and the HIV AIDS pandemic [41,42]. The symptoms of TB include cough, fever, night sweats, weight loss, *etc*., and may persist for many months [43]. On an average, an infected individual can transmit the disease up to 12-15 persons within a year and it is believed that one out of every 10 individual develops TB in their lifetime thus making TB one of the major causes for human mortality and morbidity [44].

1.4 Chemotherapy of TB and Vaccine

With the discovery of the causative agent of tuberculosis and the effective way for its diagnosis there was a transcendent change in the therapeutic cure of the ubiquitous chronic bacterial infection. Hermann Brehmer (Germany, 1826-1889) reiterated that an immune balance and maintenance of hygienic condition, such as an environment of fresh air, good food and rest may cure a patient with consumption. This concept of *Sanatoria* was popularized and initially the patients were observed to recover. However, long term results were not encouraging although the practice of sanatoria could restrict the transmission of the disease [45]. The localized collapse therapy was then introduced and administered to patients who did not get relief from extensive rest. The collapse therapy was given by pneumothorax or thoracoplasty where clean and filtered air was introduced in the lungs. However, the pneumothorax treatment was unsuccessful as it led to further tissue infection [46,47]. In 1976, Edward Jenner (England, 1749-1823) introduced the concept of vaccine [48]. Similar to Edward Jenner, Frenchmen Albert Calmette (1863-1933) and Camille Guerin (1872-1961), discovered an attenuated strain of *M. bovis* which was proved to have protective immunity in humans especially at a young age against TB [49]. Soon the WHO, UNICEF and the Red Cross started a massive campaign against TB based on the tuberculin test followed by BCG vaccination [50-51]. The discovery of the antibiotic, streptomycin from *Streptomyces griseus* by Selman Waksman (USA, 1888-1973) was a major breakthrough in the treatment of TB. The antibiotic had potential bacteriostatic ability with low toxicity for which he was awarded the Nobel Prize in Medicine in 1952 [21, 22]. But with the emergence of the drug resistant mutant strains, the success of Streptomycin in curing TB was short lived.

At present, after the discovery of the last antitubercular drug nearly about 60 years ago, the treatment of TB includes a multidrug regimen which is administered over a period of 6-9 months under directly observed therapy (DOT) with a cure rate of approximately >95%. The treatment includes two phases. In the first phase the patient is given a combination of four drugs (isoniazid, rifampicin, pyrazinamide and ethambutol) for 2 months. It is then followed by 4 months phase which includes treatment with isoniazid plus rifampicin (Table 1.2) [52,53].

Table 1.2: Chemotherapy for the treatment of Active TB

Time	Usual daily dose
Initial two months (Inz+ Rif+ Eth+Pyz)	
Isoniazid	300mg
Rifampin	600mg
Pyrazinamide	20mg/kg(max 2g)
Ethambutol	15mg/kg(max 2.5g)
Final 4 months (Inz +Rif)	
Isoniazid	300mg
Rifampin	600mg

However, several factors are responsible for the millions of deaths per year caused by the chronic infectious disease, TB. The long term chemotherapeutic treatment, high toxicity of the existing drugs and non-compliance of the patients have slowed down the progress in global TB control. The long duration of tuberculosis treatment and the non-conformity of the patients have thus led to the evolution of the multidrug resistant (MDR), extensively drug resistant (XDR) and total drug resistant (TDR) *M. tuberculosis* strains. Patients diagnosed with MDR TB (without a previous MDR infection) is administered for an intensive 8 months phase therapy which includes four second line drugs (Table 1.3). XDR TB requires a longer treatment period and the drug regimen includes the administration of the third line anti-TB drugs which are toxic and more expensive in comparison to the first line and second line anti-TB drugs. The management of TB control is aggravated by the occurrence of infection by HIV (Human immunodeficiency virus) along with pulmonary TB. In this context, continued research aimed at developing new effective anti-TB drugs with bactericidal mechanisms and less toxicity different from those of presently available agents is urgently needed. It has been almost more than 50 years after the discovery of Rifampicin; no new potent anti-TB drug has been discovered. Thus, there is a greater need for shorter, less toxic treatment regimens along with identification of potent drug targets.

