List of Publications

1. **Yutika Nath, Suvendra Kumar Ray, Alak Kumar Buragohain.** Essential role of the ESX-3 associated *eccD3* locus in maintaining the cell wall integrity of *Mycobacterium smegmatis*, *International Journal of Medical Microbiology,* 2018. https://doi.org/10.1016/j.ijmm.2018.06.010 (I.F. 3.29).

2. Ranjan Dutta Kalita, **Yutika Nath**, Martins E. Ochubiojo and Alak Kumar Buragohain. Extraction and Characterization of Microcrystalline Cellulose from fodder grass; Setaria glauca (L) P. Beauv, and its potential as a drug delivery vehicle for isoniazid, a first line antituberculosis drug, *Colloids and Surfaces B: Biointerfaces*, 108, 85-89, 2013 (I.F 3.99).

Manuscript under communication

1. **Yutika Nath**, Alak Kumar Buragohain, Deletion of ESX3 secretion system leads to saturation of the mycolic acid essential for *Mycobacterium tuberculosis* pathogenesis. (Communicated)

Presentations in Conference/ Seminars

1. **Yutika Nath***, Alak Kumar Buragohain and Suvendra Kumar Ray, Functional analysis of EccD3 protein of ESX-3 TVII Secretion System in *Mycobacterium smegmatis*, **International Symposium under Advance DNA Technology "Technological Intervention in Microbial Resource"** held at Tezpur University, India on 4th & 5th February, 2018.

2. **Yutika Nath,** Suvendra Kr Ray and Alak Kumar Buragohain, Understanding the Functional Role of Type VII Secretion System in *Mycobacterium smegmatis*, in the 57th Annual Conference and International Symposium of Association of Microbiologists of India, Microbes and Biosphere, Whats New Whats Next held at Gauhati University from Nov- 24-27, 2016, **(Best Poster Award under Medical and Veterinary Microbiology Section).**

3. **Yutika Nath,** Suvendra Kr Ray and Alak Kumar Buragohain "Understanding the Functional Role of Type VII Secretion System in

maintaining the cell wall integrity in Mycobacterium smegmatis", National Seminar on "Science and Technology for Human Welfare held on 27th February, 2017 at IASST, Guwahati, India.

International Journal of Medical Microbiology xxx (2018) xxx-xxx

Contents lists available at ScienceDirect

International Journal of Medical Microbiology

journal homepage: www.elsevier.com

Essential role of the ESX-3 associated *eccD3* locus in maintaining the cell wall integrity of *Mycobacterium smegmatis*

Yutika Nath[a], Suvendra Kumar Ray[a], Alak Kumar Buragohain[a, b, *]

[a]Department of Molecular Biology and Biotechnology, Tezpur University, Napaam, Tezpur, Assam, 784028, India
[b]Dibrugarh University, Dibrugarh, Assam, 786004, India

ARTICLE INFO

Keywords:
Tuberculosis
Cell wall
Permeability
ESX-3
Secretion system

ABSTRACT

Mycobacterial pathogens have evolved a unique secretory apparatus called the Type VII secretion system (T7SS) which comprises of five gene clusters designated as ESX1, ESX2, ESX3, ESX4, and ESX5. Of these the ESX3 T7SS plays an important role in the regulatory uptake of iron from the environment, thereby enabling the bacteria to establish successful infection in the host. However, ESX3 secretion system is conserved among all the mycobacterial species including the fast-growing nonpathogenic species *M. smegmatis*. Although the function of ESX3 T7SS is known to be absolutely critical for establishing infection by *M. tuberculosis*, its conserved nature in all the pathogenic and nonpathogenic mycobacterial species intrigues to explore the additional functional roles in *Mycobacterium* species through which potent targets for drugs can be identified and developed. In the present study, we investigated the possible role of EccD3, a transmembrane protein of the ESX3 T7SS in *M. smegmatis* by deleting the entire *eccD3* gene by efficient allelic exchange method. The preliminary investigations through the creation of knockout mutant of the *eccD3* gene indicate that this secretory apparatus has an important role in maintaining the cell wall integrity which was evident from the abnormal colony morphology, lack of biofilm formation and difference in cell wall permeability.

1. Introduction

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 claims about 1.4 million lives annually making it among the top 10 leading causes of mortality from an infectious disease worldwide, ranking above the human immunodeficiency virus (HIV) (http://www.who.int/tb/publications/global_report/gtbr2016).

Mycobacteria have a unique cell-envelope structure that insulates the bacteria from the extracellular environment and plays a critical role in protecting the bacteria from the host immune system. However, this extremely hydrophobic and thick barrier also possesses a unique problem in the exchange of metabolites between the bacterium and the environment during chronic intracellular infection in the host. Like other plant and animal pathogens, Mycobacterial pathogens have also evolved generalized and specialized secretion systems and pathways to acquire as well as transport molecules across the thick cell wall. In addition to the two specialized secretion systems: the Twin-arginine translocase (Tat) export system, and the accessory Sec A2 system, *Mycobacterium* genome encodes a unique secretory system called the Type VII secretion system (T7SS) responsible for transport of mycobacterial protein products (Champion and Cox, 2007; Ligon et al., 2012). The expression of the secretory proteins and their subsequent secretion by this unique secretion system are the two most crucial functions of pathogenic mycobacterial species in establishing pathogenesis in the host (Pym et al., 2002). *Mycobacterium sp*. has five different T7SS, designated as ESX1 to ESX5 however, the non-pathogenic strains harbors a lower number of this secretion system. The best characterized ESX-1 genes are responsible for the secretion of virulence factors, *viz*; the early secretory Ag target 6 (ESAT-6) protein and the Culture Filtrate Protein (CFP10) which play a crucial role in pathogenesis by *M. tuberculosis* (Champion et al., 2009), while in the non-pathogenic strain *M. smegmatis*, ESX1 is involved in conjugal transfer of DNA (Flint et al., 2004; Coros et al., 2008). 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 (Gey Van Pittius et al., 2001). According to the present understanding, although the putative role(s) of ESX2 and ESX4 are very limited yet the conservation of the

* Corresponding author: Department of Molecular Biology and Biotechnology, Tezpur University, Napaam, Tezpur, Assam, 784028, India.
Email addresses: yutikanath@gmail.com (Y. Nath); suven@tezu.ernet.in (S.K. Ray); alak@tezu.ernet.in (A.K. Buragohain)

https://doi.org/10.1016/j.ijmm.2018.06.010
Received 8 February 2018; Received in revised form 21 May 2018; Accepted 24 June 2018
Available online xxx
1438-4221/

ESX4 in all the mycobacterial species suggests its importance in maintaining the metabolism of the bacteria (Newton-Foot et al., 2016). ESX3, a paralogous system present in all Mycobacterial species has been reported to be essential for iron sequestration in *M. tuberculosis* and *M. bovis* while it is involved in the regulated uptake of iron and zinc in *M. smegmatis* (Serafini et al., 2009). Another well-characterized T7SS is the ESX-5 secretion system which is reported to be essential for growth and virulence in *M. tuberculosis* (Bottai et al., 2012). 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 significant roles in pathogenesis (Unnikrishnan et al., 2017).

The five T7SS in Mycobacteria is a protein complex of ~1.5 MD that shares a conserved inner membrane and a cytosolic apparatus. Out of the five T7SS, the ESX3 consists of 11 gene loci. The core channel is composed of the membrane proteins EccB3, EccC3, EccD3, and EccE3. The functions of EccB3 and EccE3 within the secretion apparatus are less clear. EccC3 is a member of the FtsK/SpoIIIE-like ATPase family and possesses an ATPase cytoplasmic domain that provides the energy to transport proteins across the Mycobacterial membrane(s) (Ramsdell et al., 2015; Rosenberg et al., 2015). EccD3 is predicted to contain an N-terminal cytoplasmic domain followed by 11 predicted transmembrane helices forming the central channel in the cytoplasmic membrane of the bacterium through which cargo proteins are secreted. This core channel complex is associated with the mycosin MycP3, a membrane protease that has been implicated in substrate processing. In the cytoplasm, two accessory proteins facilitate substrate secretion: EccA3, an ATPase from the AAA + family156, and EspG3, which bind to the substrate and presumably function as chaperones to guide the substrate to the secretion apparatus imperative for the establishment of pathogenesis in the host by the *Mycobacterium*, thus making the entire ESX secretion system an attractive target for antitubercular drug discovery.

