Studies on the stress responses of
M. tuberculosis: tmRNA and α-crystallins

PhD Thesis
Submitted by
Salim Al-Hashmi
Dept. of Microbial and Cellular Sciences
University of Surrey
March 31st 2016
This thesis and the work to which it refers are the results of my own efforts. Any ideas, data, images or text resulting from the work of others (whether published or unpublished) are fully identified as such within the work and attributed to their originator in the text, bibliography or in footnotes. This thesis has not been submitted in whole or in part for any other academic degree or professional qualification. I agree that the University has the right to submit my work to the plagiarism detection service TurnitinUK for originality checks. Whether or not drafts have been so-assessed, the University reserves the right to require an electronic version of the final document (as submitted) for assessment as above.
Abstract

*Mycobacterium tuberculosis* remains a major human health problem killing millions of people around the world. Therefore, the need for an in depth understanding of its pathogenicity is very important to enable rational development of new control strategies. Not all *M. tuberculosis* infected individuals progress to active disease at the time of primary infection as some carry an asymptomatic persistent infection that may reactivate later in life to cause disease. Despite a great deal of *M. tuberculosis* research, the ability of *M. tuberculosis* to cause long-term persistent infection in immune-competent hosts is poorly understood. When *M. tuberculosis* is inside the body it faces many environmental stress conditions including hypoxia, starvation and oxidative stress that damage proteins, DNA and other molecules. Gene expression studies have identified many bacterial genes that are differentially regulated when *M. tuberculosis* is subjected to such environmental stresses. This thesis focusses on three of these genes, the *ssrA* gene, which encodes the tmRNA molecule involved in ribosome recycling and degradation of denatured proteins, and two genes encoding α-crystallin molecular chaperones (*acr1* and *acr2*).

This study was designed to achieve two aims: (1) to examine the role of tmRNA in translational control and protein homeostasis in stressed mycobacteria; and (2) to understand the roles of Acr1 and Acr2 in the stress response of *M. tuberculosis* and to reveal the degree of redundancy between them.

In this thesis it was shown that *ssrA*/tmRNA is essential for bacterial viability as it was not possible to delete the gene unless a second fully functional copy was introduced.
elsewhere in the genome. The results suggested that the protease tagging function of tmRNA is essential alongside its role in ribosome recycling. A recombinant His-tagged tmRNA was expressed in the mycobacteria in an attempt to identify if tmRNA is directly involved in the translation of stress proteins. Expression of the His-tagged tmRNA was detrimental to the cell and appeared to preclude successful tagging of tmRNA substrate polypeptides. Thus there was insufficient evidence to support the hypothesis.

Ultrastructural localisation of Acr1 and Acr2 by immuno-electron microscopy and Western blotting of subcellular fractions of mycobacteria showed that Acr1 and Acr2 were localised in different parts of the cell. Assay of the phenotypes of single and double deletion mutants of Acr1 and Acr2 in different in vitro conditions failed to show any evidence that the two chaperones are functionally redundant. Indeed, experiments on intracellular infection of macrophages showed no phenotypic consequences resulted from loss of Acr1 but deletion of Acr2 resulted in an altered cytotoxic effect on the host cell.
Acknowledgements

First of all, I would like to thank the government of Sultanate of Oman represented in the Ministry of Health. I would like to thank my supervisor Prof. Graham Stewart and my co-supervisor Dr. Tom Mendum for their constructive comments without which this project would not have taken shape. I would also like to thank all the TB group and microbiology molecular laboratory staff and researchers namely; Dr Dany Best, Dr Jane Newcombe, Dr Rachel Butler, Dr Kerstin Williams, Dr Noel Wardell, Dr Suzie Hingley-Wilson, Dr Riccardo Balhana, Dr Mishaal Alanazi, Sue Wall, Tim Baker, Jenny Spinks, Mohd Al Braki, Rebecca Clarke, Anna Stedman, Jai Mehat, Johanna Hernandez, Radika Pothi, Alex Smith, Winifred Nyinoh, Puva Kuppusamy, Jade Passey and all fellow PhD researchers. Also Dr Julian Thorpe for cutting the ultra thin section and Dr Robert Francis for his help with the electron microscope. Finally, I would like to dedicate this work to my dear parents, invaluable wife and precious children.
# Table of content

ACKNOWLEDGEMENTS........................................................................................................................................................................V