Table 1.3: Categories of antituberculosis drugs [54]

Group 1: first-line oral anti-TB drugs	Group 2A: second line fluoroquinolones	Group 2B: second line injectable anti-TB drugs	Group 2C: Other core second line agents
Isoniazid (5 mg/kg daily dose)	Ofloxacin (15 mg/kg daily dose)	Streptomycin (15 mg/kg daily dose)	Ethionamide/ Prothionamide
Rifampicin (10 mg/kg daily dose)	Levofloxacin (15 mg/kg daily dose)	Kanamycin (15 mg/kg daily dose)	Cycloserine
Ethambutol (15–25 mg/kg daily dose)	Moxifloxacin (7·5–10 mg/kg daily dose)	Amikacin (15 mg/kg daily dose)	Linezolid
Pyrazinamide (30 mg/kg daily dose)		Capreomycin (15 mg/kg daily dose)	Clofaximine

1.5 Drug targets in *Mycobacterium tuberculosis*

The proliferation of the tubercle bacilli beyond a critical threshold can accelerate active TB in an infected individual and also enhance the spread of the bacteria. The bacterial enzymes, metabolites, regulatory protein and RNA play crucial roles in the proliferation of the tubercle bacilli. These essential substrates and functions can serve as potent targets for the anti-TB drugs such that the bacilli get killed or their proliferation gets arrested. Hence, the identification of potential and novel drug targets is crucial for the development of new antimycobacterial drugs.

M. tuberculosis can remain in an inactive state where the metabolic activity of the bacterium is shut down or it can progress in the host body progressively. For the complete inhibition of growth and eradication of the bacilli, the TB drug regimen should target these biological processes in the bacterium which help the bacteria to survive and proliferate in the host. Of these, the cell wall biosynthesis pathway, cellular respiration, protein processing, *etc*., have been characterized as possible drug targets. Also the drugs should target the essential pathways which are indispensible for the bacteria to persist and tolerate the harmful effects of the anti-TB drugs in the dormant stage such as the isocitrate lyase. The loss of this enzyme activity abolishes the bacteria's ability to persist [55]. Similarly, the proteasomal complex of the bacteria appears as a potent target as inhibition of the proteasomal complex increases

M. tuberculosis susceptibility to the reactive nitrogen intermediates secreted by the macrophages upon *M. tuberculosis* infection [56]. Although several chemotherapeutic agents have been developed targeting several essential genes in the pathogenic *Mycobacterium sp.*, mutations in the target genes have resulted in emergence of the resistant strains thus contributing to the ineffectiveness of the present drugs against the resistant strains (Table 1.4).

L, D-transpeptidase involved in the 3, 3-peptide linkage of the peptidoglycan appears to be a potent target. The loss of the activity of this enzyme attenuates the growth, virulence and persistence ability of the bacteria [57]. With the progress in identifying the molecular mechanisms of dormancy of bacteria, the genes regulating this distinctive feature appeared to be potent target for developing drugs against bacteria in dormancy. The expression of the DosR transcription factor increases with the entry of the bacteria into hypoxic condition. Although the DosR expression is not essential for the bacterial survival but it is the master regulator of dormancy and hence can be an attractive target [58].

Table 1.4: Mechanism of action and resistance of anti-TB drugs [59]

Anti-TB Drug	Gene	Mechanism of action	Mechanism of resistance
Isoniazid	*katG, inhA, ahpC*	Inhibits the synthesis of mycolic acid	gene mutations in *katG* and *inhA*
Rifampicin	*rpoB*	Binds to the β-subunit of the RNA polymerase, inhibiting the elongation of mRNA.	Mutation in the *rpoB* gene
Ethambutol	*embB*	Inhibits the synthesis of arabinogalactan of the cell wall	Mutation in position embB306 of the *embB* gene
Pyrazinamide	*pncA*	Disrupts the bacterial membrane	Mutation in the gene *pncA*
Streptomycin	*rpsL*	Inhibits initiation of protein translation	Substitution from lysine to arginine in codon 43 of *rpsL*

Flouroquinolones	*gyrA/gyrB*	Inhibits DNA gyrase and topoisomeraseIV	Chromosomal mutation in *gyrA* and *gyrB*
Kanamycin, Capreomycin, Amikacin, Viomycin	*Rrs, eis, tlyA*	Inhibits protein synthesis	Mutations in the corresponding genes
Ethionamide	*inhA*	Interferes in the synthesis of mycolic acid	Mutations in *etaA*/*ethA*, *ethR* and *inhA*,
p-amino salicylic acid	*thyA*	Inhibits synthesis of folate	Mutation in *folC*
Cycloserine	*cycA*	Inhibits D-alanine racemase	Point mutation in *cycA*

1.6 The Mycobacterial cell wall as a target

The mycobacterial bacillus has a very unique cell envelope that insulates the bacteria from the extracellular environment and protects the bacteria from the harsh environment of the host immune system. The cell envelope consists of a regular inner peptidoglycan membrane to which arabinogalactan is attached which is thereby esterified with mycolic acids (MAs) forming the outer mycomembrane [60]. This remarkable cell wall is essential for the mycobacterial survival and also helps in maintaining the basal structure of the bacterium (Figure 1.2).