In earlier studies, several workers demonstrated the indispensable role of ESX3 in the regulated acquisition of iron for normal growth of the *Mycobacterium*. *M. smegmatis* with the deleted copy of the ESX3 locus could synthesize mycobactin but were unable to use the bound iron which resulted in the poor growth of the Mycobacteria (Siegrist et al., 2009). In another study, the contribution of each of the components of the ESX3 system in the acquisition of iron bound mycobactin was demonstrated (Siegrist et al., 2014). Our 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 its role in the acquisition of iron has been found out. Using the efficient allelic exchange method, the complete copy of the *eccD3* gene was deleted from the *M. smegmatis* genome. Our present study provides an essence that other than regulated uptake of iron, ESX3 is also engaged in maintaining the physiological aspects such as the maintenance of colony morphology, motility, ability to form biofilm, permeability of the cell wall *etc.* of the nonpathogenic *Mycobacterium sp*.

2. Materials and methods

2.1. Bacterial 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 (100 μg/ml for *E. coli*), Kanamycin (25 μg/ml for *E. coli* and *M. smegmatis*) and Gentamicin (10 μg/ml for *E. coli* and *M. smegmatis*) were used as appropriate. Growth conditions for *E. coli* were fixed at 37 °C for 12 h, 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, 4 g 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 10 g of Chelex100 resin (Sigma). The medium was filtered, and 1 M of $MgSO_47H_2O$ was added as a sterile solution. M63 salts medium supplemented with 2% glucose, 0.5% Casamino Acids, 1 mM $MgSO_4$, and 0.7 mM $CaCl_2$ (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.

2.2. Mycobacterial mutant construction

Efficient allelic exchange method was used to generate the ESX3 *eccD3* deletion mutant. A DNA fragment of 975bp upstream of the *eccD3* gene was amplified by Polymerase Chain Reaction (PCR) using the primers listed in Supplementary Datasheet1. The amplified PCR product was digested with XbaI and EcoRI (Fermentus) and cloned into the cloning vector pGEM-7Zf(+). The pGEM-7Zf(+) vector having the fragment upstream of the *eccD3* gene was named pD3UP. A DNA fragment of 1kb downstream of the *eccD3* gene was amplified and digested with EcoRI and HindIII (Fermentus) and ligated into EcoRI-HindIII digested pD3UP. The resultant plasmid consisting of the upstream and the downstream fragment was named as pD3DN. The deletion of the *eccD3* gene was achieved by the replacement of the *eccD3* gene by the kanamycin resistance (*kan*^{r}) cassette. The vector pUC4K was digested with EcoRI to release a 1282 bp long kanamycin resistant (*kan*^{r}) gene insert. The insert was purified and ligated into the unique EcoRI site of pD3DN. The clones were confirmed by checking the gain of resistance to kanamycin (25 μg/ml) by the ampicillin resistant parent vector; along with restriction digestions and named pD3UPKanDN. The recombination cassette containing the upstream, *kan*^{r} and the downstream region was digested with XbaI and cloned into the suicide shuttle vector pPR27 and named pPRD3KO. The pPR27 vector is a shuttle vector having a *sacB* gene, a temperature sensitive origin of replication (*ts-OriM*), an *OriE*, a gentamycin resistance cassette on its vector backbone. The *sacB* gene provides the cells sensitivity towards sucrose medium; *ts-OriM* allows the cell harboring the plasmid to grow below 30 °C and the gentamycin resistance cassette renders resistance to gentamycin. The construct was then confirmed by sequencing using insert-specific primers (Supplementary Fig. 3 and Fig. 4).

2.3. Transformation of M. smegmatis

1–5 μg of DNA was added to electrocompetent *M. smegmatis* cells and incubated on ice for 5 min. It was then introduced into Bio-rad cuvettes and subjected to electroporation with Bio-rad electroporator at 1.5 kV/mm. The cells were then mixed with 5 ml liquid MB-7H9 containing 2% glucose and 0.05% Tween 80, for recovery and grown till 6 h at 30 °C. 1 ml of the culture was pelleted after 6 h and plated onto MB-7H9 agar plates with gentamycin (10 μg/ml) and 2% glucose. The plates were incubated at 30 °C for 5 days till colonies were observed. The colonies obtained after transformation were restreaked on a gentamycin (10 μg/ml) and 2% glucose containing MB-7H9 agar plate and grown at 30 °C to obtain patches. Colonies from one of the patches were inoculated into liquid MB7H9 medium containing kanamycin (25 μg/ml), 2% glucose and 0.05% Tween-80 and grown in a shaker in-

cubator at 30 °C for 36 h. This step was essential to increase the plasmid copy number in the cells and to allow for allelic exchange to take place. In order to screen for double recombinants, the cultures were plated onto MB-7H9 agar plates containing 2% glucose, 0.05% Tween – 80, 10% sucrose and kanamycin (25 μg/ml) at different dilutions and incubated at 39 °C. The colonies obtained on these plates were then again screened for gentamycin sensitivity by plating on MB7H9-agar plates with glucose, sucrose and gentamycin (10 μg/ml). Effectively, colonies exhibiting kanamycin resistance, sucrose resistance and gentamycin sensitivity should be the double recombinants and hence the *eccD3* deleted mutants. The putative clones were subjected to PCR for screening the clones with *eccD3* deletion using the primers listed in Supplementary Datasheet 3.

2.4. Mutant complementation

To complement the *eccD3* mutant, the wild-type *eccD3* gene was PCR amplified using the primers listed in Supplementary Datasheet4. The PCR product was cloned at the PvuII-HindIII sites in pMV261hyg, a derivative of an episomal plasmid pMV261 containing a Hygromycin (*hyg*^{r}) resistance cassette instead of the kanamycin resistance cassette. The resulting plasmid was electroporated into *eccD3* mutant strain, and transformants were selected on MB-7H10 agar containing hygromycin and kanamycin.

2.5. Isolation of RNA

RNA was isolated from the *M. smegmatis* strains as described (Rustad et al., 2009). Briefly, *M. smegmatis* cells were grown early to mid-log phase up to an OD_{600} of 0.1 to 0.2 at 37 °C. Cells were pelleted at 3000 rpm for 10 mins. The cells were resuspended in 1 ml Trizol (Sigma). The cells were then bead beaten for 3 s with intermittent cooling. The glass beads were separated by centrifugation at 12,000 rpm for 1 min. The homogenized samples were incubated at room temperature for 5 mins. 300 μl of chloroform: isoamyl alcohol (24:1) was added and the tubes were vigorously shaken for 15 s. The samples were then subjected to centrifugation at 12,000 rpm for 15 mins at room temperature. The aqueous phase was transferred to a fresh tube and 0.8 volumes of isopropanol were added. The samples were incubated at room temperature for 10 mins at 4 °C and centrifuged at 12,000 rpm for 10 mins at 4 °C. The supernatant was removed, and the RNA pellet was washed with75% ethanol. The pellet was air dried and resuspended in 50 μl of RNase free water. The isolated RNA was treated with DNAse (Thermo Scientific) to remove DNA contamination, followed by DNAse inactivation at 85 °C with 0.5 M EDTA. The RNA was further purified by half the volume (500 μl) of Trizol as mentioned previously. To check for the absence of DNA in the isolated RNA, PCR was done using the primers for the upstream gene of *eccD3*, MSMEG_0622. Absence of a band corresponding to ~ 1kb of the amplified product confirmed the absence of any contaminating DNA. The primers used are listed in Supplementary Datasheet6 in the supplementary data.

2.6. Preparation of cDNA

cDNA was prepared using the Verso cDNA synthesis kit (Thermo Scientific). Briefly equal amount of RNA was taken from mc^2155, Ms0622 and Ms0622/pEccD3 as a template. Random hexamers were used as the RNA primer. The cDNA was synthesized for 60 mins at 42 °C followed by the inactivation cycle for 2 mins at 95 °C.

2.7. Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was performed as described (Roy et al., 2004) using the primers listed in Supplementary Datasheet5. The absence of DNA contamination in the RNA samples was confirmed by PCR using the RNA samples as the template. For semiquantitative results, *eccD3* was amplified along with *rrsA* as the internal control using primers listed in Supplementary Datasheet5. PCR was carried for 31 cycles. The semi-quantitative RT-PCR bands were quantified on 1% agarose gel using Gel QuantNet software.

2.8. 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 of 0.005 in fresh complete MB-7H9 media supplemented with 0.5% glycerol and 0.05% Tween80. Cultures were incubated at 37 °C 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 hr to 120 hrs at an interval of every 3 h after growth initiation. Experiments were performed in triplicates, and the mean values were used to generate growth curves.

2.9. 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. 10 μl 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 °C for 2–3 days and observed under the compound light microscope (Olympus CX21I).

2.10. Sliding motility assay

Motility assays were carried out as described previously (Martínez et al., 1999). Briefly, cells were cultured in MB-7H9 broth to mid-logarithmic phase (OD_{600} = 0.8–1.0) and 2 μl aliquots were spotted onto motility medium consisting of M63 salts supplemented with 0.5% Casamino Acids, 0.2% glycerol, 1 mM $MgCl_2$, and 10 μM $FeCl_2$, pH 6.5 solidified with 0.3% (wt/vol) agarose. The plates were incubated for 24 h at 37 °C under humidified conditions.