CHAPTER 1 ......................................................................................................................................................................................... 1

1.1 Introduction ................................................................................................................................................................................... 2
1.2 Mycobacterial Strains causing Tuberculosis .......................................................................................................................... 3
1.3 Epidemiology .................................................................................................................................................................................. 4
1.4 Immunopathogenesis of M. tuberculosis .................................................................................................................................... 5
1.5 M. tuberculosis Cell Wall Interaction with the Immune System ................................................................................................. 7
1.6 Dormancy ....................................................................................................................................................................................... 8
1.7 Reactivation and Resuscitation .................................................................................................................................................. 11
1.8 Heat Shock Proteins ....................................................................................................................................................................... 12
1.9 Small Heat Shock Proteins ............................................................................................................................................................ 13
  1.9.1 Mycobacteria α-crystallins .................................................................................................................................................... 14
  1.9.2 Role of Acr1 and Acr2 in Infection ..................................................................................................................................... 17
1.10 Regulation of Gene Expression .................................................................................................................................................... 19
  1.10.1 Transcriptional Regulation of Gene Expression in the M. tuberculosis Complex ................................................................... 20
  1.10.2 Sigma Factors of the M. tuberculosis Complex .................................................................................................................. 21
  1.10.3 Two Component Systems ..................................................................................................................................................... 24
    1.10.3.1 PhoP/PhoR (Rv0757/Rv0758) ........................................................................................................................................... 26
    1.10.3.2 MprA/MprB .................................................................................................................................................................. 26
    1.10.3.3 DosS-DosT/DosR ............................................................................................................................................................ 28
1.11 Transcriptional Repressors/Activators ........................................................................................................................................ 30
  1.11.1 HspR and HrCA .................................................................................................................................................................... 31
  1.11.2 ClgR the clp Gene Regulator .......................................................................................................................................... 32
1.12 Proteases .......................................................................................................................................................................................... 33
1.13 Translational Regulation of Gene Expression in Bacteria ......................................................................................................... 34
1.14 Regulation of Acr1 and Acr2 Gene Expression .......................................................................................................................... 34
1.15 Stress Response of M. tuberculosis .......................................................................................................................................... 36
  1.15.1 Heat Shock .............................................................................................................................................................................. 37
  1.15.2 Response to Acid pH ......................................................................................................................................................... 37
  1.15.3 Response to Oxidative Stress ........................................................................................................................................... 38