The peptidoglycan layer of *Mycobacterium sp.* contains muramic acid residues which are composed of a mixture of N-acetyl and N-glycolyl derivatives. The presence of the glycolyl containing residues helps in the formation of potential hydrogen bond thus providing extra strength to the mesh like structure of the peptidoglycan and protecting the bacteria from the lysis action of lysozyme [61-64]. The cross linking of 3-4 di-aminopimalic acid (DAP) and D alanine is comparatively more in *Mycobacterium* compared to other prokaryotes and also there is an extra 3-3 linkage between two DAPs [65,66]. In *Mycobacteria* the muramic acids are used as attachment sites for the galactan group of the arabinogalactan whereby the 6-OH of muramic acid form phosphodiester linkages with the α-l-rhamnopyranose–(1→3)-α-

d-GlcNAc (1→P) linker unit of arabinogalactan [67]. Several enzymes play vital roles in the assembly of the peptidoglycan. Of these the Alr and Ddl proteins that provide l-alanine its racemase activity and d-alanine:d-alanine ligase activity, respectively appear to be attractive targets for anti-TB drugs. It has been reported that the mechanism of action of a second line drug p-cyclserine is by inhibition of the Ddl protein [68, 69]. In actively replicating mycobacteria the peptidoglycan layer consists of the 3→4 crosslinking but as soon as the bacteria enter into a dormant phase, the peptidoglycan reverts to the nonclassical 3→3 linkages. There are at least five nonclassical l,d-transpeptidases (Ldt), which are responsible for generating 3→3 linkages in the peptidoglycan [70].

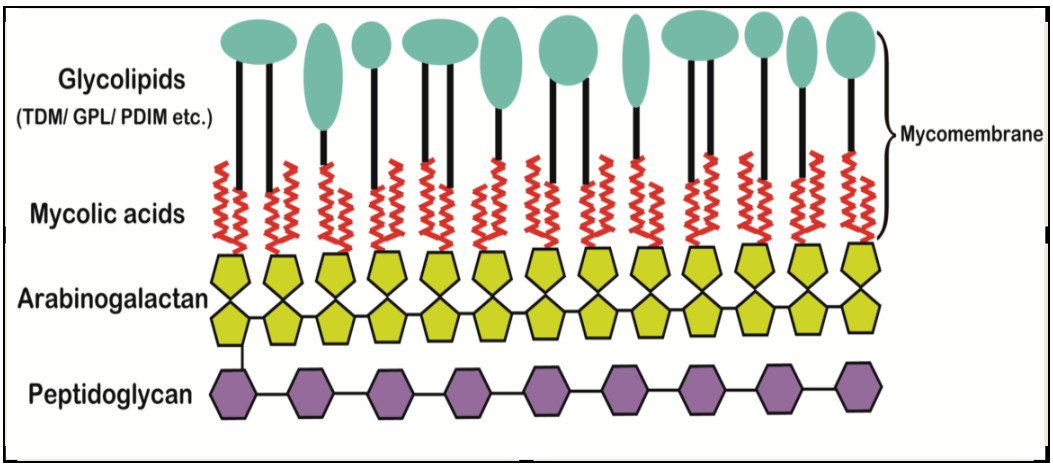

Figure 1.2: Cell wall structure of *Mycobacterium sp.* [71].

To the muramic acid residues of the peptidoglycan layer another layer of a heteropolysaccharide, Arabinogalactan (AG) is attached by phosphodiester bonds. The peptidoglycan and the AG layer form a macro polymer between the cytoplasmic membrane of the bacterium and the outer mycolic acid layer. The AG is composed of arabinose and galactose and both the sugars are present in their rare form, d-galactofuranosyl (Galf) and d-arabinofuranosyl (Araf) respectively [72-74]. The biosynthetic pathway of formation of the AG which involves the linker unit that joins the AG to the peptidoglycan layer and the presence of Rhamnose sugar which is absent in human make it a potential target for effective drug discovery [75].

The remarkable architecture, membrane fluidity and impermeability of *Mycobacterium sp.* cell wall is attributed to the unique MAs [76, 77]. The MAs act as a potent barrier to the obnoxious compounds that the host immune system presents to Mycobacteria and also to the antibiotics [60, 78]. Since this outer mycomembrane layer is the major defense mechanism of the bacteria to survive in the hostile

environment, the chemistry and biosynthesis of mycolic acids are of greater importance. MAs are the hallmark of the *Mycobacterium sp*. and display a wide variety of chain lengths and chemical functions. It is composed of 2-alkyl, 3-hydroxy long-chain fatty acids [R-CH(OH)-CH(R')COOH] synthesized by condensation of two preformed fatty acids; one constitutes the α unit, or branched portion [CH(R)COOH], and the other becomes the β-unit, or straight portion [R-CH(OH)] [79-82]. Mostly three different classes of MAs are present in the *Mycobacterium sp*. They differ primarily from one another by the nature of the chemical groups at the so-called 'proximal' and 'distal' positions of their main meromycolic chain. The most apolar MAs known as 'α' MAs contain 74–80 carbon atoms and generally two double bonds (of *cis*- or *trans*-configuration) or two *cis*-cyclopropyl groups located in the meromycolic chain. The second type of mycolate is less apolar and are known as 'α′' MAs having 60- 64 carbon atoms with one cis double bond and the third one is the methoxy or the keto group with a methyl group on the vicinal carbon atom [81,83,84]. Each of the different MA subclass plays a significant role in the virulence of the tuberculous bacilli *M. tuberculosis*. The oxygenated α MAs present in *M. tuberculosis* is required for the successful infection in the host [85]. Similarly, the cyclopropanation of the α MAs and the *cis-trans* configuration of the cyclopropyl ring influences the virulence of *M. tuberculosis* [86, 87]. With the emergence of multidrug-resistant and total drug resistant mycobacterial strains, the metabolic pathway of MA synthesis represents potential targets for the development of anti-tuberculosis drugs.

