Characterization of the morphological and physiological role of eccD3 gene.

4.1 Introduction

Tuberculosis (TB) is an age old disease and it infects about one third of the world's population latently [1]. The devastating scenario of the deaths caused by TB is aggravated with the occurrence of AIDS caused by HIV infection. The annual deaths caused by TB in HIV-negative people were about 1.3 million and coincidence of TB and AIDS accounted for about 374 000 deaths [2]. Out of these about 60% of the people infected with TB were either not diagnosed or treated. Another major hurdle in achieving control over TB is the treatment procedure. The chemotherapy of TB includes a regimen of several first line drugs that has to be continued for several months. Even in the case of latent TB where the clinical symptoms of the disease are completely absent, the chemotherapy includes a drug regimen of isoniazid, rifapentine and rifampicin for a period of about 3-9 months [3]. Although the antituberculous drugs that have been used so far for the treatment of TB have helped the patients to recover to some extent, the side effects and the underlying toxicities of these drugs are undesirable. The toxicity of these drugs is so prominent that it is reported to affect the eight cranial nerve, resulting in vestibular (equilibrium) and cochlear (auditory) damage [4]. In addition to neurological damage, anti-TB drugs are considered to be the most common cause of hepatotoxicity worldwide ranging from appearance of elevation in the liver enzymes to unstable liver inadequacy [5]. The long term drug regimen and the associated toxicity of the present drugs result in noncompliance by the patients. This situation is additionally provoked with the emergence of the multidrug and extensively drug resistant strains. Thus new drugs targeting potential biosynthetic and metabolic pathways in the bacterium with a define mechanism of action with less toxicity can solve the problem of latent TB infection, coinfection of TB and AIDS and MDR and XDR tuberculosis.

A major quantum leap was achieved in the control and treatment of tuberculosis with the discovery of the attenuated strain of *M. bovis* in 1921which was used as the anti-TB vaccine [6]. The Bacille-Calmette-Guérin (BCG) vaccine is the only vaccine developed so far for the immunization against tuberculosis. Although the administration of BCG vaccines in children is effective in providing protection against TB infection, its success rate in the adults is not encouraging [7-9]. Moreover, the presence of the attenuated *M. bovis* in the BCG vaccines interferes in the diagnosis of latent tuberculosis. The genome of *M. bovis* and *M. tuberculosis* shares a

high percentage of similarity and hence, populations vaccinated with the BCG strain and infected with latent tuberculosis is difficult to differentiate with the tuberculin skin test.

With the advance in the genomic research, the discovery of the absence of the region of difference (RD1) from the BCG strain established a milestone in TB research [10]. This discovery led to the development of T-cell-based immunodiagnostics in which the release of Interferon-gamma (IFN-Ɣ) by the host immune cells in response to the RD1 encoded antigens ESAT6 and CFP10 is measured [11]. Later, it was found that the RD1 region is present in the ESX1 locus of the Type VII secretion system (T7SS) of *M. tuberculosis* [12]. *M. tuberculosis* harbors five different chromosomal ESX clusters of conserved gene that comprises the unique T7SS found only in the Mycobacteriaceae family [13]. The complementation of the wild type copy of the ESX1 loci in the BCG strain resulted in the secretion of the immune modulatory antigens ESAT6 and CFP10 thereby inducing specific host immune response [14-16]. The various studies on T7SS indicates that attenuated mycobacterial strains with variation in expression of these specialized T7SS can be used as a promising strategy in the development of anti TB vaccine and drugs [16-18].

In addition to the ESX1 cluster the T7SS consists of four ESX loci designated as ESX2, ESX3, ESX4 and, ESX5. Out of these five gene clusters usually two or all the five gene clusters are present in the mycobacterial species. But the ESX3 T7SS is conserved among all the species of *Mycobacterium* [13,19]. The presence of ESX3 in all the mycobacterial species thus indicates its essential and crucial role in *Mycobacterium sp*. Various studies have reported the involvement of the ESX3 in the regulated sequestration of iron and zinc in *M. tuberculosis* and *M. smegmatis*. The expression of the ESX3 locus is regulated by the different levels of iron and zinc present in the host during infection [20-22]. In case of persistent TB infection, the expression of the ESX3 in the lungs of the infected mice was observed to increase suggesting it to play a crucial role in pathogenesis [20]. Besides these reports, there is a paucity of the conserved role of the ESX3 secretion system in the pathogenic and the non-pathogenic strains.

In light of the earlier studies the present study relates to an investigation on the functional role of the ESX3 secretion apparatus, the *eccD3* gene in particular that

forms the central channel in the cell membrane in *M. smegmatis*. Till date no report of the function of ESX3 other than in the acquisition of iron has been divulged. The present study provides an essence that other than regulated uptake of iron, ESX3 is also engaged in maintaining the physiological aspects of the nonpathogenic *Mycobacterium sp.*

4.2 Materials and methods

4.2.1 Bacteria strain, plasmid, and growth conditions.

The wild-type *M. smegmatis* mc^2155 and the mutant bacteria were grown in Middlebrook 7H9 medium (MB-7H9, HiMedia) supplemented with 0.05% Tween80 and 0.2% glycerol or Middlebrook 7H10 (MB-7H10, HiMedia) plates supplemented with 0.5% glycerol. Luria-Bertani (LB, HiMedia) medium was used for the of *E. coli* strains. The antibiotics were added in the following concentrations, Ampicillin (100µg/ml for *E. coli*), Kanamycin (25µg/ml for *E. coli* and *M. smegmatis*) and Gentamicin (10µg/ml for *E. coli* and *M. smegmatis*) were used as appropriate. Growth conditions for *E. coli* were fixed at 37^0C for 12 hours, whereas, growth conditions for *M. smegmatis* was modified as per requirement. Chelated Sauton's medium (HiMedia) was used as an alternative minimal media for mycobacterial growth. It consisted of 60 mL of glycerol, 0.5 g of KH_2PO_4, 2.2 g of citric acid monohydrate, 4g of asparagine, and 0.5% Tween-80. After adjustment of the pH to 7.4, the medium was stirred 1–2 days at room temperature with 10g of Chelex100 resin (Sigma). The medium was filtered, and 1M of $MgSO_47H_2O$ was added as a sterile solution. M63 salts medium supplemented with 2% glucose, 0.5% Casamino Acids, 1mM $MgSO_4$, and 0.7mM $CaCl_2$ (biofilm medium) was used to assay for biofilm formation. The sliding-motility plates contained 0.3% ultrapure agarose (Sigma) as a solidifying agent in M63 salts supplemented with 0.2% glucose.

4.2.2 Bacterial growth curves

The strains were grown to mid-log phase and bacterial density was recorded at OD_{600}. Cells were diluted to an OD_{600} of 0.005 in fresh complete MB-7H9 media supplemented with 0.5% glycerol and 0.05% Tween80. Cultures were incubated at 37^0C with shaking throughout the entire growth phase. Samples from all the three strains, the wild type, mutant and the complemented strain were collected at the same time, and the OD_{600} values were measured starting from 0 hour to 120 hours at an

interval of every 3 hours after growth initiation. Experiments were performed in triplicates, and the mean values were used to generate growth curves.

4.2.3 Optical microscopy

In order to observe the colony morphology, the wild-type *M. smegmatis* (mc^2155) and the mutant strain (Ms0622) along with the complemented strain (Ms0622/pEccD3) were grown in MB-7H9 media (supplemented with 0.5% glycerol and 0.05% Tween80), at pH 6.8. 10µL of each strain was spotted onto MB-7H9 agar plates (supplemented with 0.5% glycerol and 0.05% Tween80). The plates were incubated at 37^0C for 2–3 days and observed under the compound light microscope (Olympus CX21I).