2.11. Pellicle formation assay

Pellicle formation by different strains was monitored by growing the cultures of *M. smegmatis* without shaking at 37 °C for 48 h in MB-7H9 medium devoid of Tween 80 (Chen et al., 2006).

2.12. Biofilm formation assay

An assay for biofilm formation was carried out as described previously with minor modifications (Ojha and Hatfull, 2007). 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 °C for 72 h. After biofilm formation, the medium above the surface of the biofilm was removed and the free-floating cells were washed with deionized water. 1 ml of 1% Crystal Violet, CV (HiMedia) was added to the biofilm and incubated for 10 mins. The CV was removed, and the biofilm was gently washed three times with phosphate-

buffered saline, PBS (137 mM NaCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.0). CV was then extracted with 1 ml of 95% ethanol. The absorption of the extracted CV was measured at 570 nm 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 media was used (Siegrist et al., 2009).

2.13. Disk diffusion method

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

2.14. Ethidium bromide (EtBr) and Nile Red uptake assay

The accumulation of EtBr and Nile Red was measured using the methods previously described (Li et al., 2016). Both the wild-type and the mutant 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. 200 µl 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 2 µM and 6 µM respectively for 15 mins, 30 mins and 60 mins. 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.

2.15. 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 °C and 37 °C for 20 min., then serially diluted in PBS and spotted onto 7H9 agar plates. For acid challenge studies, the bacteria were incubated in low pH medium (pH 3 and pH5) for 6 h, then serially diluted in PBS and spotted onto MB-7H9 agar plates after 48 h.

2.16. 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 MB-7H9 medium containing 0.001% and 0.01% SDS and incubated in a shaker incubator at 37 °C for 4 h. Aliquots were removed at 0 hr and after 4 h. Cells were pelleted and washed with PBS, serially diluted and plated in MB-7H10 plates and incubated at 37 °C. The number of CFU was recorded. The survival of each strain was calculated by dividing the CFU after 4 h of SDS exposure by the CFU at 0 hr and multiplying by 100. The results are mean ± SD of three independent experiments.

2.17. Spot tests

The sensitivity of the wild-type and the mutant strains were determined as previously described (Li et al., 2016). Briefly, cells were grown to an OD_{600} of 0.8–1.0 and plated on MB-7H9 agar media containing different antitubercular drug disks. The concentrations of antibiotics used for spot tests were isoniazid (4 µg/ml), tetracycline (50 µg/ml), ethambutol (50 µg/ml), rifampicin (50 µg/ml), ampicillin (100 µg/ml).

2.18. 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.

3. Results

3.1. Construction of eccD3 mutant strains

The present work describes the construction of a suicide delivery vector for the replacement of *eccD3* with *aph* gene encoding for kanamycin resistance using pPR27 suicide vector thereby transforming the wild-type *M. smegmatis* to generate *eccD3* deletion mutant through homologous recombination. A fragment of DNA upstream and downstream of *eccD3* was cloned in the pGEM-7Zf(+) vector. Between the upstream and downstream fragment, an *aph* (*kan*^{r})gene coding for the kanamycin resistance was cloned. The entire recombinant cassette, the fragment of DNA upstream of *eccD3*, *kan*^{r} and the downstream fragment of *eccD3* was finally cloned in the suicide vector, pPR27. The recombinant suicide vector was successfully electroporated in the wild-type *M. smegmatis*. Effectively, colonies exhibiting kanamycin resistance, sucrose resistance and gentamycin sensitivity should be the double recombinants and hence the *eccD3* disruption mutants (Fig. 1B). Gene knockout was confirmed by PCR screening using primers listed in Supplementary Datasheet3. One of the strains with the correct deletion of the *eccD3* gene was isolated and conserved for further studies.

3.2. Complementation of eccD3 mutant strain

The *eccD3* mutant strain was complemented by introducing a replicative plasmid pMV261hyg containing the wild-type *eccD3* gene cloned downstream from the hsp60 promoter (Stover et al., 1991). The semi-quantitative expression of the *eccD3* gene in the complemented strain, Ms0622/pEccD3 showed less expression in comparison to the wild type *M. smegmatis* strain ($P < 0.05$) (Fig. 2). Complementation of the *eccD3* mutant showed partial restoration of wild-type phenotypic characteristics such as the pellicle formation, biofilm formation, growth kinetics sensitivity to various environmental stress conditions and permeability of the cell wall. The fact that the mutant strain was partially complemented by *eccD3* on a multicopy plasmid, confirms that the *eccD3* gene is responsible for the aforesaid phenotypes.

3.3. 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 slight wrinkled edges (Fig. 3A). 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 clump-

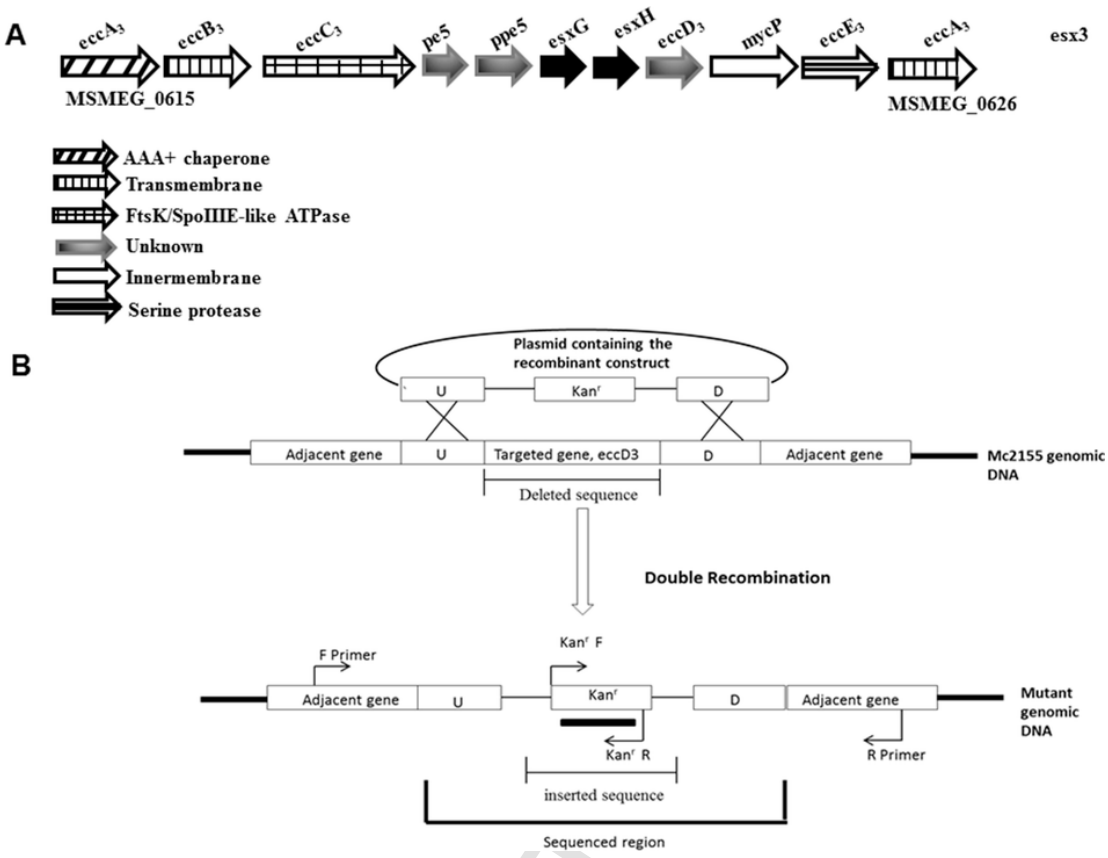

Fig. 1. Construction of *M. smegmatis eccD3* mutants. (A) Schematic representation of the esx-3 loci from *Mycobacterium smegmatis* and (B) strategies used to generate the *M. smegmatis eccD3* deletion mutant.