1.7 Protein secretion system

The *Mycobacterium sp.* is an environmental saprophyte. Few *Mycobacterium sp. viz., M. tuberculosis* and *M. leprae* shifted from their original environment and established themselves as obligate pathogens. Evolution is a dynamic process and it allows an organism to establish itself and adapt to the changing environment [88]. *M. tuberculosis* might have also defended itself from the environmental toxins which in the due course of time provided resistance to the host immune cells and the antibiotics. According to the Darwinian theory, the virulence of an organism is a random event caused because of genetic variations such as deletion, horizontal gene transfer, polymorphism, *etc.* [88]. The study of the differences between the pathogenic and the non-pathogenic species of Mycobacteria and the orthologous

genes present in these species may reveal the virulence mechanisms of the pathogenic strains which they deploy to establish pathogenicity in the host and can be used as potential drug targets. Moreover, the comparative study would enable to understand why certain hosts show complete resistances to this obligate pathogen. On analysis of the bacterial genomes for pathogenicity islands, it was found that few genes showed similarity with the genes known to cause virulence [89]. These included four copies of an operon for invasins, the iron sequestration system, several phospholipases C and other lipases, esterases, cutinases and proteases that might act on cellular or vesicular components responsible for virulence. The genome of *M. tuberculosis* has undergone extensive horizontal gene transfer which resulted in the decrease of the genome size and evolved as a specialized human pathogen without retaining the original environmental niche [90].

Mycobacteria have a unique cell-envelope structure which insulates the bacteria from the extracellular environment. The cell wall plays a critical role in protecting the bacteria from the host immune system. However, this extremely hydrophobic and thick barrier also poses a unique problem for the export of bacterial products. *Mycobacteria* secrete virulence proteins across this thick barrier that can facilitate its colonization and persistence in the host [91]. The expression of the virulent proteins and their subsequent secretion by unique secretion system are the two most crucial functions of *Mycobacteria* in pathogenesis. Like any other prokaryotes, *Mycobacteria* secrete the unfolded proteins having the N-terminal signal sequence through a general secretory pathway (GSP) known as the Sec pathway. The Sec system comprises of several membrane components such as SecD, SecE, SecF, SecG and, SecY which recognizes the signal sequence of secreted proteins. *Mycobacterium* also possesses a non-essential SecA homologue known as the SecA2 secretion system [92]. A SecA2 *M. smegmatis* mutant was found to be growth defective whereas mutation of the same secretion system in *M. tuberculosis* resulted in impaired growth and viability in mice [93,94]. The SecA2 requirement during infection reflects the fact that the substrates of the Sec pathway might be involved in the virulence of the bacteria. *Mycobacteria* secrete the folded protein through a Sec independent secretion system known as the Tat secretory pathway. In addition to the N-terminal signal sequence, the substrates secreted by the Tat pathway consist of a double arginine motif followed by two uncharged residue [95]. The mycobacterial Tat

system is homologous to other bacterial Tat system and is functional in both *M. tuberculosis* and *M. smegmatis* [96,97].

In addition to the Sec and Tat secretion system, *Mycobacterium sp.* exchange materials across its thick lipid-rich highly impermeable membrane essential for its survival through a unique secretory apparatus, known as the Type VII secretion system (T7SS), conserved among all *Mycobacterium sp*. [98,99]. Several studies revealed that two virulence proteins that play a pivotal role in pathogenesis, *viz*., early secreted antigenic target 6 (ESAT6) and culture filtrate 10 (CFP10) are secreted by a unique secretion system present in *Mycobacteria* known as early antigen 6 target (ESAT6) or ESX or Type VII secretion system [100-103]. Deletion in the regions encompassing the ESX1 locus also known as Region of difference 1 (RD1) region have led to the development of the attenuated strains of BCG [104]. The genome of *M. tuberculosis* has five copies of gene cluster known as the ESX gene cluster region namely, ESX-1 (Rv3866-Rv3883c locus), ESX-2 (Rv3884c-Rv3895c locus), ESX-3 (Rv0282- Rv0292 locus), ESX-4 (Rv3444c-Rv3450c locus) and ESX-5 (Rv1782-Rv1798 locus) (Figure 1.3) [99].