4.2.4 Sliding motility assay

Motility assays were carried out as described previously [23]. Briefly, cells were cultured in MB-7H9 broth to mid-logarithmic phase (OD_{600} = 0.8 to 1.0) and 2µl aliquots were spotted onto motility medium consisting of M63 salts supplemented with 0.5% Casamino Acids, 0.2% glycerol, 1mM MgCl2, and 10µM FeCl2, pH 6.5 solidified with 0.3% (wt/vol) agarose. The plates were incubated for 24 hours at 37^0C under humidified conditions.

4.2.5 Pellicle formation assay

Pellicle formation by different strains was monitored by growing the cultures of *M. smegmatis* without shaking at 37^0C for 48 hours in MB-7H9 medium devoid of Tween 80 [24].

4.2.6 Biofilm formation assay

An assay for biofilm formation was carried out as described previously with minor modifications [25]. The M63 medium was added to 24-well polyvinyl chloride (PVC) plates and inoculated with cells to an OD_{600} = 0.03. The plates were incubated at 37^0C for 72 hours. After biofilm formation, the medium above the surface of the biofilm was removed and the free-floating cells were washed with deionized water. 1ml of 1% Crystal Violet, CV (HiMedia) was added to the biofilm and incubated for 10mins. The CV was removed, and the biofilm was gently washed three times with phosphate-buffered saline, PBS (137 mM NaCl, 4.3 mM Na2HPO4, 1.4 mM KH2PO4, pH 7.0). CV was then extracted with 1ml of 95% ethanol. The absorption

of the extracted CV was measured at 570nm on a spectrophotometer (MultiskanGO, Thermo Fisher Scientific). For the study of biofilm formation in the *eccD3* deleted strain at different concentration of iron, chelated Sauton's minimal media was used (Siegrist et al., 2009)

4.2.7 Disk diffusion method

The differences in H_2O_2 sensitivities between wild-type, mutant and complemented *M. smegmatis* strains were qualitatively measured using the disk diffusion method. Mid-exponential phase cultures were used to prepare the lawns of cells as previously described. Whatman filter disks (5.5 mm-diameters) were placed on the bacterial lawn and 1% of H_2O_2 was spotted onto it. After incubating overnight at 37^0C, the diameter of the zone of complete inhibition was measured. The experiments were performed in triplicates.

4.2.8 Ethidium bromide (EtBr) and Nile Red uptake assay

The accumulation of EtBr and Nile Red was measured using the methods previously described [26]. All the three *M. smegmatis* strains were grown in MB-7H9 to an OD_{600} = 1.0, washed twice with PBS and resuspended in such a way that the OD of the cells was 0.8 corresponding to 1×10^8 cfu/ml. 200µl of the cell suspension was added in triplicate to a 96-well black fluoroplate and EtBr and Nile red was added to a final concentration of 2µM and 6µM, respectively for 15mins, 30mins and 60mins. The accumulation of these dyes was measured by fluorescence using a Tecan Infinite M200 spectrofluorometer (Eppendorf) with an excitation of 540 nm and emission of 630 nm for Nile Red and an excitation of 545 nm and emission of 600 nm for EtBr.

4.2.9 Heat shock and acid challenge

The wild-type and mutant strains were grown in 7H9 media till mid-exponential phase and washed with PBS (pH 7.0), and then diluted OD_{600} = 0.5. For heat shock, the bacteria were incubated at 52^0C and 37^0C for 20mins, then diluted serially in PBS and spotted onto MB-7H9 agar plates. For acid challenge studies, the bacteria were incubated in low pH medium (pH 3 and pH5) for 6 hours, then serially diluted in PBS and spotted onto 7H9 agar plates after 48 hours.

4.2.10 SDS sensitivity assay

SDS sensitivity assay was done in liquid MB-7H9 medium, in which the wild-

type, mutant and complemented strains were grown to early log phase before exposure to SDS. Cells were diluted to an OD_{600} of 0.5 and inoculated in fresh 7H9 medium containing 0.001%, and 0.01% SDS and incubated in a shaker incubator at 37^0C for 4hours. Aliquots were removed at 0h and after 4hours. Cells were pelleted and washed with PBS, serially diluted and plated in MB-7H11 plates and incubated at 37^0C. The number of CFU was recorded. The survival of each strain was calculated by dividing the CFU after 4hours of SDS exposure by the CFU at 0h and multiplying by 100. The results are mean ± SD of three independent experiments.

4.2.11 Spot tests

The sensitivity of the wild-type and the mutant strains were determined as previously described [26]. Briefly, cells were grown to an OD_{600} of 0.8 - 1.0 and plated on 7H9 media containing different antitubercular drug disks. The concentration of antibiotics used for spot tests: isoniazid (4µg/ml), tetracycline (50µg/ml), ethambutol, (50µg/ml), rifampicin (50µg/ml), ampicillin (100µg/ml).

4.2.12 Statistical analysis

Data from at least three biological replicates were used to calculate means and standard deviation (SD) for graphing purposes. The statistical analysis (ONE WAY ANOVA) and t- test was done using ORIGIN 8.5 software, and a p value, $P \leq 0.001$, 0.005, and 0.05 was considered significant.

4.3 Results

4.3.1 Colony morphology, Pellicle formation, and Sliding motility

To understand the physiological role of *eccD3*, the phenotypic changes associated with the knockout mutant strain of *M. smegmatis* genome were examined. The *M. smegmatis* strain with the *eccD3* gene deletion showed altered colony morphology. The mutant strain showed flat, smooth and moist colonies with an even colony surface in contrast to the wild-type with rough, wrinkled and dry colonies with uneven edges. The wild-type phenotype could not be restored in the complemented strain. The colony morphology of the complemented strain too was flat, moist but with wrinkled edges (Figure 4.1).

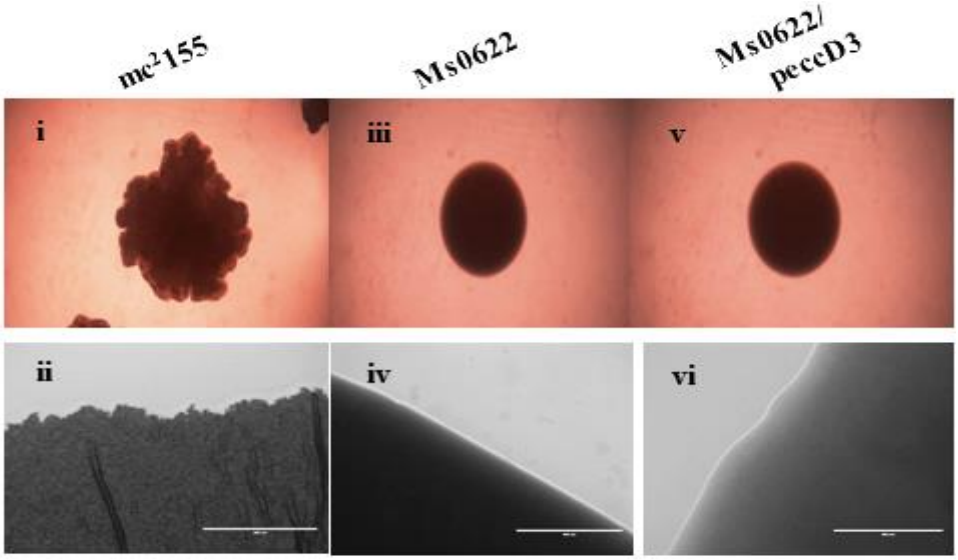

Figure 4.1 Effect of *eccD3* deletion on colony morphology of (i) mc^2155, wild type; (iii) Ms06622, *eccD3* deleted mutant and (v) Ms06622/pEccD3, *eccD3* complemented strain. The edge of all the three strains were observed under the compound microscope, (ii) mc^2155, wild type exhibited rough edges; (iv) *eccD3* deleted mutant, Ms0622 exhibited very smooth colony morphology with even edges and (vi) while in the complemented strain, Ms0622/pEccD3, the abnormal phenotype was not completely restored.

