

CONTENTS

| <u>SUBJECT</u> | <u>PAGE NO.</u> |
|--|-----------------|
| ABSTRACT | i-iv |
| DECLARATION BY THE CANDIDATE | v |
| CERTIFICATE OF SUPERVISOR | vi |
| CERTIFICATE OF CO-SUPERVISOR | vii |
| CERTIFICATE OF EXTERNAL | viii |
| ACKNOWLEDGMENT | ix-xi |
| TABLE OF CONTENTS | xii-xvii |
| LIST OF TABLES | xviii |
| LIST OF FIGURES | xix-xxiv |
| LIST OF ABBREVIATIONS AND SYMBOLS | xxv-xxvii |
| | |
| CHAPTER 1 INTRODUCTION AND REVIEW OF | 1-36 |
| LITERATURE | |
| 1.1 Tuberculosis - An Ancient Affliction | 2-4 |
| 1.2 The Genus <i>Mycobacterium</i> | 4-5 |
| 1.3 <i>Mycobacterium tuberculosis</i> | 5-6 |
| 1.4 Chemotherapy of TB and Vaccine | 7-9 |
| 1.5 Drug targets in <i>Mycobacterium tuberculosis</i> | 9-11 |
| 1.6 The <i>Mycobacterial</i> cell wall as a target | 11-13 |
| 1.7 Protein secretion system | 13-20 |
| 1.8 <i>Mycobacterium smegmatis</i> , A surrogate model for <i>M. tuberculosis</i> | 20-21 |
| 1.9 Objectives | 21 |
| References | 22-36 |
| | |
| CHAPTER 2 Construction of an in-frame deletion of | 37-69 |
| <i>eccD3</i> in <i>M. smegmatis</i> by efficient allelic | |
| exchange method | |
| 2.1 Introduction | 38-40 |
| 2.2 Materials and Methods | 40-49 |

| | | |
|---------|---|-------|
| 2.2.1 | Sequence alignment of <i>msm</i> MSMEG_0623 and <i>mtb</i> Rv0290 | 40 |
| 2.2.2 | Bacterial strains and growth conditions | 40 |
| 2.2.3 | Plasmids and primers used in the study | 41-42 |
| 2.2.4 | Construction of a suicide delivery vector for the replacement of <i>eccD3</i> with <i>kan^r</i> using pPR27 vector. | 42-43 |
| 2.2.4.1 | Cloning of the upstream 1kb region of <i>eccD3</i> into pGEM-7Zf(+) vector | 43-44 |
| 2.2.4.2 | Cloning of the 1.2 kb region downstream of <i>eccD3</i> into pD3UP | 44-45 |
| 2.2.4.3 | Cloning of <i>kan^r</i> gene into pD3UPDN | 45 |
| 2.2.4.4 | Cloning of the recombination cassette into pPR27 | 45 |
| 2.2.5 | Transformation of <i>M. smegmatis</i> with rpPR27 | 45-46 |
| 2.2.6 | Screening for clones with targeted disruption of <i>eccD3</i> with <i>kan^r</i> in <i>M. smegmatis</i> | 46 |
| 2.2.7 | Mycobacterial genomic DNA extraction | 46-47 |
| 2.2.8 | Sequence analysis | 47 |
| 2.2.9 | PCR based confirmation of the mutants. | 47 |
| 2.2.9.1 | Amplification using Set-1 primers: KanrFor and Kanr Rev | 47 |
| 2.2.9.2 | Amplification using Set-2 primers: Ms0621For and KanrRev | 47-48 |
| 2.2.9.3 | Amplification using Set-3 primers: KanrFor and Ms0625Rev | 48-49 |
| 2.3 | Results | 50-63 |
| 2.3.1 | Homology between the <i>M. tuberculosis</i> and <i>M. smegmatis eccD3</i> gene | 50-51 |
| 2.3.2 | Cloning of the upstream 1kb region of <i>eccD3</i> into pGEM-7Zf(+) vector | 51-52 |
| 2.3.3 | Sequencing of the upstream fragment cloned in | 53-54 |

| | | |
|------------------|--|--------------|
| | pGEMT7Zf(+) | |
| 2.3.4 | Cloning of the 1.2 kb region downstream of <i>eccD3</i> into pD3UP | 55 |
| 2.3.5 | Sequencing of the upstream fragment cloned in pGEMT7Zf(+) | 56-57 |
| 2.3.6 | Cloning of <i>kan^r</i> gene into pD3UPDN | 57-58 |
| 2.3.7 | Cloning of the recombination cassette into pPR27 | 58-59 |
| 2.3.8 | Screening for clones with targeted deletion of <i>eccD3</i> with <i>kan^r</i> in <i>M. smegmatis</i> | 60-61 |
| 2.3.9 | PCR based identification of the <i>eccD3</i> disruption mutant in <i>M. smegmatis</i> | 62 |
| 2.3.9.1 | PCR amplification using Set-1: kanrFor and kanrRev primers | 62 |
| 2.3.9.2 | PCR amplification using Set 2: Ms0621For and KanrRev | 62-63 |
| 2.3.9.3 | Set 3: KanrFor and Ms0625Rev | 63 |
| 2.4 | Discussion | 63-65 |
| | References | 66-69 |
| CHAPTER 3 | Complementation of the wild copy of <i>eccD3</i> gene in the mutant strain of <i>M. smegmatis</i> with complete deletion of the <i>eccD3</i> gene | 70-83 |
| 3.1 | Introduction | 71-72 |
| 3.2 | Materials and Methods | 72-74 |
| 3.2.1 | Bacteria strain, plasmid, and growth conditions | 72 |
| 3.2.2 | Primers used in the study | 72-73 |
| 3.2.3 | Mutant complementation | 73 |
| 3.2.4 | Isolation of RNA | 73 |
| 3.2.5 | Preparation of cDNA | 74 |
| 3.2.6 | Semi-quantitative RT-PCR | 74 |

