A STUDY ON THE FUNCTIONAL ROLE OF ESX-3 TYPEVII SECRETION SYSTEM IN *MYCOBACTERIUM SMEGMATIS*

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Yutika Nath

Registration No: TZ133618 of 2013

Department of Molecular Biology and Biotechnology

Tezpur University

Tezpur-784028

Assam, India

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ABSTRACT

The genus *Mycobacterium,* belonging to the family Mycobacterieceae and the order Actinomycetales, comprises of over 190 species. Most of these are environmental saprophytes. The genus of *Mycobacterium* can be classified into three classes, *viz.*, the fast growing Mycobacteria, the slow growing Mycobacteria and the unculturable Mycobacteria. The fast growing *Mycobacterium sp.* includes mostly the non-pathogenic and environmental saprophytes, *viz, M. smegmatis, M. chelonae, M. fortuitum, M. aurum, M. agri, M. phlei, etc.* The slow growing Mycobacteria are the pathogenic species and comprise the *Mycobacterium tuberculosis* complex (MTB) that includes *M. microti* and *M. leprae* which cause leprosy and *M. bovis, M. africanum, M. tuberculosis*, the causative agent of tuberculosis (TB).

TB is one of the oldest infectious diseases known to mankind. Infection caused by *M. tuberculosis* accounts for almost 1.3 billion deaths per year fueled by HIV/AIDS pandemic, poverty and the emergence of multi-drug resistant and total-drug resistant strains. However, the present pathogenic *M. tuberculosis* strain evolved from an ancestral environmental saprophytic *Mycobacterium*. Through time immemorial this pathogenic *Mycobacterium* had undergone several evolutionary modifications which ultimately resulted in the emergence of a very clever pathogenic bacterium that can adapt to any environmental stress condition and have the capability to evade the host immune system. Various evolutionary mechanisms such as horizontal gene transfer (HGT), gene duplication and gene deletion have led to the downsizing of the mycobacterial genome as can be seen in the case of *M. tuberculosis* (4.4Mb) in comparison to *M. marinum* (6.6Mb) and *M. kanasii* (6.4Mb). During the divergence of *M. tuberculosis* from *M. marinum* and *M. kanasii,* the pathogenic bacterium acquired several genes such as the transferases and the genes that were essential for the bacterium to survive in an anaerobic environment. It is interesting to observe that the pathogenic *Mycobacterium* also harbors certain clusters of genes that are conserved in almost all the *Mycobacterium sp.* One amongst these genes, encoding a secretory apparatus known as the Type VII secretion system (T7SS), is unique to the *Mycobacterium sp.*

The re-emergence of TB in the under developed, developing and even in the developed countries has stimulated extensive efforts in mycobacterial research to understand the pathogenesis, identification of potential drug targets and development

of vaccines. The comparative study of the pathogenic and non-pathogenic *Mycobacterium sp*. could help in determining the potent drug targets. Moreover, such study would help in identifying the conserved sequences in both the pathogenic and non-pathogenic *Mycobacterium sp.* which are essential for the survival of the bacteria thus allowing a better understanding of the virulence mechanism of the pathogenic species.

The mycobacterial bacilli have a remarkable lipid rich thick cell wall which insulates the bacteria from the extracellular environment. This extremely hydrophobic and thick barrier facilitates the secretion of the bacterial products that play a pivotal role in virulence of the pathogenic *Mycobacterium*, *M. tuberculosis* through a novel secretory apparatus known as T7SS. Although this secretory apparatus is reported to play an important role in the establishment of pathogenesis, T7SS is also conserved and present in the non-pathogenic species such as *M. smegmatis*. There are five gene clusters in the T7SS- classified as ESX1, ESX2, ESX3, ESX4, and ESX5. Out of the five ESX clusters, ESX3 is present in all the mycobacterial species. This suggested that the ESX3 was the first duplicated ESX cluster in the mycobacterial genome.