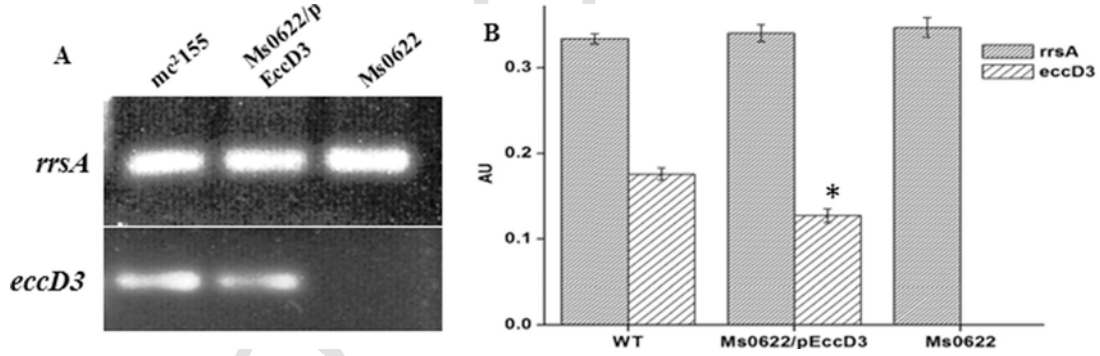

Fig. 2. Expression profile of *eccD3* in Wild type (WT), Mutant (Ms0622), and Complement (Ms0622/pEccD3) detected by semi-quantitative RT-PCR. *rrsA* was taken as the internal control. A) Representative 1% agarose gel of RT-PCR products corresponding to each gene. B) The bar diagram represents average values of three independent quantitations derived from three independent RT-PCR experiments using different RNA samples of *M. smegmatis* strains. The expression of *rrsA* gene, internal control was not significantly different in all the three strains while the expression of the *eccD3* gene in the Ms0622/pEccD3 was different ($P < 0.05$) (2B). Error bars depict mean ± standard deviation and values that are significantly different ($P < 0.05$) are indicated by a single asterisk.

ing 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 grow in a surfactant-free liquid medium culture (*e.g.*, Tween 80) form a surface pellicle at the air-media interface in standing liquid media (Ojha et al., 2008). The wild-type formed thick and robust (1 cm height) pellicles that persisted for more than 10 days in LB media. On the other hand, cells from the mutant strain were observed to be defective in pellicle formation (Fig. 3C).

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 (Fig. 3B), indicating reduced sliding motility.

3.4. 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 quan-

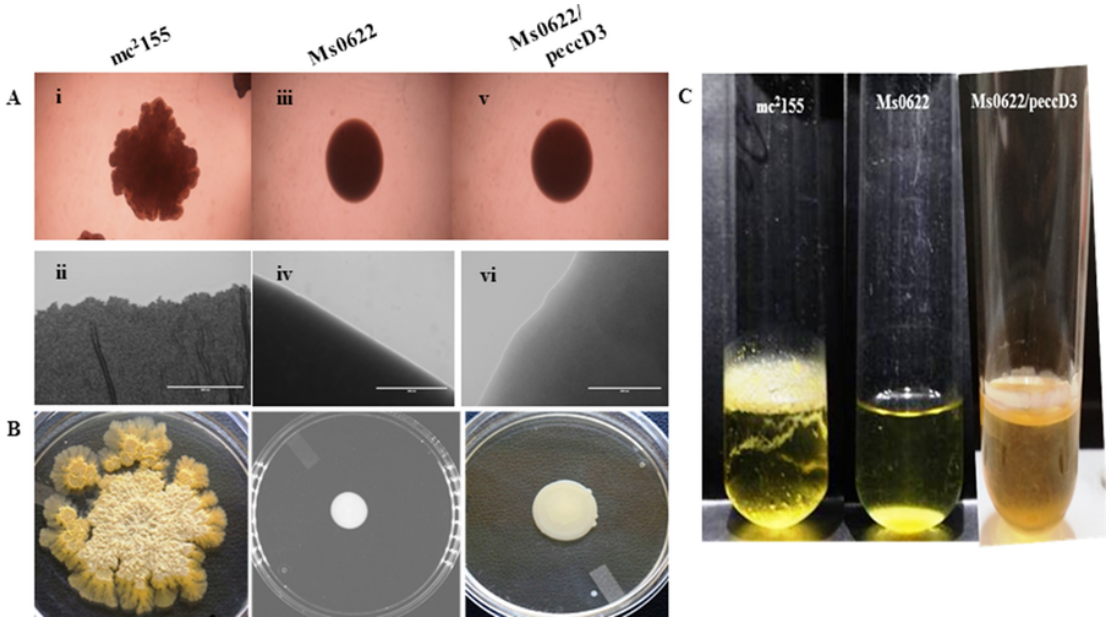

Fig. 3. (A) **Deletion of *eccD3* in *M. smegmatis* displays abnormal colony morphology** (i) mc²155, wild type; (iii) Ms06622, *eccD3* deleted mutant and (v) Ms06622/pEccD3, *eccD3* complemented strain. The edge of all the three strains were observed under the compound microscope, (ii) mc²155, wild 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. (B) **Macroscopic spreading analysis on the surface of a motility agar plate.** mc²155, Ms0622 and Ms0622/pEccD3 was grown in M63 medium with 0.3% agar. (C) **Pellicle formation of mc²155, Ms0622 and Ms0622/pEccD3.** mc²155 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.

tified 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* (Chen et al., 2006). 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 570 nm. Biofilm formation was significantly reduced in the mutant strain compared to that of the control cells ($P < 0.001$). Although, the phenotype could not be completely restored in the complemented strain its ability to form biofilm was comparatively more than that in the mutant strain ($P < 0.05$) (Fig. 4A, B). Quantification by crystal violet staining assay revealed that the mutant strain was unable to form the biofilm.

Biofilm formation by the rapidly growing mycobacteria depends on the availability of nutrient level (Williams et al., 2009). 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 50 μM iron (Ojha and Hatfull, 2007). 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, mc²155 produced relatively same amount of biofilm after 72 h (Supplementary Fig. 1). 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 ($P < 0.001$) (Fig. 4C,D). 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 (Esteban and García-Coca, 2018), the *eccD3* gene might also play significant role in biofilm formation.

3.5. 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 (Siegrist et al., 2009). 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 T_1 and T_2 for the wild type (Supplementary Fig. 2). 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 (Widdel, 2007). Similarly, the generation time was calculated for the *eccD3* deleted mutant and the complemented strain, Ms0622/pEccD3. The generation time of the mutant was 10.2 h as compared to 2.7 h in the wild type whereas the complemented strain had a generation time of 4.6 h (Fig. 5). 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 ($P < 0.05$).

3.6. eccD3 deletion leads to increased sensitivity to H_2O_2 stress

Voskuil et al. (2011), 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 (Voskuil et al., 2011). ESX 3 has been reported to play an important role in the oxidative stress reponse pathway of *M. smegmatis* (Derese Siyum, 2015). In the

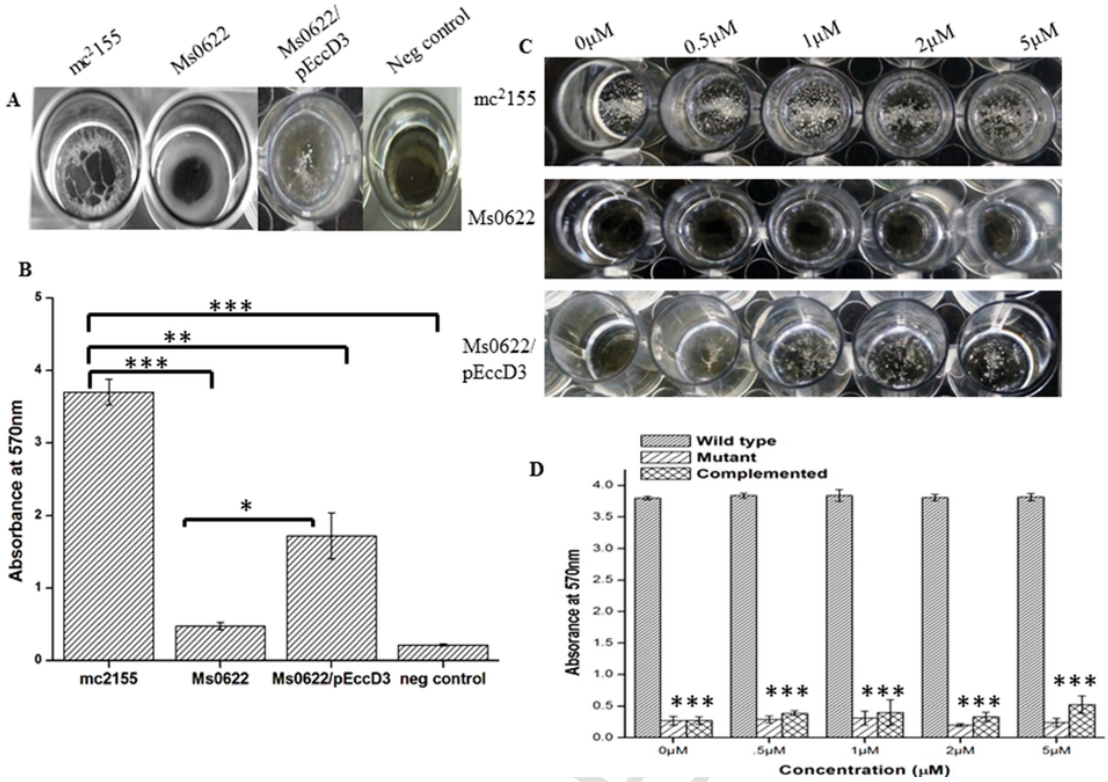

Fig. 4. Effect of *eccD3* deletion on biofilm formation. (A) Growth of mycobacterial biofilm in biofilm media by mc²155, wild type *M. smegmatis*; Ms0622, *eccD3* deleted mutant; Ms0622/pEccD3, *eccD3* complemented strain; neg control, negative control with no bacterial cells and (C) in increasing concentrations of iron (0, 0.5, 1, 2 and 5μM). (B) Biofilm formation assayed using the crystal violet (CV) staining assay. Cells of mc²155, Ms0622 and Ms0622/pEccD33 in biofilm media, M63 salts were grown on polyvinyl chloride plates for 72 h and (D) in Sauton's minimal media in the presence of increasing concentration (0, 0.5, 1, 2 and 5μM) of iron. Results are representative of at least three independent experiments and error bars represent the standard deviations from the triplicates (*, $P < 0.5$; **, $P < 0.005$; ***, $P < 0.001$).