In silico analysis led to the suggestion that the genes surrounding esxBA, the operon which encodes for CFP-10 and ESAT-6, may be important for secretion of these proteins [99, 106,107]. This was proved to be true when individual genes at the RD1 locus were identified to define virulence genes of *M. tuberculosis* [102,103,108,109]. Disruption of individual genes (Rv3870, Rv3871 and Rv3877) within this locus prevented secretion of ESAT-6 and CFP-10, providing the first genetic evidence that this region encodes for a secretion system [102, 103]. Rv3870, Rv3871 or Rv3877 mutant strains are phenotypically similar to RD1 deletion strains and are also attenuated for growth in macrophages and also elicit an altered host immune response [102]. The ESX-1 locus is conserved in several pathogenic and non-pathogenic *Mycobacterium* species. Rv3868, Rv3878 and Rv3879 were shown to be required for the secretion of ESAT-6/CFP-10 in *M. marinum* [110]. In *M. smegmatis*, the homologues of Rv3866, Rv3869, Rv3882c and MycP1 were shown to be required for ESAT-6/CFP-10 export [111]. Thus there are many genes, identified in a number of distinct mycobacterial species, which are required for the export of ESAT-6 and CFP-10 virulent proteins. These findings suggest that the ESX-1 system is very complex and requires multiple protein complexes for its proper functioning (Figure

1.4). It was shown that ESAT-6 and CFP-10 interact to form a tight dimer, and these two proteins are interdependent on each other for stability [102,112].