  1.15.4 Hypoxia .................................................................................................................................................................................. 40
  1.15.5 Role of Toxin AntiToxin Systems ..................................................................................................................................... 41
  1.15.6 Role of tmRNA .................................................................................................................................................................... 43
1.16 Protein Synthesis ............................................................................................................................................................................ 44
1.17 Transfer Messenger RNA ............................................................................................................................................................ 47
  1.17.1 Structure of tmRNA ............................................................................................................................................................ 49
  1.17.2 SmpB Function .................................................................................................................................................................... 51
  1.17.3 Trans-Translation Process ............................................................................................................................................... 52
  1.17.4 Trans-Translation Independent Ribosomal Rescue ......................................................................................................... 54
1.18 STUDY RATIONALE ................................................................................................................................................................... 57
1.19 AIDS AND OBJECTIVES ........................................................................................................................................................... 59
## METHODS AND MATERIALS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1. Bacterial strains and growth conditions</td>
<td>61</td>
</tr>
<tr>
<td>2.2. PCR</td>
<td>62</td>
</tr>
<tr>
<td>2.3. Ligation</td>
<td>62</td>
</tr>
<tr>
<td>2.4. E. coli transformation</td>
<td>62</td>
</tr>
<tr>
<td>2.5. Construct screening</td>
<td>65</td>
</tr>
<tr>
<td>2.6. Gene replacement of ssrA and acr2</td>
<td>65</td>
</tr>
<tr>
<td>2.6.1. Preparing H37rv competent cells</td>
<td>65</td>
</tr>
<tr>
<td>2.6.2. Electroporation for knockout and plating</td>
<td>66</td>
</tr>
<tr>
<td>2.6.3. Complementation of ssrA gene and plating</td>
<td>67</td>
</tr>
<tr>
<td>2.6.4. Complementation of acr1 and acr2 genes and plating</td>
<td>67</td>
</tr>
<tr>
<td>2.6.5. M. tuberculosis H37rv transformants screening</td>
<td>67</td>
</tr>
<tr>
<td>2.7. Producing BCG str. Pasteur merodiploid harbouring M. tuberculosis H37rv ssrA-his variant</td>
<td>68</td>
</tr>
<tr>
<td>2.7.1. Preparing BCG str. Pasteur competent cells</td>
<td>68</td>
</tr>
<tr>
<td>2.7.2. Preparing M. smegmatis groEL1ΔC competent cells</td>
<td>68</td>
</tr>
<tr>
<td>2.7.3. Electroporation of BCG str. Pasteur with ssrA&lt;sup&gt;His&lt;/sup&gt; variant</td>
<td>68</td>
</tr>
<tr>
<td>2.7.4. Electroporating of M. smegmatis groEL1ΔC with ssrA&lt;sup&gt;His&lt;/sup&gt; variant</td>
<td>68</td>
</tr>
<tr>
<td>2.8. M. tuberculosis H37rv genomic DNA (gDNA) preparation</td>
<td>69</td>
</tr>
<tr>
<td>2.9. BCG STR. PASTEUR CYTOSOLIC AND MEMBRANE BOUND PROTEIN EXTRACTION</td>
<td>70</td>
</tr>
<tr>
<td>2.10 ssrA-His variant tagged protein extraction and purification</td>
<td>71</td>
</tr>
<tr>
<td>2.10.1. Protein extraction</td>
<td>71</td>
</tr>
<tr>
<td>2.10.2. ssrA-His variant tagged protein extraction purification</td>
<td>72</td>
</tr>
<tr>
<td>2.11 Western blot</td>
<td>72</td>
</tr>
<tr>
<td>2.11.1 Protein sample preparation</td>
<td>72</td>
</tr>
<tr>
<td>2.11.2 Protein gel preparation</td>
<td>73</td>
</tr>
<tr>
<td>2.11.2.1 Resolving gel (12%)</td>
<td>73</td>
</tr>
<tr>
<td>2.11.2.2 Stacking gel (3%)</td>
<td>73</td>
</tr>
<tr>
<td>2.11.3 Protein sample loading and Electrophoresis</td>
<td>74</td>
</tr>
<tr>
<td>2.11.4 Protein gel staining</td>
<td>74</td>
</tr>
<tr>
<td>2.11.5 Protein gel blotting</td>
<td>74</td>
</tr>
<tr>
<td>2.11.6 Membrane blocking</td>
<td>75</td>
</tr>
<tr>
<td>2.11.7 Primary Antibodies Treatment</td>
<td>75</td>
</tr>
<tr>
<td>2.11.8 Secondary Antibodies (HRP conjugate) Treatment</td>
<td>75</td>
</tr>
<tr>
<td>2.11.9 Western Blotting Development</td>
<td>75</td>
</tr>
<tr>
<td>2.12 In Vitro Stress Assays</td>
<td>76</td>
</tr>
<tr>
<td>2.12.1 Hypoxia</td>
<td>76</td>
</tr>
<tr>
<td>2.12.2 Heat shock</td>
<td>76</td>
</tr>
<tr>
<td>2.12.3 pH</td>
<td>76</td>
</tr>
<tr>
<td>2.12.4 H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>77</td>
</tr>
<tr>
<td>2.13 THP-1 cell infections</td>
<td>77</td>
</tr>
<tr>
<td>2.13.1 THP-1 cell preparation</td>
<td>77</td>
</tr>
<tr>
<td>2.13.2 Infection with M. tuberculosis</td>
<td>78</td>
</tr>
</tbody>
</table>
CHAPTER 3 .................................................. 91