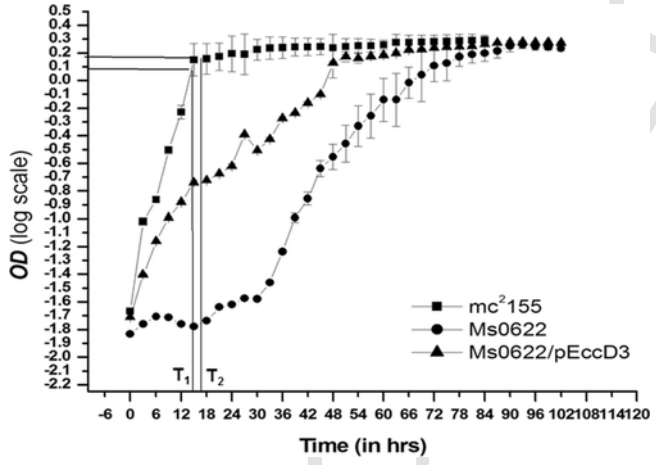

Fig. 5. . Growth kinetics of the wild type *M. smegmatis* mc²155 solid squares; *eccD3* mutant Ms0622 solid circles and complemented strain Ms0622/pEccD3 solid triangles. The generation time was calculated from the equation $t_d = 0.693/\mu$, where $\mu = 2.303$ (log OD2-log OD1)/T2-T1. The experiments were performed 3 times in triplicate. Error bars represent the standard deviations from the replicates and the values are significantly different ($P < 0.05$).

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 (Fig. 6A, Fig. 6B). Significantly the H_2O_2 stress defect in the mutant was partly reversed by genetic complementation of the *eccD3* gene in the mutant strain.

3.7. Increased sensitivity of the eccD3 deleted strain to the cationic detergent SDS

The wild-type and the *eccD3* deleted strains were treated with different concentrations of the cationic detergent, 0.001% and 0.01% for 4 h. 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 (Fig. 6C). 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.

3.8. Sensitivity to pH and heat shock

Exponentially growing wild-type and the mutant cells $OD_{600} = 0.8$ were incubated at 37 °C and 52 °C and at low pH condition, pH3 and pH5 for 20 min s. The cells were pelleted and washed in PBS buffer. Subsequently, cells were serially diluted from 10^{-1} to 10^{-7} and spotted on MB-7H9 agar plates. It was observed that the mutant cells grew normally as the wild-type cells at 37 °C. However, the mutant cells were unable to grow at 52 °C 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 at pH5 but were unable to grow after the exposure at a much lower pH, *i.e.*, at pH3 (Fig. 6D and Fig. 6E).

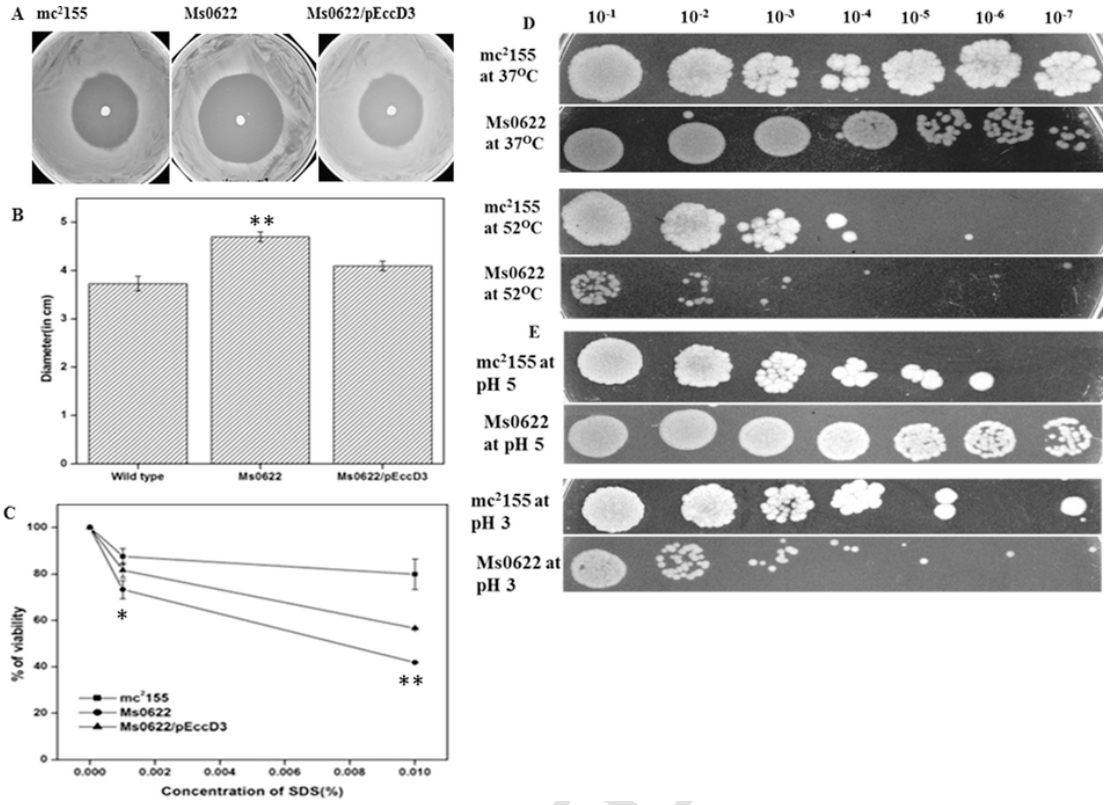

Fig. 6. Sensitivity of the *eccD3 M. smegmatis* mutant to various environmental stress. (A) Increased sensitivity of the *eccD3* mutant to H_2O_2. Disc diffusion assay was performed using discs containing 1% H_2O_2. **(B)** The diameter of zone of complete inhibition was measured (**, $P < 0.005$. (C) Effect of SDS treatment on bacterial survival. Representative data are shown for n = 3, error bars represent the standard deviations from the replicates. Survival of mc^2155 and Ms0622 after treatment with heat shock after treatment at 52 °C and 37 °C for 20 min. (D) and low pH- pH3 and pH5 for 20 min. (E). Symbols: closed square, mc^2155; closed circle, Ms0622; and closed triangle Ms0622/pEccD3.

3.9. 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 in the wild-type strain ($P < 0.001$), indicating a decrease in cell wall permeability (Fig. 7A, Fig. 7B). Complementation of the mutant strain with a wild-type copy of the *eccD3* gene partly rescued this phenotype ($P < 0.5$), as evident from the partial increase in the accumulation of EtBr and the Nile red in the complemented strain.

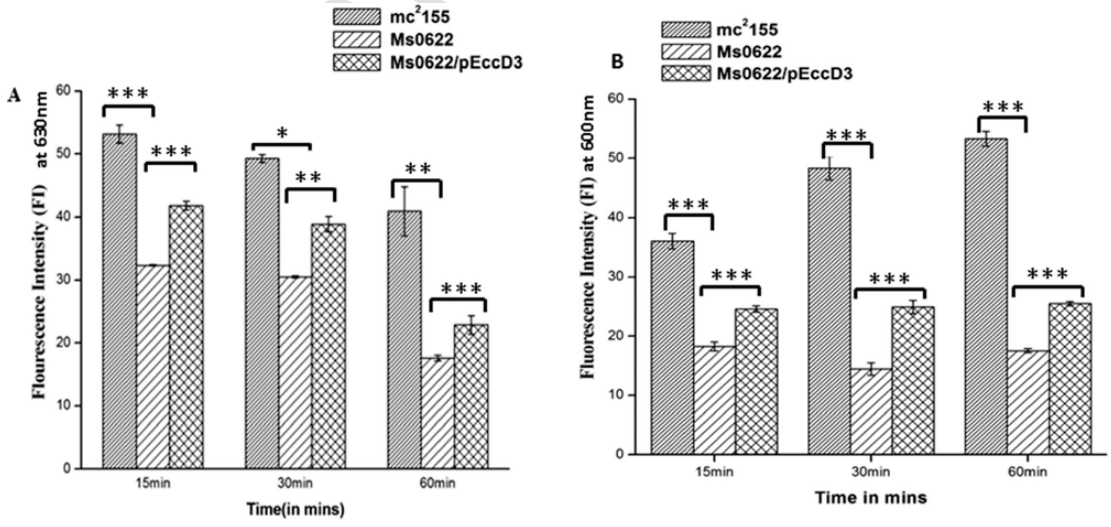

Fig. 7. Deletion of *eccD3* in *M. smegmatis* results in decreased cell wall permeability.1×10^8 cfu/ml of wild type, mutant and complemented strains were incubated in PBS with 2 μM ethidium bromide (A) and 6 μM Nile red stain (B). The experiment was performed at least three times in triplicate. Error bars indicate the standard deviation of the proportions and the results are significantly different (*, $P < 0.5$; **, $P < 0.005$; ***, $P < 0.001$).