4.3.2 Pellicle formation

The wild-type and the mutant showed different macroscopic aspects in broth cultures both in shaken and static growth conditions. Under agitation and in the absence of Tween 80, the mutant did not form any aggregate or clumping whereas, in the wild-type, excessive cell clumping and aggregation had been observed. Since the *eccD3* mutant forms drastically different colonies on agar plates and aggregates less than that in the wild-type strain in liquid media, any defect in pellicle formation was also determined. Because of their high mycolic acid content, mycobacteria growing in a surfactant-free liquid medium culture (e.g., Tween 80) form a surface pellicle at the air-media interface in standing liquid media [27]. The wild-type formed thick and robust (1 cm height) pellicles that persisted for more than 10 days on LB media. On the other hand, cells from the mutant strain were observed to be defective in pellicle formation (Figure 4.2).

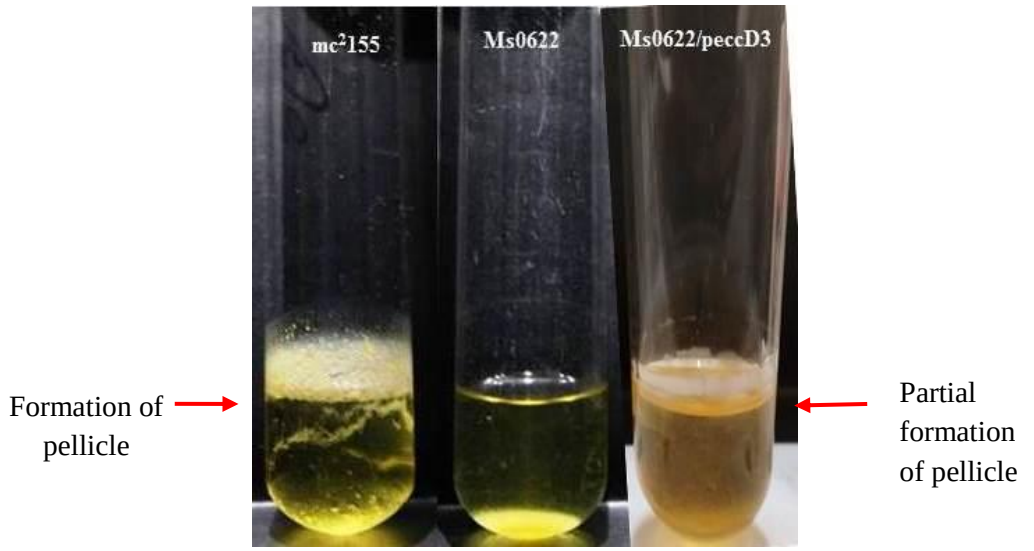

Figure 4.2 Effect of *eccD3* deletion on formation of Pellicle. Wild-type *M. smegmatis*, mc^{2}155 forms a thick pellicle at the air-liquid interface of the standing MB-7H9 culture. The *eccD3* mutant, Ms0622 is defective in pellicle formation, but pellicle formation is partially restored upon complementation with an intact *eccD3* gene in Ms0622/pEccD3.

4.3.3 Sliding motility

In addition to the absence of pellicle formation, the sliding motility of the mutant strain with the deleted copy of *eccD3* also showed an altered phenotype on a motility agar plate. The diameter of the sliding halo in the mutant showed a 4-fold reduction in size (Figure 4.3), indicating reduced sliding motility.

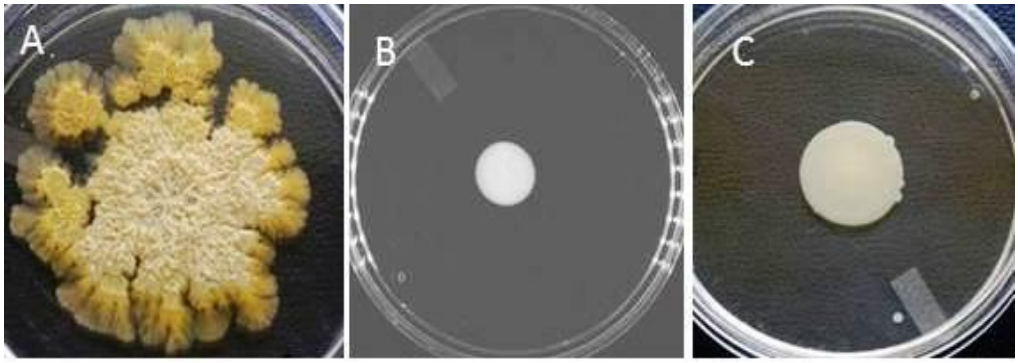

Figure 4.3 Macroscopic spreading analyses on the surface of a motility agar plate. (A) mc^{2}155, (B) Ms0622 and (C)Ms0622/pEccD3 was grown in M63 medium with 0.3% agar. The plates were sealed with parafilm and incubated at 37^{0}C for 2 weeks.

4.3.4 The growth of eccD3 gene deleted mutant of M. smegmatis.

In 2009, Siegrist et al. (2009) demonstrated that the deletion of the entire locus of ESX3 is responsible for the inability of *M. smegmatis* and *M. bovis* to survive in iron-limiting conditions [28]. In our study, we found that deletion of a single gene of the ESX3 locus, *eccD3*, encoding a transmembrane protein is responsible for growth

of the bacterium in MB-7H9 medium. The generation time was calculated from the growth curve. The growth curve was obtained by plotting a semilogarithmic curve with log of OD in the Y-axis vs. Time (in mins) in X-axis. The exactly doubled points OD1 and OD2 was chosen from the linear slope (linear increase) in the semilogarithmic plot which represents the Log-phase in the growth curve. The straight-line equation "y = 0.1125x-1.532" was used to calculate the corresponding T1 and T2 for the wild type (Figure 4.4 inset). The generation time was then calculated using the equation, Generation time (t_d) = 0.693/μ, where μ= 2.303 (log OD2-log OD1)/T2-T1 [29]. Similarly, the generation time was calculated for the *eccD3* deleted mutant, Ms0622 and the complemented strain, Ms0622/pEccD3. The generation time of the mutant was 10.2 hours as compared to 2.7 hours in the wild type whereas the complemented strain had a generation time of 4.6 hours (Figure 4.4). This suggested that the ESX3 associated *eccD3* locus of the T7SS played a crucial role in the normal growth of the bacterium. The experiment was done in triplicates and the values were significantly different (P<0.05).