The ESX3, a multimeric protein structure comprises of components *viz.*, EccA3, EccB3, EccC3, EccD3, EccE3, mycP3, espG3 and the secreted effectors ESXG, ESXH and Proline Glutamic acid 5/ Proline Proline Glutamic acid 4 (PE5/PPE4) protein pairs. Various works have been carried out to decipher the role of the paralogous ESX3 T7SS which is conserved in all the mycobacterial species. ESX3 reportedly plays a critical role in the regulated uptake of iron, crucial for vital biological processes in the pathogenic species *M. tuberculosis* and the non-pathogenic species *M. smegmatis*. Previous studies demonstrated the essentiality of the core components of the ESX3 locus such as EccC3, EspG3 and EccD3 in mycobactin mediated iron acquisition and secretion of the ESXG and ESXH effectors.

Studies on the role of the core components, EccC3, EspG3 and EccD3 of the ESX3 secretion system in *M. smegmatis* are limited. There is also lack of direct evidence till date about the physiological and morphological roles of the EccD3 component, a transmembrane protein that forms the central channel of the ESX3 T7SS in *M. smegmatis*. The EccD3 protein shares about 61% homology with that in the *M. tuberculosis* counterpart. Although it is speculated to have functional roles related to the cell wall and the cellular processes of the bacteria, its role has not yet been deciphered and defined.

Chapter 1 of the work embodied in the thesis, comprises of Introduction and Review of Literature which describe the propensity of the infectious disease caused by *M. tuberculosis*. The chapter describes the history of the tuberculosis, the current scenario of our understanding of the disease and the drug regimens developed for global TB control till date. It describes the drug targets in the bacterium against which several lines of drugs have been developed and also the limitations of the drugs discovered so far. With the emergence of drug resistant *M. tuberculosis* strains, the importance of exploring new drug targets in the bacterium has also been highlighted with special reference to the gene clusters conserved in both the pathogenic and non-pathogenic *Mycobacterium sp*. Of the conserved gene clusters, the ESX3 T7SS has been described in details. The core components and the contribution of each component of the ESX3 T7SS have been discussed. The chapter reviews the function of the ESX3 T7SS in *Mycobacterium sp*. The chapter raises the research questions based on the gaps in our understanding of the ESX3 T7SS and states the objectives required to address the research questions.

Chapter 2 describes the method for the creation of the *eccD3* knockout *M. smegmatis* mutant in order to study its functionally conserved role in the non-pathogenic *Mycobacterium sp*. The chapter begins with an introduction of the core components of the ESX3 secretion system. It describes the modern molecular tools and the fundamental approaches used to construct a gene mutant and thereby elucidating its functional role. The efficient allelic exchange method by which in frame deletion of the *eccD3* gene in *M. smegmatis* has been created is described. Further, the selection of the putative clones with the deleted *eccD3* gene and the approaches by which the deletion mutation has been confirmed is also described.

Chapter 3 explains the complementation of the wild copy of *eccD3* gene in the mutant *M. smegmatis* strain. The approach for generating an *eccD3* complementing *M. smegmatis* and its confirmation by semi-quantitative PCR has been described. The chapter also demonstrates in details the lack of any polar effect due the deletion of the *eccD3* gene.

Chapter 4 describes the atypical phenotypes exhibited by the *eccD3* deleted *M. smegmatis* mutant and restoration of the same upon complementation. It describes the morphological and physiological anomalies such as abnormal colony morphology, defective biofilm formation and decreased cell wall permeability, attributed to the deletion of the *eccD3* gene.

Chapter 5 describes the mechanism by which the *eccD3* plays a role in the cell wall permeability. It explains the structural role of *eccD3* in determining the correct cell wall architecture of *M. smegmatis*.

Finally, Chapter 6 presents the conclusions of the work and proposes the Future Work to be done to completely decipher the role of this conserved secretion system.

The last part of the thesis contains the Appendices and publication list.