3.10. Susceptibility of *M. smegmatis eccD3* mutant to β- 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 significantly increased susceptibility ($P < 0.001$) to the β-lactam antibiotic, ampicillin. The mutant strain showed complete resistance to the first line antitubercular drug, Isoniazid 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 also less than the wild-type strain ($P < 0.001$) (Fig. 8).

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 (Chipperfield and Ratledge, 2000; Serafini et al., 2013). 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 (Siegrist et al., 2014). 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 roles 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 created for the study. This was achieved by in-frame deletion of the gene using the efficient allelic exchange method. The *eccD3* gene in *M. smegmatis* was replaced with the *aph* gene conferring kanamycin resistance (*kan*^{r}) to the mutant strain with the help of the pPR27 suicide vector. We used pPR27 suicide vector for availing the advantage of efficient screening offered by the *sacB* gene and ts-oriM present in the vector backbone (Stover et al., 1991). The wild-type cells transformed with the recombinant pPR27 (pPRD3KO) are capable of growing at temperatures below 30 °C and are resistant to both kanamycin and gentamycin. Moreover, the *sacB* gene from the plasmid backbone renders these cells sensitive to sucrose. With the advent of the single crossover event, the recombinant pPR27 would integrate into the chromosome ensured by its ability to grow at 30 °C and death in the sucrose-containing medium. Whereas, a double recombination event ensures the loss of gent^r and *sacB* genes from the vector backbone. Thus, the clones growing in sucrose containing media and exhibiting resistance to kanamycin and sensitivity to gentamycin are the putative *eccD3* deleted mutants, thereby satisfying all conditions of a successful targeted deletion event. The putative clones were then subjected to PCR reactions to verify the mutants which exhibited the desired band patterns.

The complementation of the wild copy of the *eccD3* gene in the mutant strain using the pMV261hyg episomal plasmid could partially restore the expression of the *eccD3* gene in the complemented strain. The comparatively less expression of the *eccD3* gene in the complemented strain might be attributed to the instability of the expression plasmids. The structural instability of an expression plasmid is common in many Gram-positive microorganisms (Lopez et al., 1984). The instability of the plasmids in these organisms might be due to the genetic rearrangements occurring in the plasmid because of the homologous or non- homologous recombination events (Haeseleer, 1994). Hong et al. (2008), also demonstrated the loss of the pMV261hyg from the host after several generations resulting in the less expression of the cloned gene (Hong and Hondalus, 2008). This might be the reason for the partial restoration of phenotype by the complemented strain in our study.

Our current 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 wall peptidoglycan and esterified with mycolic acids that particularly contribute to the hydrophobicity of the cell wall and thus play a structural role, important for the integrity of the mycobacterial cell wall (Barry et al., 1998). Previous studies have demonstrated that the resulting appearance of the 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 (Barkan et al., 2012). 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 (Chen et al., 2006). A similar smooth colony phenotype was observed in a *mbtE M. smegmatis* mutant which

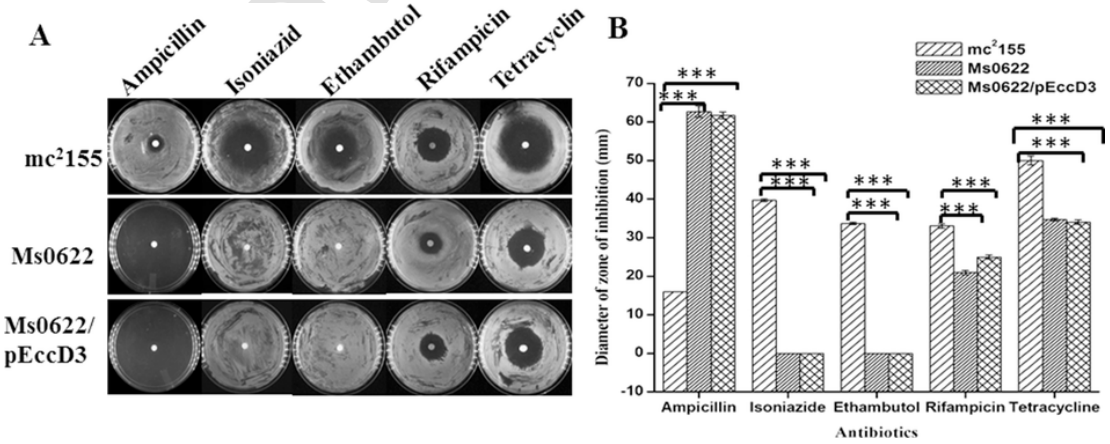

Fig. 8. Antibiotic susceptibility of *eccD3* mutant by disk diffusion assays. (A) Disk diffusion assay was done using antibiotics, Ampicillin(amp), Tetracycline(tet), Ethambutol(eth), Rifampicin(rif), 100 μg ml^{-1}); and Isoniazid(inz), 4 μg ml^{-1}. (B) A bar diagram showing susceptibility of the wild type, mc^{2}155; the eccD3 deleted mutant, Ms0622 and the complemented strain, Ms0622/pEccD3. The experiments were performed 3 times in triplicate. Error bars represent the standard deviations from the replicates and the values are significantly different (*, $P < 0.001$).

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 (Reddy et al., 2013).The appearance of the smooth colony morphology with an even edge and defect in the formation of pellicle in the *eccD3* deleted mutant might therefore be attributed to the change in the cell wall integrity, which is highly regulated by the components that comprise the cell wall.

Growth defect in the *eccD3* deleted mutant strain was observed in our study. The survival and growth of *Mycobacterium* largely depends upon the unique cell wall that comprises of 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 (Stephan et al., 2005). The mycobacterial outer membrane is responsible for the low fluidity permeation barrier of the cell wall (Liu and Nikaido, 1999). A study demonstrated severe growth defect in *M. tuberculosis* and *M. bovis* mutant that lacked a proper outer membrane (Yuan et al., 1998).

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 (Branda et al., 2005). Biofilm formation by mycobacteria is advantageous in a number of ways- (a) for protection against external chemicals and antibiotics (b) amelioration through cooperative nutritional capacities and (c) avoiding detachment from the surface by shear forces (Young, 2006). Mycobacterial species including *M. tuberculosis* require iron and mobile mycolate component for biofilm formation (Ojha et al., 2005; Wolff et al., 2015). Iron sequestration is one of the most important factors in *M. smegmatis* that plays a crucial role in the formation of biofilm (Ojha and Hatfull, 2007). *M. smegmatis* acquires iron by two pathways- (i) the exochelin pathway and (ii) the mycobactin pathway (Ratledge and Dover, 2000; Ratledge and Ewing, 1996). 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 (Siegrist et al., 2009). Later, the same group demonstrated the role of various components of the ESX3 in acquisition of iron by the mycobactin pathway (Siegrist et al., 2014). In reference to previous reports, *M. smegmatis* with an intact exochelin pathway for iron sequestration and in the presence of higher concentration of iron should have formed normal biofilm (Ojha and Hatfull, 2007) 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 (Ojha et al., 2005). *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 for the first time 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 very efficient permeability barrier to harmful substances and also limits the accessibility of the drugs to their respective targets in the bacterium (Jankute et al., 2015; Liu and Nikaido, 1999). It is because of this efficient 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 (Purdy et al., 2009). 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 (Olmsted and Kearns, 1977). 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 (Chuang et al., 2015). In our 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 (Stephan et al., 2005; Wolschendorf et al., 2007). Overexpression of *M. smegmatis* porin protein MspA in *M. tuberculosis* increased its susceptibility to the hydrophilic drugs, isoniazid, and ethambutol (Mailaender et al., 2004). 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 (Gey Van Pittius et al., 2001; Jones and Niederweis, 2010). 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 (Li et al., 2016). 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 remarkable increase in the sensitivity 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 (Bisson et al., 2012; Jarlier et al., 1991). 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 sensitivity 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 sensitivity of the mutant towards ß- lactam drugs (Lun et al., 2014). 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 hypersensitivity of these strains to the penicillin-type ß- lactam antibiotics (Flores et al., 2005).