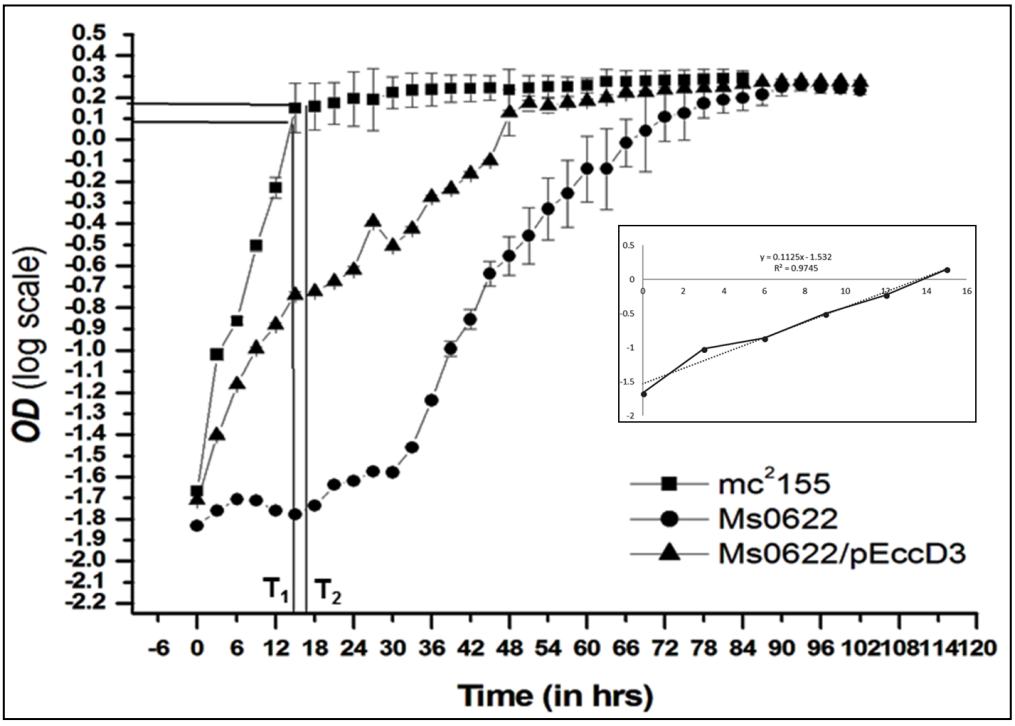

Figure 4.4: Effect of *eccD3* deletion on growth kinetics of the wild type *M. smegmatis* mc^2155 solid squares; *eccD3* mutant, Ms0622 solid circles and complemented strain Ms0622/pEccD3 solid triangles. The generation time was calculated from the equation t_d = 0.693/μ, where μ= 2.303 (log OD2-log OD1)/T2-T1. The experiments were performed 3 times in triplicate. Error bars represent the standard deviations from the replicates and the values are significantly different (P< 0.05).

4.3.5 Biofilm formation

A strong correlation exists between pellicle formation and the ability of a microorganism to form biofilm. As the mutant strain was deficient in pellicle formation, the amount of biofilm formation was quantified in the wild-type, mutant, and the complemented strain. To quantify the defect of biofilm formation in the *eccD3* deleted mutant, assays were performed on PVC plates using M63-based liquid medium, which was previously used to assay biofilm formation of *M. smegmatis* [24]. Quantification of biofilm formation on PVC plate was performed by extracting the biofilm-associated crystal violet dye with ethanol and measuring the optical density at 570nm. Biofilm formation was significantly reduced in the *eccD3* deleted mutant strain compared to that of the parental wild type ($P<0.001$). Although the phenotype could not be completely restored in the complemented strain its ability to form biofilm was comparatively more than the mutant strain ($P<0.05$) (Figure 4.5[A]). Quantification by crystal violet staining assay revealed that the mutant strain was unable to form the biofilm (Figure 4.5[B]).

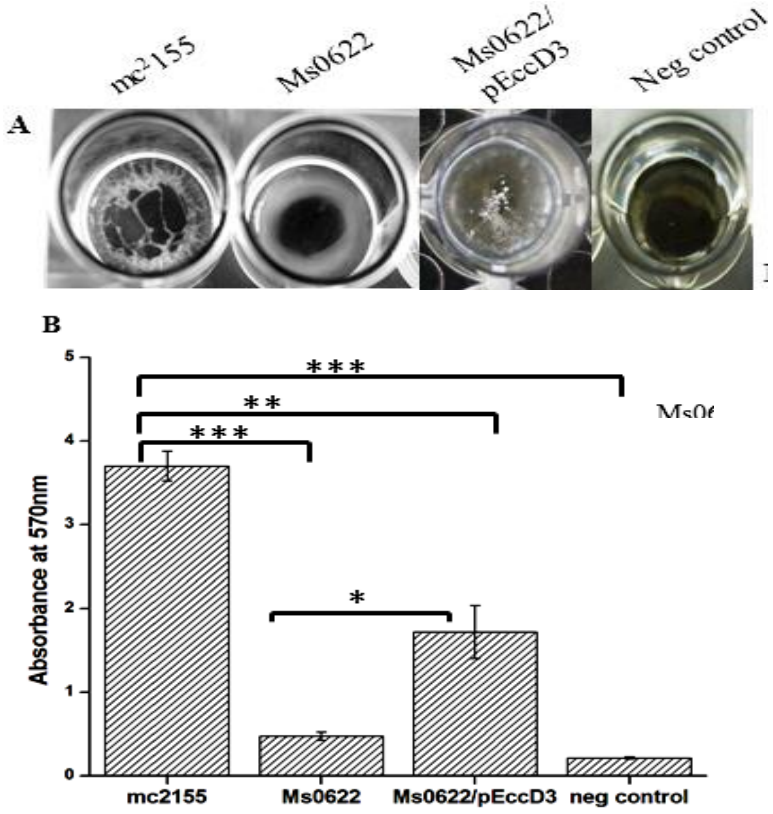

Figure 4.5: Effect of *eccD3* deletion on biofilm formation. [A] Growth of mycobacterial biofilm in biofilm media by mc^2155, wild type *M. smegmatis*; Ms0622, *eccD3* deleted mutant; Ms0622/pEccD3, *eccD3* complemented strain; neg control, negative control with no bacterial cells [B] crystal violet (CV) staining assay was used for assaying biofilm formation. Cells of mc^2155, Ms0622 and Ms0622/pEccD33 in biofilm media, M63 salts were grown on polyvinyl

chloride plates for 72 hours. Results are representative of at least three independent experiments and error bars represent the standard deviations from the triplicates (*, $P< 0.5$; **, $P< 0.005$; ***, $P< 0.001$).

Biofilm formation by the rapidly growing mycobacteria depends on the availability of nutrient level [30]. Studies by Ojha *et al.* 2007, revealed that mutation in the exochelin synthesis, important for iron acquisition and uptake pathways resulted in normal planktonic growth but, were significantly deficient in the formation of biofilm. Substantially, the phenotype was restored after the addition of 50µM iron [25]. In the context of the reported evidence, a strong correlation exists between biofilm formation and the sequestration of iron. In our study, biofilm formation in the wild type and in the mutant *M. smegmatis* strains was investigated under conditions with and without iron supplementation in the Chelated Sauton's minimal media. It was observed that in the absence of iron (0µM) and in presence of iron (5 µM), the parental strain, mc^2155 produced relatively same amount of biofilm after 72hours (Figure 4.6). Whereas the *eccD3* deleted *M. smegmatis* mutant exhibited about 14-fold less biofilm formation in comparison to the wild type even in the presence of increasing concentration of iron ($P<0.001$) (Figure 4.7 [A], Figure 4.7 [B]). This indicated that the sequestration of iron is not the sufficient condition for the formation of biofilm in *M. smegmatis*. In addition to factors, such as nutrients, ions, carbon sources and glycopeptidolipids that influence the formation of mycobacterial biofilm [31], the *eccD3* gene might also play significant role in biofilm formation.

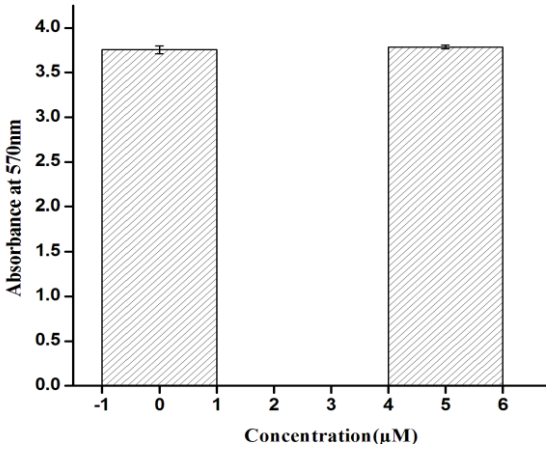

Figure 4.6: Biofilm formation assayed using the crystal violet (CV) staining assay in the wild type *M. smegmatis*, mc^2155 in the presence of 0µM and 5µM iron in the Biofilm growth media.