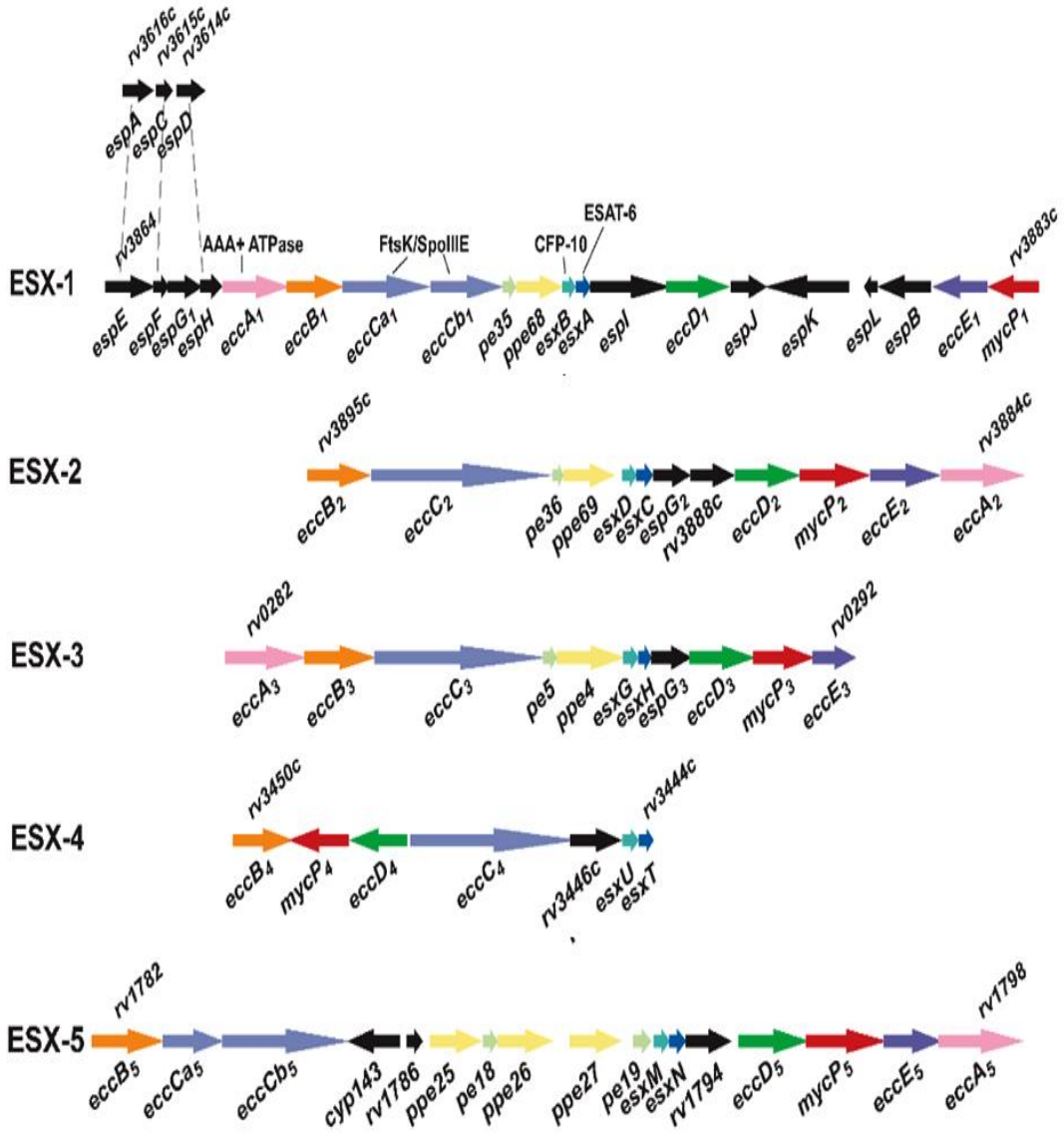

Figure 1.3: The five ESX T7SS loci present in *M. tuberculosis* [105]

Yeast two-hybrid experiments revealed that Rv3870 interacts with Rv3871, a cytosolic protein, and function as an AAA ATPase of the SpoIIIE/FtsK family. Rv3871 also interacts with CFP-10, and was hypothesized to escort CFP-10 and ESAT-6 to Rv3870 and Rv3877, a multi trans membrane protein, which may make up the pore that spans the cytosolic membrane [113-117]. Therefore, it is likely that Rv3871 functions to recognize the CFP-10/ESAT-6 substrate pair, and deliver it in an ATP-dependent manner to Rv3870 which is at the membrane.

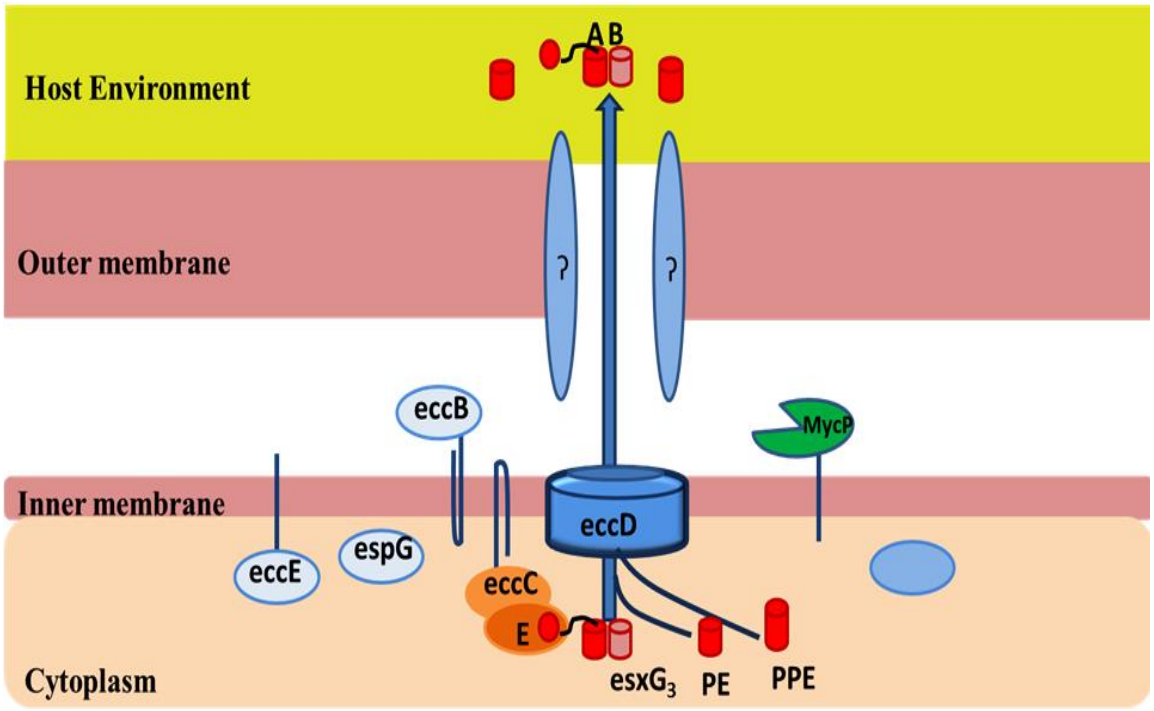

Figure 1.4: A model for ESX-1 mediated secretion in *Mycobacteria* [118].

Clearly, this system is a major determinant of Mycobacterial pathogenesis. Many groups have suggested that this system functions to modulate early events during *M. tuberculosis* infection. ESX-1 mutants are attenuated for growth during the first few days of infection of mice and cultured macrophages [119,102,103]. ESX-1 is responsible for the elicitation of the cytokine interferon beta and the resulting induction of a set of interferon responsive genes by wild-type *M. tuberculosis* [120]. Hsu *et al*., 2003 suggested that ESAT-6 functions as a toxin to directly lyse cellular membranes [108]. This is consistent with studies demonstrating that the RD1 region is required for tissue necrosis in lungs of infected mice [121].