It is suggested that the absence of the *eccD3* gene resulted in the limited capability of the mutant cells to survive in multiple stress con-

ditions 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 (Zahrt and Deretic, 2002). H_2O_2 is a natural oxidant and induces oxidative stress (Imlay, 2013). 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 (Li et al., 2015; Singh et al., 2015). 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 (Kocincova et al., 2004; Deng et al., 2015). 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 the *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 architecture of the bacterium might have been 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 conditions of stress. 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 transmembrane 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 (Watt et al., 1992; Keener, 2014). This study provides information about the functional role of the *eccD3* gene of the ESX3 secretion system in not only maintaining the integrity of the cell wall in *M. smegmatis* but also as an integral part of the normal physiology of the bacterium as well.

Data availability

The datasets generated during the current study are available from the

Competing financial interests

There are no competing financial interests or other interests that might be perceived to influence the results and discussion reported in this paper.

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Author contributions

A.K.B and S.K.R conceptualized the research problem; Y.N designed and carried out the experiments. Y.N wrote the manuscript, A.K.B and S.K.R proofread and A.K.B edited the manuscript.

Acknowledgments

We thank Prof Dipankar Chatterji, Molecular Biophysics Unit, Indian Institute of Science, India for providing the pPR27 and pUCk vectors and Dr. Ramandeep Singh, VIDRC, Translational Health Science and Technology Institute, India for providing the pMV261 vector. Department of MBBT, TU is supported by UGC-SAP, DST FIST, DBT Strengthening, DBT Hub, and DBT-BIF.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijmm.2018.06.010.

References

Barkan, D., Hedhli, D., Yan, H.G., Huygen, K., Glickman, M.S., 2012. Mycobacterium tuberculosis lacking all mycolic acid cyclopropanation is viable but highly attenuated and hyperinflammatory in mice. Infect. Immun. 80, 1958–1968.

Barry, C.E., Lee, R.E., Mdluli, K., Sampson, A.E., Schroeder, B.G., Slayden, R.A., Yuan, Y., 1998. Mycolic Acids: Structure, Biosynthesis and Physiological Functions Progress in Lipid Research 37, 143–179.

Bisson, G.P., Mehaffy, C., Broeckling, C., Prenni, J., Rifat, D., Lun, D.S., Burgos, M., Weissman, D., Karakousis, P.C., Dobos, K., 2012. Upregulation of the phthiocerol dimycocerosate biosynthetic pathway by rifampin-resistant, rpoB mutant mycobacterium tuberculosis. J. Bacteriol. 194, 6441–6452.

Bottai, D., Di Luca, M., Majlessi, L., Frigui, W., Simeone, R., Sayes, F., Bitter, W., Brennan, M.J., Leclerc, C., Batoni, G., Campa, M., Brosch, R., Esin, S., 2012. Disruption of the ESX-5 system of mycobacterium tuberculosis causes loss of PPE protein secretion, reduction of cell wall integrity and strong attenuation. Mol. Microbiol. 83, 1195–1209.

Branda, S.S., Vik, S., Friedman, L., Kolter, R., 2005. Biofilms: the matrix revisited. Trends Microbiol. 13, 20–26.

Champion, P.A., Cox, J.S., 2007. Protein secretion systems in mycobacteria. Cell. Microbiol. 9, 1376–1384.

Champion, P.A., Champion, M.M., Manzanillo, P., Cox, J.S., 2009. ESX-1 secreted virulence factors are recognized by multiple cytosolic AAA ATPases in pathogenic mycobacteria. Mol.Microbiol. 73, 950–962.

Chen, J.M., German, G.J., Alexander, D.C., Ren, H., Tan, T., Liu, J., 2006. Roles of Lsr2 in colony morphology and biofilm formation of mycobacterium smegmatis. J. Bacteriol 188, 633–641.

Chipperfield, J.R., Ratledge, C., 2000. Salicylic acid is not a bacterial siderophore: a theoretical study. Biometals 13, 165–168.

Chuang, Y.M., Bandyopadhyay, N., Rifat, D., Rubin, H., Bader, J.S., Karakousis, P.C., 2015. Deficiency of the novel exopolyphosphatase Rv1026/PPX2 leads to metabolic downshift and altered cell wall permeability in mycobacterium tuberculosis. MBio. 6, e02428.

Coros, A., Callahan, B., Battaglioli, E., Derbyshire, K.M., 2008. The specialized secretory apparatus ESX-1 is essential for DNA transfer in mycobacterium smegmatis. Mol. Microbiol. 69, 794–808.

Deng, W., Zeng, J., Xiang, X., Li, P., Xie, J., 2015. PE11 (Rv1169c) selectively alters fatty acid components of mycobacterium smegmatis and host cell interleukin-6 level accompanied with cell death. Front. Microbiol. 6, 613.

Derese Siyum, Y, 2015. Role of Esx-3 Secretion System and Stress Response in Mycobacteria Smegmatis.

Esteban, J., García-Coca, M., 2018. Mycobacterium biofilms. Frontiers Microbiol. 8, 2651.

Flint, J.L., Kowalski, J.C., Karnati, P.K., Derbyshire, K.M., 2004. The RD1 virulence locus of mycobacterium tuberculosis regulates DNA transfer in mycobacterium smegmatis. Proc. Natl. Acad. Sci. U. S. A. 101, 12598–12603.

Flores, A.R., Parsons, L.M., Pavelka Jr, M.S., 2005. Genetic analysis of the +¦-lactamases of Mycobacterium tuberculosis and Mycobacterium smegmatis and susceptibility to +¦-lactam antibiotics. Microbiology 151, 521–532.

Gey Van Pittius, N.C., Gamieldien, J., Hide, W., Brown, G.D., Siezen, R.J., Beyers, A.D., 2001. The ESAT-6 gene cluster of Mycobacterium tuberculosis and other high G+C Gram-positive bacteria. Genome Biol. 2, RESEARCH0044.

Haeseleer, Françoise., 1994. Structural instability of recombinant plasmids in mycobacteria. Res. Microbiol. 145 (9), 683–687.

Hong, Yang, Hondalus, Mary K., 2008. Site-specific integration of Streptomyces ΦC31 integrase-based vectors in the chromosome of Rhodococcus equi. FEMS Microbiol. Lett. 287 (1), 63–68.

Imlay, J.A., 2013. The molecular mechanisms and physiological consequences of oxidative stress: lessons from a model bacterium. Nat. Rev. Microbiol. 11, 443–454.

Jankute, M., Cox, J.A., Harrison, J., Besra, G.S., 2015. Assembly of the mycobacterial cell wall. Annu. Rev. Microbiol. 69, 405–423.

Jarlier, V., Gutmann, L., Nikaido, H., 1991. Interplay of cell wall barrier and beta-lactamase activity determines high resistance to beta-lactam antibiotics in Mycobacterium chelonae. Antimicrob. Agents Chemother. 35, 1937–1939.

Jones, C.M., Niederweis, M., 2010. Role of porins in iron uptake by Mycobacterium smegmatis. J. Bacteriol. 192, 6411–6417.

Keener, A.B., 2014. Oldie but goodie: Repurposing penicillin for tuberculosis. Nat. Med. 20, 976–978.

Kocincova, D., Sonden, B., Mendonca-Lima, L., Gicquel, B., Reyrat, J.M., 2004. The Erp protein is anchored at the surface by a carboxy-terminal hydrophobic domain and is important for cell-wall structure in Mycobacterium smegmatis. FEMS Microbiol.Lett. 231, 191–196.

Li, X., Wu, J., Han, J., Hu, Y., Mi, K., 2015. Distinct responses of Mycobacterium smegmatis to exposure to Low and High levels of hydrogen peroxide. PLoS.One. 10, e0134595.