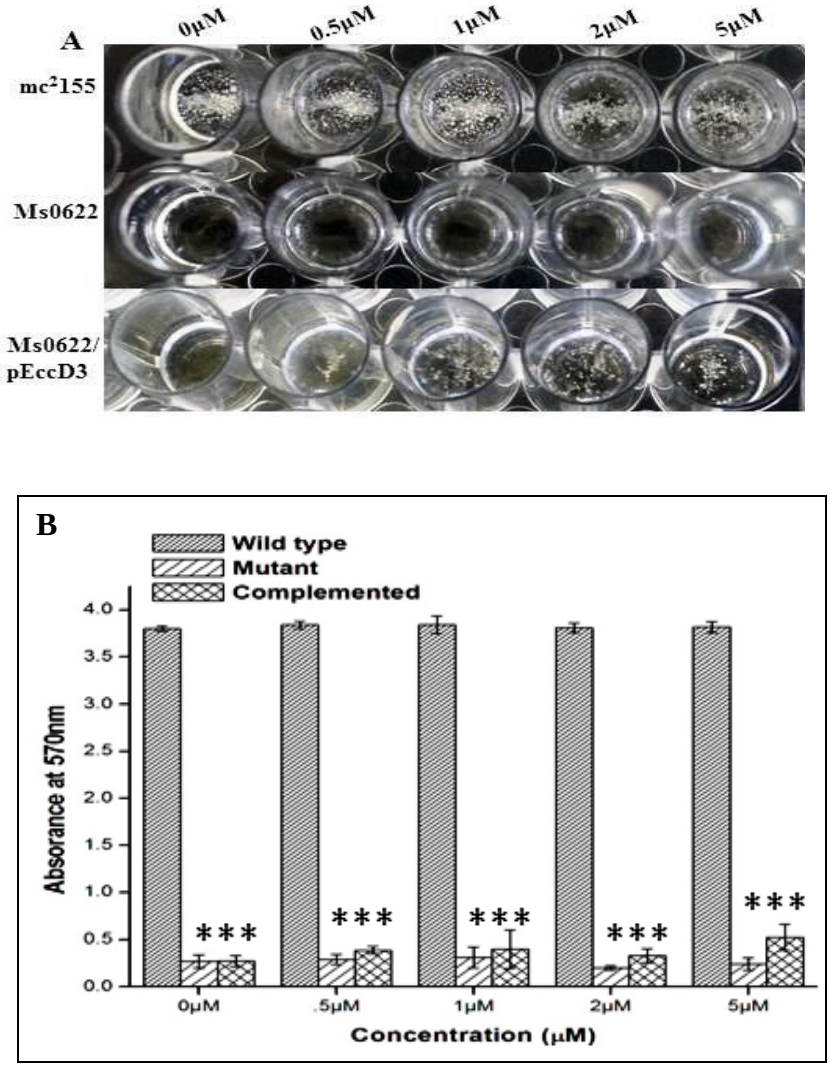

Figure 4.7 Growth of mycobacterial biofilm in presence of increasing concentration (0, 0.5, 1, 2 and 5 μM) of iron. [A] Growth of mycobacterial biofilm by the parental strain, mc^{2}155, *eccD3* deleted strain, Ms0622 and the complemented strain, Ms0622/pEccD3 in Chelated Sauton's minimal media. [B] Biofilm formation assayed using the crystal violet (CV) staining assay. Results are representative of at least three independent experiments and error bars represent the standard deviations from the triplicates (***, P< 0.001).

4.3.7 The deletion of eccD3 in M. smegmatis leads to increased sensitivity to H_2O_2 stress

Voskuil *et al.* 2007 did an exhaustive study on profiling the expression of some genes involved in the iron acquisition of mycobacteria in response to oxidative and nitrosative stresses [32]. ESX 3 has been reported to play an important role in the oxidative stress reponse pathway of *M. smegmatis* [33]. In our present study, the mutant exhibited defective biofilm growth in the presence of iron. It was therefore hypothesized that the mutant strain might be more susceptible to H_2O_2 stress. The *eccD3* deleted strain was more sensitive to oxidative stress via hydrogen peroxide

treatment relative to the wild type (P< 0.005), as the zone of inhibition was significantly larger for the mutant as compared to that in the wild type (Figure 4.8 [A, B]). Significantly the H_2O_2 stress defect in the mutant was partly reversed by genetic complementation of the *eccD3* gene in the mutant strain.

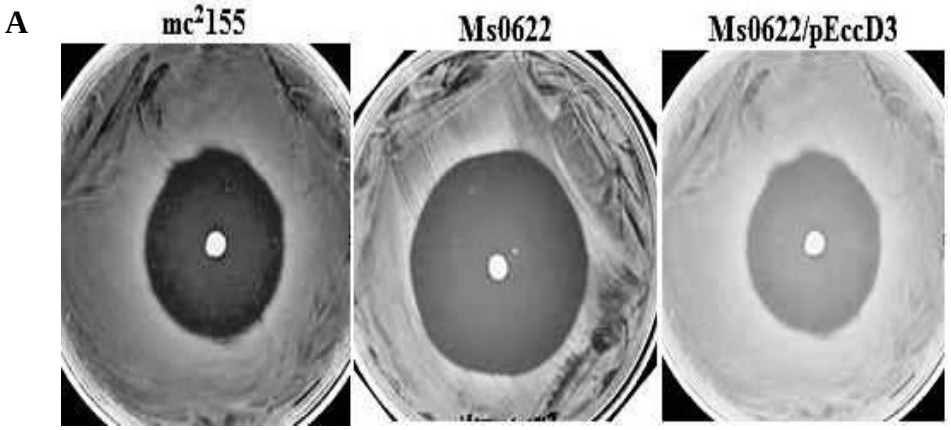

Figure 4.8: (A) Increased sensitivity of the *eccD3* mutant to H_2O_2. Disc diffusion assay was performed using discs containing 1% H_2O_2.

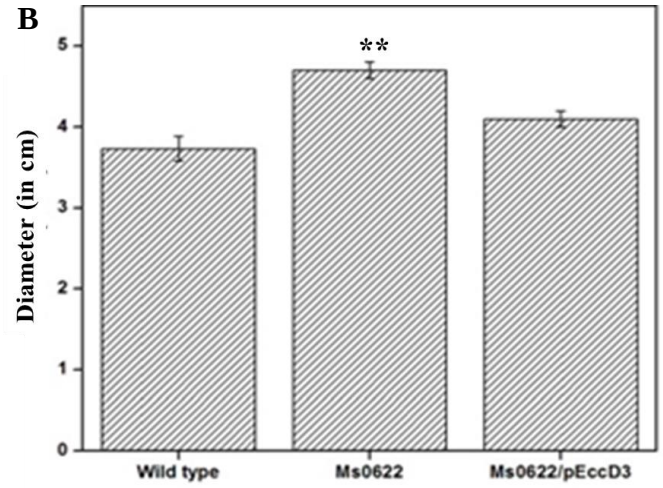

Figure 4.8: (B) Increased sensitivity of the *eccD3* mutant to H_2O_2. The diameter of zone of complete inhibition was measured. The data reported represent the means (n = 3) ± SD. (**, *P*< 0.005)

4.3.8 Sensitivity to pH and heat shock

Exponentially growing wild-type and the mutant cells OD_{600} = 0.8 were incubated at 37^0C and 52^0C and also at low pH condition, pH3 and pH5 for 20 minutes. The cells were pelleted and washed in PBS buffer. Subsequently, cells were serially diluted from 10^{-1} to 10^{-7} and spotted on 7H9 agar plates. It was observed that the mutant cells grew normally as the wild-type cells at 37^0C, however, the mutant

cells were unable to grow at 52^0C as compared to the wild-type. Their ability to survive the acidic pH environment was also measured. It was observed that the mutant could survive the lower acidic stress, pH5 but were unable to grow after the exposure at a much lower pH *i.e.* at pH3 (Figure 4.9 [A,B]).