The role of ESX-1 in pathogenesis must take into account the fact that this pathway is also present in non-pathogenic Gram-positive and mycobacterial species. One possibility is that the pathway plays a fundamental role in both pathogenic and non-pathogenic organisms, for example, in cell-to-cell communication. In support of this, Flint *et al*., 2004 reported that *M. smegmatis* ESX-1 mutants display increased conjugation efficiency compared with wild-type cells, and they present indirect evidence that ESAT-6/CFP-10 secretion in trans suppresses this hyperconjugation phenotype [122]. Alternatively, the ESX-1 pathway may be modular, allowing substrates to evolve for the particular needs of each organism [111]. It is therefore necessary to study the ESX-1 system in both pathogenic and non-pathogenic bacteria

to further elucidate the roles this system may play in addition to promoting bacterial virulence. In addition to ESX1, four of the paralogous CFP-10/ESAT-6 pairs are embedded within loci with synteny to the ESX-1 locus (ESX-2-ESX-5) [30,99]. The functions for ESX-2 and ESX-4 remain unknown, but it is believed that ESX-4 represents the most ancestral T7SS in *Mycobacteria* [99].

It has been observed that *M. tuberculosis* fails to grow in the absence of iron and has therefore developed a specialized mechanism for acquiring iron from their environment [123]. *Mycobacterium* regulates the amount of intracellular iron by sensing iron concentrations, and consequently controlling its uptake and storage. These organisms must also control the levels of intracellular iron within the cells, because excess iron may be toxic, due to its participation in reactions that generate toxic oxygen radicals from normal aerobic metabolic products [124-128]. ESX-3 has been shown to be essential for *in vitro* growth of *M. tuberculosis* and in particular, associated with iron acquisition [129]. It was determined that the ESX-3 gene cluster is regulated by iron in both *M. smegmatis* and *M. tuberculosis*, and by zinc in *M. tuberculosis* [130]. The ESX-3 gene cluster is present in all mycobacterial species and was found to be involved in growth through regulation of iron/zinc homeostasis [130,131]. ESX-3 assists in iron acquisition via the mycobactin pathway, as ESX-3 knockout in *M. smegmatis*, have been previously shown to be unable to use iron-bound mycobactins [129]. Since the ESX-3 gene cluster is transcriptionally controlled by IdeR and ZUR, ESX-3 might probably be involved in metal ion homeostasis of *Mycobacteria*. However, in *M. tuberculosis*, the ESX-3 gene cluster is thought to be regulated by both iron and zinc [132], but only by iron in, *M. smegmatis* which indicated an association of ESX-3 with zinc homeostasis also [132]. Using metabolomics approach the role of ESX-3 in cellular metabolism was investigated, by examining a *M. smegmatis* ESX-3 knockout strain in comparison to its isogenic wild type parent strain. In their study, it was found that the intracellular concentrations of several amino acids in particular, were seen to be significantly altered in the ESX-3 knockout strain comparatively [133]. Similar to the ESX1 secreted PE/PPE proteins; the ESX3 also secretes PE5/PPE4 protein and its secretion is dependent on the secretion of the ESX3 effectors ESXG and ESXH. The PE5/PPE4 proteins are essential for iron sequestration mediated by sidherophore [134].

ESX-5 secretion is the most recently evolved T7SS in *Mycobacteria* and it has been identified in MTB complex, *M. marinum*, and *M. ulcerans* [135, 136]. The ESX-

5 locus has recently been shown to operate in *M. marinum* and is the first report of a functional ESX locus besides ESX-1 [137]. ESX-5 is implicated to play an important role in virulence [98]. The duplication of ESX- 5 seems to correspond to the growth of the slow-growing species [138]. Additionally, ESX-5 also plays an important role in the secretion of PE and PPE proteins, as well as virulence [139]. PE and PPE proteins share a number of characteristics with ESAT-6 and CFP-10, in that they are secreted proteins that do not have a classical secretion signal [99] and form a tight 1:1 complex with one another [140].The RD1 encoded PPE protein Rv3873, interacts with CFP-10 and ESAT-6 [118], and this implies that Rv3873 may be secreted together with the ESAT-6-CFP-10 complex [98]. The disruption of ESX5 in *M. tuberculosis* causes loss of PPE protein secretion, change in cell wall integrity, strong attenuation and protection similar to BCG clearly indicating the impact of this system on cell wall homeostasis [139]. Interestingly, the *M. tuberculosis* ESX-5 mutant strains showed protective activity superior to that induced by BCG when administered as a vaccine in a mouse model of TB [141].