GENETIC MANIPULATION OF THE SSRA GENE TO UNDERSTAND THE FUNCTION OF THE
MYCOBACTERIAL TMRNA ................................................................. 91

3.0 INTRODUCTION ................................................................. 92

3.1 Construction of a suicide vector to knockout ssrA (PG5ssrA::hyg) .................. 95

3.2 Deletion of the ssrA gene from WT M. tuberculosis H37Rv .......................... 97

3.3 Generation of ssrA merodiploid M. tuberculosis H37Rv ............................ 99

3.4 Deleting the in situ ssrA gene from the ssrA merodiploid M. tuberculosis H37Rv strain. 101

3.5 Construction of a ssrAHis variant gene and integrating construct ............... 102

3.6 Attempt to delete the ssrA gene from the ssrAHis expressing M. tuberculosis H37Rv strain .......................................................... 106

3.7 Experiments to detect ssrA tagged substrate proteins using mycobacteria expressing a
ssrAHis .......................................................... 107

3.7.1 Purification of His-tagged proteins from (BCGHis,
PK.ssrAHis, lysate ................................................. 109

3.7.2 Detection of ssrAHis His-tagged protein in M. smegmatis groEL1ΔC harbouring PKssrAHis,
pMV261.ssrAHis and pMV361.ssrAHis expression vectors ..................................... 112

3.8 Co-expression of the smpB gene with ssrAHis ......................................... 114

3.9 Validation of ssrAHis expression in the M. smegmatis groEL1ΔC merodiploids harbouring
 pk.smpB-Rv3099c-ssrAHis or pMV261-smpB-Rv3099c.ssrAHis expression vectors ........... 117

3.10 Detection of ssrAHis variant His-tagged proteins using M. smegmatis groEL1Δ C
harbouring PK.smpB-Rv3099c-ssrAHis, pMV261.smpB-Rv3099c-ssrAHis expression vectors ............................................................................. 120

3.11 Deletion of the lon (MSMEG_3582) gene from M. smegmatis groEL1ΔC ............. 121

3.12 Detection of ssrAHis variant tagged protein using M. smegmatis groEL1ΔCΔCΔlon
harbouring PK.smpB-Rv3099c-ssrAHis,pMV261.smpB-Rv3099c-ssrAHis expression vectors 125

3.13 The construction of the ssrAHis gene construct ......................................... 126
Appendix 8.6. Checking GroEL ΔC ................................................................. 235
Appendix 8.7. acr2 deletion sequence confirmation ........................................ 237
Appendix 8.8. pK.smpB-Rv3099c-ssrA construct map .................................. 241
Appendix 8.9. pMV361-smpB-Rv3099c-ssrA<sup>9His</sup> construct map ............... 242
Appendix 8.10. ssrA<sup>9His</sup> sequence comparison with ssrA<sup>9His</sup> .................... 243

Figures

Figure 1 Domain organisation of sHsp ............................................................. 13
Figure 2 A Two Component System (TCS) signalling pathway ...................... 24
Figure 3. M. tuberculosis dormancy response to hypoxic conditions ............... 29
Figure 4. Different toxin-antitoxin systems based on their mode of action ....... 42
Figure 5. Protein synthesis ........................................................................... 45
Figure 6. Structure of tmRNA ....................................................................... 50
Figure 7. tmRNA function ............................................................................ 53
Figure 8. Alternative pathway for ribosome rescue ArfA and ArfB .................... 55
Figure 9. PG5<sup>ssrA</sup>::hyg construct ............................................................. 95
Figure 10. pG5<sup>ssrA</sup>::Hyg plasmid screening ............................................... 96
Figure 11. The <sup>ssrA</sup> deletion PCR product BglII digest schematic diagram ..... 98
Figure 12. PCR to screen the transformants for <sup>ssrA</sup> gene replacement .......... 98
Figure 13. pK.<sup>ssrA</sup> construct .................................................................... 99
Figure 14. Amplification of the <sup>aph</sup> gene to demonstrate integration of pK.<sup>ssrA</sup> construct to make <sup>ssrA</sup> merodiploid M. tuberculosis ........................................ 100
Figure 15. PCR screening of PG5<sup>ssrA</sup>::hyg.................................................. 102
Figure 16. <sup>ssrA</sup> and <sup>ssrA</sup><sup>9His</sup> variant sequence ........................................ 103
Figure 17. pK.<sup>ssrA</sup><sup>9His</sup> construct ................................................................. 104
Figure 18. Screening for pK.<sup>ssrA</sup><sup>9His</sup> M. tuberculosis H37rv merodiploid .... 105
Figure 19. Western blot and Protein gel for nickel column purified protein from BCG<sup>9HisA</sup> ......................................................................................... 110
Figure 20. Mascot result ............................................................................. 111
Figure 21. SDS-PAGE of whole cell lysates and His-tag purified proteins from M. smegmatis GroELΔC and M. smegmatis groELΔC merodiploids harbouring constructs that express <sup>ssrA</sup><sup>9His</sup> ............................................................ 113
Figure 22. Western blot for detection of the histidine tagged protein from M. smegssrA<sup>9HisB</sup> and M. smegssrA<sup>9HisA</sup> His-pull down preps. .................................................. 113
Figure 23. Schematic diagram (A) showing the position of smpB, MSMEG_2092 and MSMEG_2093 (tmRNA) and their orientation on the M. smegmatis genome obtained from http://mycobrowser.epfl.ch/smegmasearch.php?gene+name=smpB&submit=Search .... 115
Figure 24. pk-smpB-Rv3099c-ssrA<sup>9His</sup> construct .................................... 116
Figure 25. pMV261-smpB-Rv3099c-ssrA<sup>9His</sup> construct .............................. 116
Figure 26. PCR of cDNA from RT PCR of different RNA preps from *M. smegmatis groEL1AC* and *M. smegmatis groEL1AC ssrA* merodiploids harbouring either pk.*smpB-Rv3099c-ssrA* or pMV261-*smpB-Rv3099c-ssrA*.