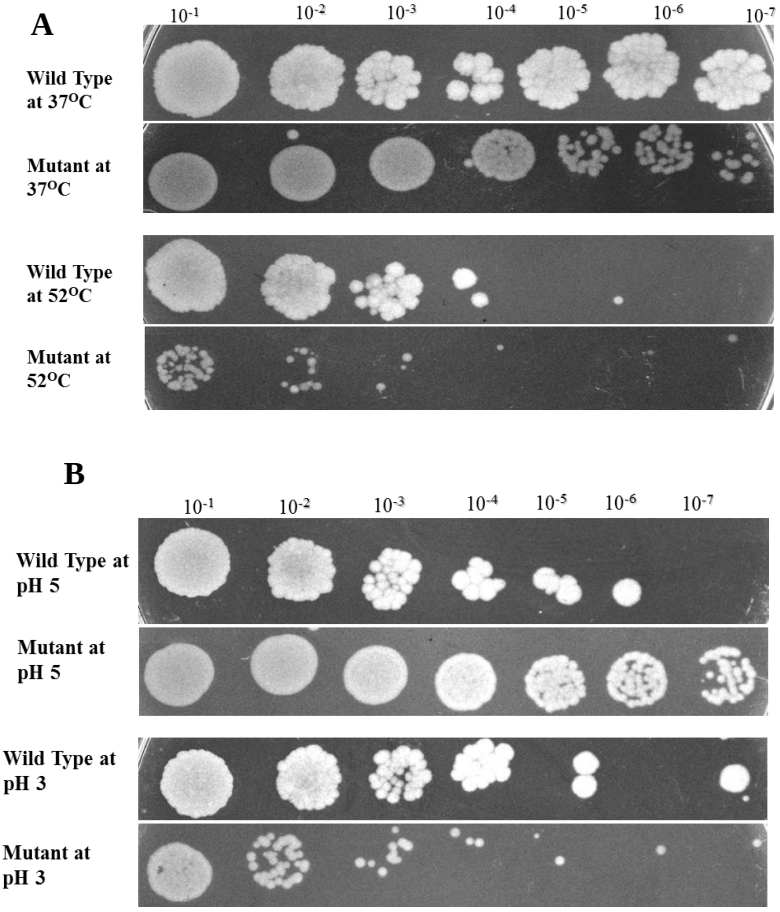

Figure 4.9 Survival of mc^{2}155 and Ms0622 after treatment with (A) heat shock after treatment at 52 ^{0}C and 37 ^{0}C for 20mins and (B) low pH- pH3 and pH5 for 20mins.

4.3.9 Increased sensitivity of the eccD3 deleted strain to the cationic detergent SDS

The wild-type, the *eccD3* deleted strain and the complemented strains were treated with different concentration of the cationic detergent SDS, 0%, 0.001% and 0.01% for 4hours. In the absence of the detergent, both the parental and the mutant strain resulted in comparable colony forming units. However, at 0.001% and 0.01%, there was a drastic decrease in the number of colony forming units by the mutant strain (Figure 4.10). Complementation of the *eccD3* mutant showed partial restoration of the SDS susceptibility phenotype. This result suggested that the cationic detergent was toxic to the mutant cells.

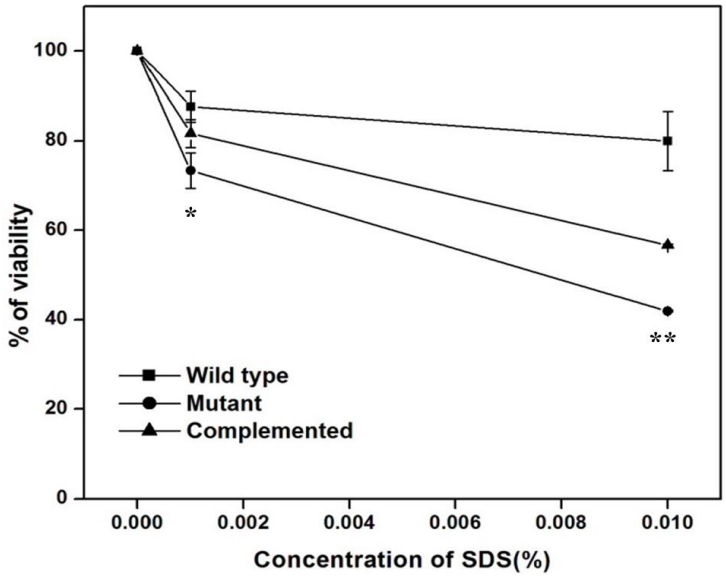

Figure 4.10: Effect of SDS treatment on bacterial survival. Symbols: closed square, mc^2155; closed circle, Ms0622; and closed triangle Ms0622/pEccD3. Representative data are shown for n=3, error bars represent the standard deviations from the replicates (*, P< 0.5; **, P< 0.005).

4.3.10 The cell permeability of the eccD3 deleted strain is altered

The cell wall integrity of the *eccD3* deleted mutant was examined by fluorescence spectroscopy to measure the whole-cell accumulations of ethidium bromide (EtBr) and Nile red, as a representative of hydrophilic and hydrophobic compounds respectively. The results showed that EtBr and the Nile red accumulated less in the mutant strain than the wild-type strain (P<0.001), indicating a decrease in cell wall permeability (Figure 4.11 [A], [B]). Complementation of mutant strain with a wild-type copy of the *eccD3* gene partly rescued this phenotype (P<0.5), as evidenced by the increase in the accumulation of EtBr and the Nile red in the complemented strain.

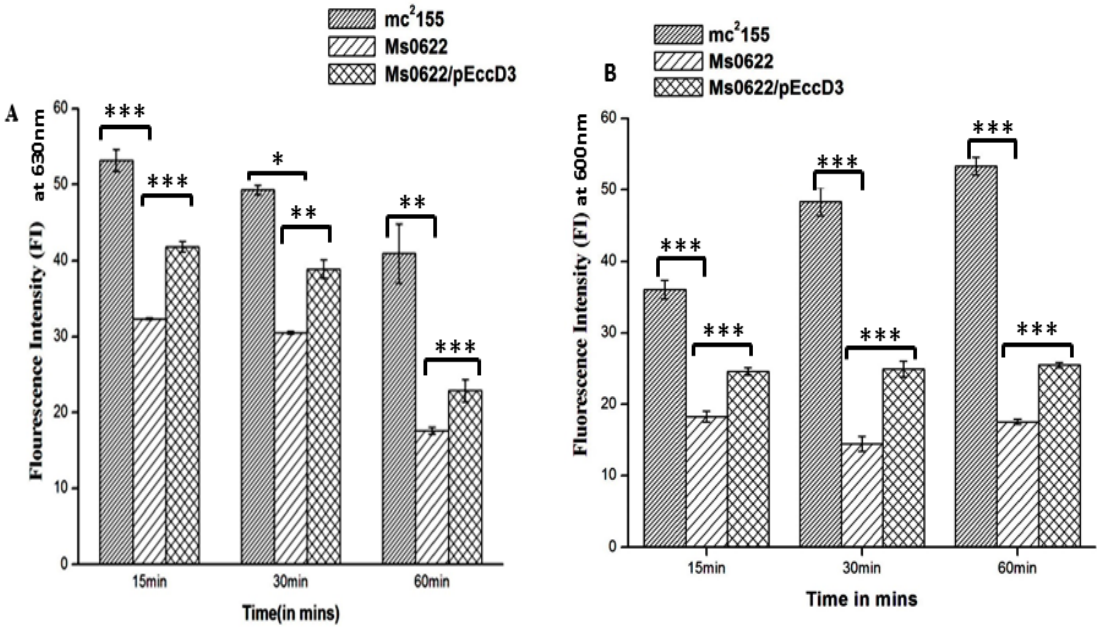

Figure 4.11: Deletion of *eccD3* in *M. smegmatis* results in decreased cell wall permeability. Mid-log-phase cultures of wild type, mutant and complemented strains (OD_{600} = 0.5) were incubated in PBS with (A) 2μM ethidium bromide and (B) 6μM Nile red stain. The experiment was performed at least three times in triplicate. Error bars indicate the standard deviation of the proportions (*, P< 0.5; **, P< 0.005; ***, P< 0.001).