Li, Q., Zhou, M., Fan, X., Yan, J., Li, W., Xie, J., 2016. Mycobacteriophage SWU1 gp39 can potentiate multiple antibiotics against Mycobacterium via altering the cell wall permeability. Sci.Rep. 6, 28701.
Ligon, L.S., Hayden, J.D., Braunstein, M., 2012. The ins and outs of Mycobacterium tuberculosis protein export. Tuberculosis. (Edinb.) 92, 121–132.
Liu, J., Nikaido, H., 1999. A mutant of Mycobacterium smegmatis defective in the biosynthesis of mycolic acids accumulates meromycolates. Proc. Natl. Acad. Sci. U. S. A. 96, 4011–4016.
Lopez, P., Espinosa, M., Greenberg, B., Lacks, S.A., 1984. Generation of deletions in pneumococcal mal genes cloned in Bacillus subtilis. PNAS 81 (16), 5189–5193.
Lun, S., Miranda, D., Kubler, A., Guo, H., Maiga, M.C., Winglee, K., Pelly, S., Bishai, W.R., 2014. Synthetic lethality reveals mechanisms of Mycobacterium tuberculosis resistance to beta-lactams. MBio. 5, e01767–14.
Mailaender, C., Reiling, N., Engelhardt, H., Bossmann, S., Ehlers, S., Niederweis, M., 2004. The MspA porin promotes growth and increases antibiotic susceptibility of both Mycobacterium bovis BCG and Mycobacterium tuberculosis. Microbiology 150, 853–864.
Martínez, A., Torello, S., Kolter, R., 1999. Sliding motility in mycobacteria. J. Bacteriol. 181, 7331–7338.
Newton-Foot, M., Warren, R.M., Sampson, S.L., van Helden, P.D., Gey van Pittius, N.C., 2016. The plasmid-mediated evolution of the mycobacterial ESX (Type VII) secretion systems. BMC.Evol.Biol. 16, 62.
Ojha, A., Hatfull, G.F., 2007. The role of iron in Mycobacterium smegmatis biofilm formation: the exochelin siderophore is essential in limiting iron conditions for biofilm formation but not for planktonic growth. Mol. Microbiol. 66, 468–483.
Ojha, A., Anand, M., Bhatt, A., Kremer, L., Jacobs Jr, W.R., Hatfull, G.F., 2005. GroEL1: a dedicated chaperone involved in mycolic acid biosynthesis during biofilm formation in mycobacteria. Cell 123, 861–873.
Ojha, A.K., Baughn, A.D., Sambandan, D., Hsu, T., Trivelli, X., Guerardel, Y., Alahari, A., Kremer, L., Jacobs Jr, W.R., Hatfull, G.F., 2008. Growth of Mycobacterium tuberculosis biofilms containing free mycolic acids and harbouring drug-tolerant bacteria. Mol.Microbiol. 69, 164–174.
Olmsted III, J., Kearns, D.R., 1977. Mechanism of ethidium bromide fluorescence enhancement on binding to nucleic acids. Biochemistry 16, 3647–3654.
Purdy, G.E., Niederweis, M., Russell, D.G., 2009. Decreased outer membrane permeability protects mycobacteria from killing by ubiquitin-derived peptides. Mol. Microbiol. 73, 844–857.
Pym, A.S., Brodin, P., Brosch, R., Huerre, M., Cole, S.T., 2002. Loss of RD1 contributed to the attenuation of the live tuberculosis vaccines Mycobacterium bovis BCG and Mycobacterium microti. Mol.Microbiol. 46, 709–717.
Ramsdell, T.L., Huppert, L.A., Sysoeva, T.A., Fortune, S.M., Burton, B.M., 2015. Linked domain architectures allow for specialization of function in the FtsK/SpoIIIE ATPases of ESX secretion systems. J. Mol. Biol. 427, 1119–1132.
Ratledge, C., Dover, L.G., 2000. Iron metabolism in pathogenic bacteria. Annu. Rev. Microbiol. 54, 881–941.
Ratledge, C., Ewing, M., 1996. The occurrence of carboxymycobactin, the siderophore of pathogenic mycobacteria, as a second extracellular siderophore in Mycobacterium smegmatis. Microbiology 142 (Pt 8), 2207–2212.
Reddy, P.V., Puri, R.V., Chauhan, P., Kar, R., Rohilla, A., Khera, A., Tyagi, A.K., 2013. Disruption of mycobactin biosynthesis leads to attenuation of Mycobacterium tuberculosis for growth and virulence. J. Infect. Dis. 208, 1255–1265.
Rosenberg, O.S., Dovala, D., Li, X., Connolly, L., Bendebury, A., Finer-Moore, J., Holton, J., Cheng, Y., Stroud, R.M., Cox, J.S., 2015. Substrates control multimerization and activation of the multi-domain ATPase motor of type VII secretion. Cell 161, 501–512.
Roy, S., Mir, M.A., Anand, S.P., Niederweis, M., Ajitkumar, P., 2004. Identification and semi-quantitative analysis of Mycobacterium tuberculosis H37Rv ftsZ gene-specific promoter activity-containing regions. Res. Microbiol. 155, 817–826.
Rustad, T.R., Roberts, D.M., Liao, R.P., Sherman, D.R., 2009. Isolation of mycobacterial RNA. Mycobacteria Protocols. Springer, 13–22.
Serafini, A., Boldrin, F., Palu, G., Manganelli, R., 2009. Characterization of a Mycobacterium tuberculosis ESX-3 conditional mutant: essentiality and rescue by iron and zinc. J.Bacteriol. 191, 6340–6344.
Serafini, A., Pisu, D., Palu, G., Rodriguez, G.M., Manganelli, R., 2013. The ESX-3 secretion system is necessary for iron and zinc homeostasis in Mycobacterium tuberculosis. PLoS. One. 8, e78351.
Siegrist, M.S., Unnikrishnan, M., McConnell, M.J., Borowsky, M., Cheng, T.Y., Siddiqi, N., Fortune, S.M., Moody, D.B., Rubin, E.J., 2009. Mycobacterial Esx-3 is required for mycobactin-mediated iron acquisition. Proc. Natl. Acad. Sci. U. S. A. 106, 18792–18797.
Siegrist, M.S., Steigedal, M., Ahmad, R., Mehra, A., Dragset, M.S., Schuster, B.M., Philips, J.A., Carr, S.A., Rubin, E.J., 2014. Mycobacterial Esx-3 requires multiple components for iron acquisition. MBio. 5, e01073–14.
Singh, A.K., Dutta, D., Singh, V., Srivastava, V., Biswas, R.K., Singh, B.N., 2015. Characterization of Mycobacterium smegmatis sigF mutant and its regulon: overexpression of SigF antagonist (MSMEG 1803) in M. smegmatis mimics sigF mutant phenotype, loss of pigmentation, and sensitivity to oxidative stress. Microbiologyopen. 4, 896–916.
Stephan, J., Bender, J., Wolschendorf, F., Hoffmann, C., Roth, E., Mailander, C., Engelhardt, H., Niederweis, M., 2005. The growth rate of Mycobacterium smegmatis depends on sufficient porin-mediated influx of nutrients. Mol. Microbiol. 58, 714–730.
Stover, C.K., De La Cruz, V.F., Fuerst, T.R., Burlein, J.E., Benson, L.A., Bennett, L.T., Bansal, G.P., Young, J.F., Lee, M.H., Hatfull, G.F., 1991. New use of BCG for recombinant vaccines. Nature 351, 456.
Unnikrishnan, M., Constantinidou, C., Palmer, T., Pallen, M.J., 2017. The enigmatic Esx proteins: looking beyond Mycobacteria. Trends Microbiol. 25, 192–204.
Voskuil, M.I., Bartek, I.L., Visconti, K., Schoolnik, G.K., 2011. The response of mycobacterium tuberculosis to reactive oxygen and nitrogen species. Front. Microbiol. 2, 105.
Watt, B., Edwards, J.R., Rayner, A., Grindey, A.J., Harris, G., 1992. In vitro activity of meropenem and imipenem against mycobacteria: development of a daily antibiotic dosing schedule tuber.. Lung Dis. 73, 134–136.
Widdel, F., 2007. Theory and measurement of bacterial growth. Di dalam Grundpraktikum Mikrobiologie 4.
Williams, M.M., Yakrus, M.A., Arduino, M.J., Cooksey, R.C., Crane, C.B., Banerjee, S.N., Hilborn, E.D., Donlan, R.M., 2009. Structural analysis of biofilm formation by rapidly and slowly growing nontuberculous mycobacteria. Appl. Environ. Microbiol. 75, 2091–2098.
Wolff, K.A., de la Pena, A.H., Nguyen, H.T., Pham, T.H., Amzel, L.M., Gabelli, S.B., Nguyen, L., 2015. A redox regulatory system critical for mycobacterial survival in macrophages and biofilm development. PLoS.Pathog. 11, e1004839.
Wolschendorf, F., Mahfoud, M., Niederweis, M., 2007. Porins are required for uptake of phosphates by Mycobacterium smegmatis. J. Bacteriol. 189, 2435–2442.
Young, K.D., 2006. The selective value of bacterial shape. Microbiol. Mol. Biol. Rev. 70, 660–703.
Yuan, Y., Zhu, Y., Crane, D.D., Barry III, C.E., 1998. The effect of oxygenated mycolic acid composition on cell wall function and macrophage growth in Mycobacterium tuberculosis. Mol. Microbiol. 29, 1449–1458.
Zahrt, T.C., Deretic, V., 2002. Reactive nitrogen and oxygen intermediates and bacterial defenses: unusual adaptations in Mycobacterium tuberculosis. Antioxid. Redox. Signal. 4, 141–159.