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

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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 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

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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 MB-7H9 medium containing kanamycin (25 μg/ml), 2% glucose and 0.05% Tween-80 and grown in a shaker incubator 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 MB-7H9-agar plates