4.3.11 Susceptibility of M. smegmatis eccD3 mutant to ß- lactam antibiotic and anti-tubercular drugs

The *eccD3* deleted mutant strain was tested by disk diffusion to determine its susceptibility to various antibiotics. The wild-type *M. smegmatis* strain was not susceptible but the *eccD3* mutant showed significant increased susceptibility (P<0.001) to the β-lactam antibiotic, ampicillin. The mutant strain showed complete resistance to the 1st line antitubercular drug, Isoniazid, Rifampicin, and Ethambutol compared to the wild-type (P<0.001). In addition, the susceptibility of the *eccD3* deleted strain towards the hydrophobic antitubercular drug, Rifampicin and tetracycline was comparable to the wild-type strain (P<0.001) (Figure 4.12).

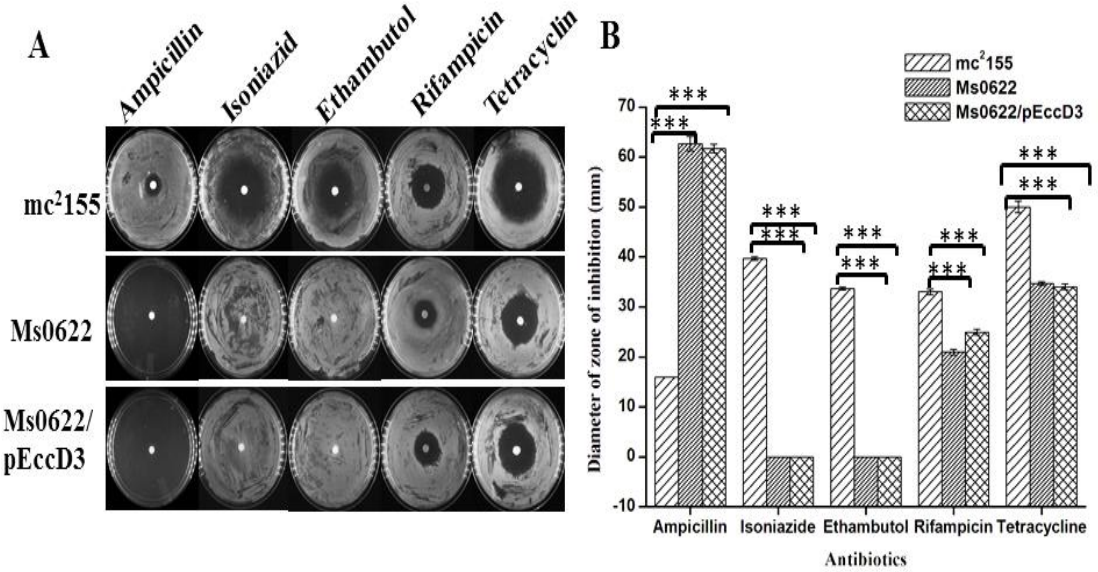

Figure 4.12: Antibiotic susceptibility of *eccD3* mutant by disk diffusion assays. Disk diffusion assay was done using antibiotics, Ampicillin (amp), tetracycline (tet), ethambutol (eth), rifampicin (rif), 100μgml^{-1}); and Isoniazid (inz), 4μgml^{-1}.

4.4 Discussion

Various works have been carried out to decipher the role of the paralogous ESX3 T7SS conserved entirely in all the mycobacterial species. ESX-3 reportedly plays a critical role in the regulated uptake of iron, which is crucial in vital biological processes, including electron transport, gene regulation, binding and transport of oxygen, and regulation of cell growth and differentiation [34, 35]. Previous studies demonstrated the essentiality of EccC3, EspG3, and EccD3, all core components of the ESX3 locus in mycobactin mediated iron acquisition and secretion of the EsxG and EsxH effectors [36]. Studies on the role of the core components, EccC3ms, EspG3ms, and EccD3ms of the ESX-3 secretion system are limited. There is also lack of direct evidence till date about the physiological and morphological role of EccD3, the transmembrane protein that forms the central channel of the ESX3 T7SS in *M. smegmatis*.

In the present study, an *eccD3 M. smegmatis* mutant has been studied. The study indicates that the *eccD3* mutant has altered colony morphology and is deficient in pellicle and biofilm formation in comparison to the wild-type. Colony morphology of *Mycobacterium sp.* is a complex phenotype which is influenced by a number of factors, involving the ability of the cells to interact with each other and maintaining the integrity of the cell wall. The unique cell envelope of mycobacteria comprises of arabinogalactan linked to the cell wall peptidoglycan and esterified with mycolic

acids that particularly contributes to the hydrophobicity of the cell wall and thus play a structural role, important for the integrity of the mycobacterial cell wall [37]. Several works have demonstrated the resulting appearance of colony morphology has a direct relation with the cell wall structure of the bacterium. The deletion of the mycolic acid methyltransferases (MAMTs) in *M. smegmatis*, responsible for the modification of mycolic acids resulted in small, smooth and doughnut shaped colonies [38]. Furthermore, *M. smegmatis* mutant deficient in Lsr2, incapable of synthesizing the α′ component of the mycolic acid resulted in the absence of pellicle and biofilm formation and appearance of a smooth colony morphology [24]. A similar smooth colony phenotype was observed in a *mbtE M. smegmatis* mutant which showed a striking change in the colony morphology that was substantiated with the change in cell wall permeability, evidenced from the increased uptake of a stain [24, 39].The appearance of the smooth colony morphology with an even edge and defect in the formation of pellicle might be attributed to the change in the cell wall integrity, which is highly regulated by the components that comprise the cell wall. The change in the colony morphology may be due to the alteration in the cell envelope of the mutant. The modification in the cell envelop may be due to a defect in transporting the trehalose meromycolates(TMM) synthesized in the cytosol of the bacterium to the outer cell wall which finally forms a thick layer of mycolic acid. It might also likely be due to the defect in exporting a protein to the cell surface or due to a defect in exporting a biosynthetic enzyme important for lipid modification. The appearance of the smooth colony morphology with an even edge and defect in the formation of pellicle in the *eccD3* deleted mutant thus might be attributed to the change in the cell wall integrity, which is highly regulated by the components that comprise the cell wall.

It was observed in our study the *eccD3* deleted strain had growth defects. The survival and growth of *Mycobacterium* largely depends upon the unique cell wall that comprises a thick outer membrane. The consistency and thickness of the outer membrane directly determines the ability of the hydrophilic and hydrophobic compounds to traverse through the thick cell wall. Studies report that expression of the porin proteins in *M. bovis* through which the hydrophilic compounds can traverse could rescue the slow growth rate of the bacterium suggesting the fact that access of the bacterium to the extracellular nutrients limits its growth rate [40]. The

mycobacterial outer membrane is responsible for the low fluidity permeation barrier of the cell wall [41]. A study demonstrated severe growth defect in *M. tuberculosis* and *M. bovis* mutant that lacked a proper outer membrane [42].