| | | |
|------------------|--|---------------|
| 3.3 | Results | 74-79 |
| 3.3.1 | Cloning of the <i>eccD3</i> into pMV261hyg vector | 74-75 |
| 3.3.2 | Sequencing of the <i>eccD3</i> gene cloned in pMV261hyg | 76 |
| 3.3.3 | Screening of clones with the wild copy of <i>eccD3</i> gene | 77 |
| 3.3.4 | Isolation of RNA | 77 |
| 3.3.5 | Semi quantitative PCR of <i>eccD3</i> gene | 77-78 |
| 3.3.6 | Absence of polar effect | 78-79 |
| 3.4 | Discussion | 79-80 |
| | References | 81-83 |
| Chapter 4 | Characterization of the morphological and physiological role of <i>eccD3</i> gene | 84-113 |
| 4.1 | Introduction | 85-87 |
| 4.2 | Materials and methods | 87-90 |
| 4.2.1 | Bacteria strain, plasmid, and growth conditions. | 87 |
| 4.2.2 | Bacterial growth curves | 87-88 |
| 4.2.3 | Optical microscopy | 88 |
| 4.2.4 | Sliding motility assay | 88 |
| 4.2.5 | Pellicle formation assay | 88 |
| 4.2.6 | Biofilm formation assay | 88-89 |
| 4.2.7 | Disk diffusion method | 89 |
| 4.2.8 | Ethidium bromide (EtBr) and Nile Red uptake assay | 89 |
| 4.2.9 | Heat shock and acid challenge | 89 |
| 4.2.10 | SDS sensitivity assay | 89-90 |
| 4.2.11 | Spot tests | 90 |
| 4.2.12 | Statistical analysis | 90 |
| 4.3 | Results | 90-101 |
| 4.3.1 | Colony morphology, Pellicle formation, and Sliding motility | 90-91 |

| | | |
|------------------|--|----------------|
| 4.3.2 | Pellicle formation | 91-92 |
| 4.3.3 | Sliding motility | 92 |
| 4.3.4 | The growth of <i>eccD3</i> gene deleted mutant of <i>M. smegmatis</i> . | 92-93 |
| 4.3.5 | Biofilm formation | 94-96 |
| 4.3.7 | The deletion of <i>eccD3</i> in <i>M. smegmatis</i> leads to increased sensitivity to H ₂ O ₂ stress | 96-97 |
| 4.3.8 | Sensitivity to pH and heat shock | 97-98 |
| 4.3.9 | Increased sensitivity of the <i>eccD3</i> deleted strain to the cationic detergent SDS | 98-99 |
| 4.3.10 | The cell permeability of the <i>eccD3</i> deleted strain is altered | 99-100 |
| 4.3.11 | Susceptibility of <i>M. smegmatis eccD3</i> mutant to β- lactam antibiotic and anti-tubercular drugs | 100-101 |
| 4.4 | Discussion | 101-106 |
| | References | 107-113 |
| Chapter 5 | Elucidation of the role of <i>eccD3</i> in <i>M. smegmatis</i> cell wall structure | 114-149 |
| 5.1 | Introduction | 115-120 |
| 5.2 | Materials and methods | 121-123 |
| 5.2.1 | Bacteria strain, plasmid, and growth conditions. | 121 |
| 5.2.2 | Confocal Laser Scanning Microscopy | 121 |
| 5.2.3 | Scanning electron microscopy (SEM) | 121 |
| 5.2.4 | Extraction of Mycobacterial Mycolic Acids | 121-122 |
| 5.2.5 | Thin layer chromatography (TLC) | 122 |
| 5.2.6 | Purification of the mycolic acid subspecies | 122 |
| 5.2.7 | Differential Scanning Calorimetry | 123 |
| 5.2.8 | Gas Chromatography-Mass Spectrometry of MAMES | 123 |
| 5.2.9 | NMR Analysis of MAMES | 123 |
| 5.3 | Results | 123-135 |

| | | |
|-------------------|--|----------------|
| 5.3.1 | Deletion of <i>eccD3</i> leads to conversion of mycobacterial rods to coccus shape | 123-124 |
| 5.3.2 | Cell wall lipid analysis | 125 |
| 5.3.3 | Effect of <i>eccD3</i> deletion on cell wall fluidity | 126-127 |
| 5.3.4 | Gas chromatographic/mass spectrometric analysis | 127-132 |
| 5.3.5 | NMR analysis | 132-135 |
| 5.4 | Discussion | 135-140 |
| | References | 141-149 |
| Chapter 6 | Conclusion | 150-155 |
| 6.1 | Conclusion | 151-155 |
| 6.2 | Future works | 155 |
| Appendices | | 156-158 |