Figure 27. The pG5Lon::*hyg* construct for gene-replacement of *M. smegmatis groEL1AC*.

Figure 28. Lon gene homologous recombination schematic diagram.

Figure 29. PCR amplification and *BglII* digest to determine gene-replacement of *lon*.

Figure 30. *ssrA* gradient variant sequence compared to *ssrA* gradient variant.

Figure 31. *M. smegmatis groEL1 ΔC, M. smegmatis groEL1AC Δlon* and *M. smegmatis groEL1AC Δlon* (*ssrA* gradient variant merodiploid) growth curves by OD 600.

Figure 32. *M. smegmatis groEL1AC, M. smegmatis groEL1AC Δlon* and *M. smegmatis groEL1AC Δlon* (*ssrA* gradient variant merodiploid) growth curves by cfu.

Figure 33. *M. smegmatis groEL1AC, M. smegmatis groEL1AC Δlon* and *M. smegmatis groEL1AC Δlon* (*ssrA* gradient variant merodiploid) growth curves by OD 600.

Figure 34: Detection of Acr1 by Western in different BCG culture fractions.

Figure 35. Detection of Acr2 by Western blotting in different BCG culture fractions.

Figure 36. Immunogold electron micrograph of *M. tuberculosis H37rv* stained with non-immune rabbit serum (specificity control).

Figure 37. Acr1 immunogold electron micrograph.

Figure 38. Acr2 immunogold electron micrograph.

Figure 39. Schematic diagram illustrating the position of *acr1* in the *M. tuberculosis H37rv* genome and PCR primers for verifying the deletion of *acr1*.

Figure 40. Deletion of *acr1* gene - confirmation by PCR.

Figure 41. Genome position of the *acr2* gene and the PCR based strategy for verification of *acr2* gene replacement.

Figure 42. PCR screening for the *acr2* gene deletion.

Figure 43. Confirmation of *acr2* gene deletion by *BglI* digest of PCR products encompassing the *acr2* region.

Figure 44. Western detection of Acr1 and Acr2 in different knockout strains and WT *M. tuberculosis H37Rv*.

Figure 45. pK-*acr1* construct.

Figure 46. pK-*acr1acr2* construct.

Figure 47. Confirmation of pK-*acr1*, pK-*acr2* and pK-*acr1&2* plasmid construction by restriction digest.

Figure 48: Western blot detection of Acr1 showing complementation of Acr1 expression in null mutants.

Figure 49. Western blot detection of Acr2 showing complementation of Acr2 expression in null mutants.