In the present investigation, we found that deletion of the *eccD3* gene resulted in the complete absence of biofilm formation. *Mycobacterium sp*. is reported to form robust biofilms on hydrophobic surfaces [43]. Biofilm formation by mycobacteria is propitious in a number of ways- (a) for protecting themselves against external chemicals and antibiotics (b) ameliorate from cooperative nutritional capacities and (c) avoiding detachment from the surface by shear forces [44]. Mycobacterial species including *M. tuberculosis* require iron and mobile mycolate component for biofilm formation [45, 46]. Iron sequestration is one of the most important factors in *M. smegmatis* that play a crucial role in the formation of biofilm [25]. Iron sequestration is one of the most important factors in *M. smegmatis* that play a crucial role in the formation of biofilm [25]. *M. smegmatis* acquire iron by the two pathways- (i) the exochelin pathway and (ii) the mycobactin pathway [47, 48]. Siegrist et al. (2009), demonstrated that deletion of the ESX3 resulted in a defective mycobactin pathway essential for iron acquisition. A severe growth defect in the ESX3 mutants was observed in the absence of iron. But the growth defect was restored upon addition of high concentration of iron [22]. Later, the same group demonstrated the role of various components of the ESX3 in acquisition of iron by the mycobactin pathway [36]. In reference to previous reports, *M. smegmatis* with an intact exochelin pathway for iron sequestration should have formed normal biofilm [25] but in our study we found that deletion of the ESX3 associated *eccD3* locus resulted in loss of biofilm formation even in the presence of increasing concentration of iron. On the other hand the wild type strain, mc^2155 with a functioning exochelin and mycobactin pathway together with the felicitous mycomembrane could form biofilm even in the absence of iron. This result was suggestive of the fact that in addition to the iron sequestration pathways, additional factors such as the explicit cell wall played a critical role in the formation of biofilm. Several studies have reported biofilm formation deficiency in the GroEL1 *M. smegmatis* mutants having an altered mycolic acid profile [45]. *eccD3* gene in the ESX3 locus played a crucial role in maintaining the precise construction of the cell wall thereby playing an important role in biofilm formation thus serving the bacteria to adapt themselves to unfavorable conditions.

We show in this study that deletion of the *eccD3* gene from the ESX3 locus leads to changes in cell wall permeability manifested by decreased uptake of the hydrophilic and hydrophobic compounds. A distinctive feature of the *Mycobacterium sp*. is its intricate cell wall. This highly integrated cell wall acts as a highly efficient permeability barrier to harmful substances and also limits the accessibility of the drugs to their respective targets in the bacterium [41, 49]. It is because of the highly conducive permeability barrier, that the pathogenic Mycobacteria is able to survive inside the host phagosome instead of its exposure to the bactericidal host factors such as oxidative radicals and antimicrobial peptides [50]. Lack of a highly organized cell wall may account for the difference in permeability of the bacterium to various polar and non-polar compounds. In the present study decrease in the accumulation of both the dyes in the *eccD3* deleted strain was observed in comparison to the wild type. It was also found that the accumulation of EtBr decreased with the increase in time. This phenomenon can be attributed to the fluorescence decay behavior of ethidium bromide [51]. In a report it was demonstrated that the knockdown of the *ppx2* gene led to an altered cell wall permeability which accounted for decreased sensitivity towards the antibiotics [52]. In this study also, a decreased accumulation of the hydrophobic and hydrophilic compound, EtBr and Nile red respectively in the *eccD3* mutant cells clearly correlates with the reduced uptake of antibiotics thereby resulting in increased resistance to the drugs such as Rifampicin, Tetracyclin, Isoniazid and Ethambutol. It is assumed that at least two general diffusion pathways across the mycobacterial outer membrane exist: the hydrophobic (or lipid) pathway, which is characterized by the nature and the interactions of the membrane lipids; and the hydrophilic (or porin) pathway, whose properties are determined by water-filled channel proteins, the porins, which span the outer membrane. The deletion of porin proteins from *M. smegmatis* results in reduced outer membrane permeability, reduced growth rate and influx of hydrophilic compounds [40, 53]. Overexpression of *M. smegmatis* porin protein MspA in *M. tuberculosis* increased its susceptibility to the hydrophilic drugs, isoniazid, and ethambutol [54]. EccD3 is a transmembrane protein that traverses the outer cell wall through the inner membrane of the *Mycobacterium* might act as a porin protein in the cell wall which accounts for the resistance to the hydrophilic drug isoniazid and ethambutol due to its deletion [13, 55]. On the other hand permeability of the non-polar compound, Nile red is directly related to the susceptibility of the bacteria towards the lipophilic drug [26]. In our study, the

resistance acquired by the *eccD3* deleted strain to the lipophilic drug, Rifampicin and Tetracyclin directly correlates with the decrease in the uptake of Nile red.

It was interesting to observe the susceptibility of the *eccD3* deleted mutant to the ß- lactam antibiotic, ampicillin. The mutant strain showed an intensive increase in the susceptibility to ampicillin in comparison to the wild-type. Generally *Mycobacterium sp.* shows resistance to ß- lactam antibiotics and this feature is associated with the interplay of the following factors: (i) correct architecture of cell wall (ii) beta-lactamase production by the *Mycobacterium sp.*, and (iii) affinity of the penicillin-binding proteins (PBPs) for the drugs [56, 57]. The hypersensitivity of the *eccD3* deleted mutant towards ampicillin might be due to the defect in the cell wall which is apparent from the altered uptake of the polar and non-polar compounds and susceptibility towards various environmental stress conditions. In a study it was reported that mutations in the genes associated with cell envelope in *M. tuberculosis* resulted in hyper susceptibility of the mutant towards ß- lactam drugs [58]. The sensitivity of the mutant strain towards ampicillin in our study might also be attributed to the inability of the *eccD3* deleted strain to secrete the beta-lactamase enzyme or inadequacy of the PBPs to bind to the antibiotic. Studies showed that deletion of the ß-lactamase by allelic exchange method in *M. tuberculosis* and *M. smegmatis* resulted in the hyper susceptibility of these strains to the penicillin-type ß-lactam antibiotics [59].

It is suggested that the absence of the *eccD3* gene resulted in the limited capability of the mutant cells to survive in multiple stress condition such as at a lower pH, H_2O_2 stress and also heat shock and exposure to the cationic detergent, SDS. During infection by *M. tuberculosis*, the pathogen encounters a defensive environment presented by the host innate immunity like chemical, oxidative, and acidic stress [60]. H_2O_2 is a natural oxidant and induces oxidative stress [61]. Various studies have reported the induction of several genes in *M. smegmatis* on exposure to H_2O_2, including the genes involved in the synthesis of the cell wall fatty acids [62, 63]. The susceptibility of the *eccD3* deleted mutant to H_2O_2 treatment implicated its probable role in maintaining the cell wall architecture. Similarly, SDS is a cationic detergent and is known for disintegrating the bacterial cell wall. The mutants defective in cell wall fatty acid component affects the integrity of the cell wall that protects the bacterium from the environmental surface stresses such as SDS and those mutants are reported to be hyper susceptible to this cationic detergent [64, 65]. All

these results exhibited by the *eccD3* mutant demonstrated its specific role in maintaining the integrity of the cell wall in *M. smegmatis*.

In summary, our findings suggest the essential role of the EccD3, the transmembrane protein of the ESX3 in maintaining the normal physiology and morphology of the bacteria. While the ESX3 has an important role both *in vivo* and *in vitro*, the molecular mechanisms are yet to be deciphered. It is interesting to witness the effect of deletion of *eccD3* as the loss of the gene completely alters the unique morphology of the mycobacteria. It might be possible that in the absence of this transmembrane protein the intricate cell wall of the bacterium might have altered. It is also apprehensible that it is indispensable for the bacteria to adapt themselves to unfavorable conditions in the context of host infection which was evident from its inability to form biofilm and survive in the external stress conditions. It was observed that the wild-type phenotypes could not be completely restored in the complemented strain Ms0622/pEccD3. This could be attributed to the fact that the expression of the *eccD3* (Figure 3.5 in Chapter 3) in the complemented *M. smegmatis* was less than the wild-type mc^2155 *M. smegmatis*. In the present scenario of the emergence of multidrug-resistant (MDR) and total drug resistant (TDR) *Mycobacterium* strains, the identification of potent drug targets and development of new therapeutic drugs against these targets is the current need of the hour. The EccD3 trans membrane protein of the ESX3 might serve as a potent drug target such that the ß-lactam antibiotics can be used in conjugation with other drugs [66, 67]. This study provides information about the functional role of the ESX3 associated *eccD3* gene in maintaining the integrity of the cell wall in *M. smegmatis* thus participating in the normal physiology of the bacterium.

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