Various studies from the phylogenetic analysis and comparative genomic studies have revealed ESX4 cluster of the T7SS to be the ancient ESX system in the *Mycobacterium sp* [99]. The most primitive PE/PPE proteins found in the ESX1 secretion system was found to be absent in the ancestral ESX4 secretion system. It appeared that during the diversification of the ESX4 to the ESX1 secretion system the certain deletion occurred in the mycobacterial genome [138,142]. The evolution and expansion of the ESX gene clusters occurred as a result of several gene duplication events following the order ESX4, ESX1, ESX3, ESX2 and ESX5. Besides mycobacteria, T7SS is also reported to be present in other species such as the *Corynebacterium diphtheriae* and *Streptomyces coelicolor*. The homologues of T7SS were also found in several members of Gram positive bacteria such as *Staphylococcus aureus*, *Bacillus anthracis*, *Streptococcus agalactiae*, *Bacillus subtilis* and were reported to play as significant role in pathogenesis [143].

Out of the five T7SS three of the ESX loci (ESX1, ESX3 and, ESX5) are most important for virulence [99,144]. The five ESX clusters of the T7SS, in general, are a protein complex comprising of a cytosolic apparatus and an inner membrane which include a tandem pair of WXG proteins, an ATPase with an Ftsk–SpoIIIE motif and several proteins with predicted transmembrane domains [145]. Each of the five ESX cluster have different number of gene loci and all the loci contained a set of core set

of proteins which were named ESX-conserved components (Ecc) [105]. The five T7SS, in general, are a protein complex comprising of a cytosolic apparatus and an inner membrane. The core components of the T7SS are EccB, EccC, EccD, and EccE form the inner channel in the membrane [145]. The role of EccB and EccE in the secretory apparatus is not very clear while the EccC functions as a coupling component and provides energy in the transfer of its protein across the mycobacterial membrane [146] while EccD forms the central channel of the secretory apparatus in the mycobacterial membrane [102]. It has been reported that disruption of the core component, EccD5 of the ESX-5 loci abolishes the stability of the secretory apparatus and is thereby engaged in maintaining the cell wall integrity of the bacterium [139].

1.8 *Mycobacterium smegmatis,* a surrogate model for *M. tuberculosis*

Mycobacterium smegmatis is a non-pathogenic, fast growing soil dwelling rod shaped bacteria and belongs to the phylum Actinobacteria and genus *Mycobacterium* [147]. *M. smegmatis* has a G+C rich 7.0 Mb genome. Of these about 68.9% of the protein codding genes are similar to the pathogenic strain [148]. One of the most remarkable features of *M. tuberculosis* is its unique cell wall. The cell wall of *M. smegmatis* is also similar to the pathogenic strain and is composed of an inner peptidoglycan layer to which the arabinogalactan layer are esterified to the peptidoglycan layer by the linker units. This assembly is thereby surrounded by a lipid rich thick wall composed of mycolic acids. The manipulations of the essential genes involved in the important biosynthetic pathways in *M. tuberculosis* can be carried out conveniently in *M. smegmatis*. This allows the researchers to decipher the physical and functional roles of the essential genes which are difficult to study in *M. tuberculosis* [149]. The *M. smegmatis* genome encodes about 500 regulatory proteins in comparison to *M. tuberculosis* genome which encodes about 180 proteins. It has been found that the same transcriptional regulator controls the expression of the same set of gene both in *M. tuberculosis* and *M. smegmatis* however, the number of genes controlled by the transcription factor in *M. smegmatis* is more than that of *M. tuberculosis* [150]. The *M. smegmatis* is a fast-growing bacterium and have a generation time of only about 4-5 hours. In addition to the less generation time the bacteria requires a biosafety level 1for its culture. Because of the similarity of *M. tuberculosis* genome, less generation time, non-pathogenicity and basic infrastructure requirement, *M. smegmatis* is used as a surrogate model for *M. tuberculosis* study.

The TMC207 is a first-in-class diarylquinoline compound with a novel mechanism of infection that can inhibit both drug sensitive and drug resistant *M. tuberculosis* strain. It is presently in the Phase II of clinical trial. It was initially screened with *M. smegmatis*. There are several strains used in the study of tuberculosis research but the most widely used is *M. smegmatis* mc^2155. It was derived from *M. smegmatis* ATCC 607 [151]. The plasmid transformation efficiency is high in this strain and is therefore very advantageous in the analysis of mycobacterial gene function, expression and replication.

1.9 Objectives

In view of the above, the aim of this study was to gain an understanding of the evolutionary significance of the ESX3 T7SS which is conserved both in the pathogenic and non-pathogenic Mycobacterium sp. With this aim, the work embodied in this thesis proceeded with the following objectives:

1.9.1 Objective 1: Construction of an in-frame deletion of *eccD3* in *M. smegmatis* by efficient allelic exchange method.

1.9.2 Objective 2: Complementation of the mutant strain with the wild copy of the *eccD3* gene.

1.9.3 Objective 3: Characterization of the morphological and physiological role of *eccD3* gene.

1.9.4 Objective 4: Elucidation of the role of *eccD3* in *M. smegmatis* cell wall structure.

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