Figure 50. Growth curve (OD 600) of WT *M. tuberculosis H37rv* and α-crystallin knockout mutants grown in 7H9 containing 10% ADC and 0.1% Tween80. Two biological replicate for each.
Figure 51. Growth curve (viability assessed by cfu) of WT *M. tuberculosis* H37rv and α-crystallin knockout mutants grown in 7H9 containing 10% OADC and 0.1% Tween80. Two biological replicate for each. ................................................................. 169
Figure 52. Heat resistance *M. tuberculosis* H37rv α-crystalline mutants ...................... 171
Figure 53. H₂O₂ resistance of *M. tuberculosis* H37RV α-crystallin mutants .............. 173
Figure 54. Percentage survival of *M. tuberculosis* H37RV α-crystallin mutants in H₂O₂ ................................................................. 175
Figure 55. Acid pH resistance of *M. tuberculosis* H37rv α-crystalline mutants .......... 177
Figure 56. Percent survival of *M. tuberculosis* H37rv α-crystallin mutants in acid stress .................................................................................. 178
Figure 57: Intracellular survival of *M. tuberculosis* H37rv α-crystallin mutants in THP-1 macrophages ........................................................................... 180
Figure 58. Cytotoxicity of infection THP-1 macrophages infected with *M. tuberculosis* H37rv α-crystallin mutants. ................................................................. 183
Figure 59: Methylene blue indication of oxygen depletion ........................................... 186
Figure 60: Growth/survival of *M. tuberculosis* H37rv α-crystallin mutants in microaerophilic culture ...................................................................................... 187
Figure 61: GroEL1 down and up-stream schematic diagram ............................................. 235
Figure 62: *M. smegmatis* groEL1Δ PCR fragment .......................................................... 235
Figure 63 *M. smegmatis* groEL1Δ sequence ................................................................ 236

Tables

Table 1. List of the complete two-component systems of *M. tuberculosis* ............... 25
Table 2. Summary of the different regulatory systems of *M. tuberculosis* α-crystalline 1 and 2 ................................................................................................................. 36
Table 3. Primers list ............................................................................................................. 63
Table 4. *M. bovis* BCG and *Mycobacterium smegmatis* groEL1Δ (M. smegmatis groEl1 ΔC) merodiploid strains harbouring different expression *ssrA*His vectors. .................. 108
# Abbreviation

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>APS</td>
<td>Ammonium persulfate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>Calcium Chloride</td>
</tr>
<tr>
<td>CaCl₂.6H₂O</td>
<td>Calcium Chloride Hexahydrate</td>
</tr>
<tr>
<td>CIAP</td>
<td>Calf-intestinal alkaline phosphatase</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>Double Distilled Water</td>
</tr>
<tr>
<td>dATP</td>
<td>Deoxy Adenosine Tri-Phosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>Deoxy Cytosine Tri-Phosphate</td>
</tr>
<tr>
<td>dGTP</td>
<td>Deoxy Guanosine Tri-Phosphate</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxy Nucleotide Tri-Phosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>dTTP</td>
<td>Deoxy Tyrosine Tri-Phosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FeSO₄.7H₂O</td>
<td>Iron (II) Sulfate Heptahydrate</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric Acid</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse Radish Peroxidase</td>
</tr>
<tr>
<td>Hyg</td>
<td>Hygromycin</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>Dipotassium phosphate</td>
</tr>
<tr>
<td>Kan</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium Chloride</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium Chloride</td>
</tr>
<tr>
<td>Chemical</td>
<td>Description</td>
</tr>
<tr>
<td>----------</td>
<td>-------------</td>
</tr>
<tr>
<td>MgCl$_2$.6H$_2$O</td>
<td>Magnesium Chloride hexahydrate</td>
</tr>
<tr>
<td>MgSO$_4$</td>
<td>Magnesium Sulfate</td>
</tr>
<tr>
<td>MnCl$_2$.4H$_2$O</td>
<td>Manganese Chloride Tetra Hydrate</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>NaH$_2$PO$_4$</td>
<td>Monosodium dihydrogen orthophosphate</td>
</tr>
<tr>
<td>NaH$_2$PO$_4$.H$_2$O</td>
<td>Monosodium di-hydrogen phosphate monohydrate</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium Hydroxide</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>Tris-Hydrochloric acid</td>
</tr>
<tr>
<td>ZnSO$_4$.7H$_2$O</td>
<td>Zinc Sulphate Heptahydrate</td>
</tr>
</tbody>
</table>

- **attP site**: Phage Attachment site in Lambda phage
- **cDNA**: Complementary DNA
- **CFU**: Colony Forming Unit
- **dsDNA**: Double Stranded DNA
- **DNA**: Deoxyribonucleic acid
- **gDNA**: Genomic DNA
- **Kan'r**: Kanamycin Resistant
- **KO**: Knockout
- **mRNA**: Messenger RNA
- **PAGE**: Polyacrylamide Agarose Gel Electrophoresis
- **PCR**: Polymerase Chain Reaction
- **PVDF**: Polyvinylidene fluoride
- **Q-PCR**: Quantitative real-time PCR
RNA: Ribonucleic Acid
RPM: Revolutions per Minute
rRNA: Ribosomal RNA
RT: Root Temperature
RT-PCR: Reverse Transcriptase Polymerase Chain Reaction
PMA: phorbol 12-myristate 13-acetate
RPMI-1640: Roswell Park Memorial Institute 1640

Units

% - percentage
$\mu F$ - microfarad
$\mu g$ - microgram
$\mu L$ - microliter
$\mu M$ - micro molar
A - Absorbance
C - Celsius
cm - centimetre
d - Days
g - Grams
g – Relative centrifugal force
g/cm$^3$ – gram/centimeter$^3$
g/L – gram/litre
h - Hours
kb - kilo base
kV - kilovolts
L - Litre
M - Molarity
mA - miliampere
mg - milligram
mg/mL – milligram/millilitre
min - minutes
mL - millilitre
Mm - millimetre
mM - milimolar
N - Normality
ng/µL – Nano gram/microlitre
nm - nanometre
Nt - nucleotides
O/N - overnight
OD – optical density
pg/µL – pictogram/microlitre
pH - potential of Hydrogen
Sec - seconds
U/mL – units/millilitre
V - Volts
v/v – volume/volume
W/V – weight/volume
Ω - ohms
Chapter 1

Introduction
1.1 Introduction
Tuberculosis (TB) is an ancient disease and has been documented since early human history (Vimlesh and Kabra, 2006); however the causative agent, *Mycobacterium tuberculosis* (*M. tb*) was not discovered until 1882 by Robert Koch (Ducati *et al.*, 2006). *M. tuberculosis* is a very successful, slow growing, non-obligatory intracellular pathogen, and TB is a worldwide issue, with a mortality rate of 42% to 50% (in some high burden countries) (WHO, 2015). Although about one-third of the world’s population is estimated to be infected with *M. tuberculosis* but, in some infected individuals this pathogen can enter an apparent dormant (viable, non-replicative) state leading to latent infection. About 10% of latently infected individual may progress to active disease (WHO, 2015). The risk of reactivation of latently infected patients has been estimated to be 2% annually; however, co-infection with HIV increases this rate to 5-10% (Dolin *et al.*, 1994). The sustained prevalence of *M. tuberculosis* highlights the need for understanding host-pathogen interactions in order to discover new strategies to overcome the ever growing problem of *M. tuberculosis* infection. Understanding how the bacterium responds to various environmental stresses it encounters within the human body is likely to be important for the development of new methods of control. For *M. tuberculosis* to survive within a macrophage it must have appropriate and effective mechanisms for adaptation to such a harsh environment. This study will investigate the response of *M. tuberculosis* to various stresses, both *in vivo* and *in vitro*, and it seeks to investigate the role of three *M. tuberculosis* genes (*α-crystallin 1*, *α-crystallin 2* and *ssrA*) in the stress response in the context of mycobacterial survival and pathogenesis. This thesis is presented in two parts; the first focuses on tmRNA encoded by the *ssrA* gene and the second on *α*-crystallins.
1.2 Mycobacterial Strains causing Tuberculosis

Taxonomically, mycobacteria belong to the order Actinomycetales, the family Mycobacteriaceae, and the genus Mycobacterium. There are around 60 species within the Mycobacterium genus with the majority existing as saprophyles, whilst a minority are pathogenic to humans, such as M. tuberculosis, M. bovis, M. africanum and M. leprae. Not all pathogenic mycobacteria species can cause human TB (Jarlier and Nikaido, 1994), and those species known to cause human tuberculosis are all closely related and are grouped within the M. tuberculosis complex, which is composed of M. tuberculosis, M. bovis, M. africanum, M. canetti and M. microti. These different species exhibit a different phenotype and level of virulence; M. africanum is not widespread and is limited to parts of Africa (Murry et al., 2005), while M. canetti is also rare and is confined to the Horn of Africa. M. bovis is no longer a common cause of human TB, especially in developed countries since the introduction of milk pasteurisation (de la Rua-Domenech, 2006); however, it is still a concern in some developing countries, especially among HIV patients (Cosivi et al., 1998). These four species, M. bovis, M. africanum, M. canetti and M. microti are relatively rare causative agents of TB in comparison to M. tuberculosis.

The complete genome sequence of the virulent M. tuberculosis strain 37rv was published in 1998. The genome size was reported as 4,411,529 bp with 4000 genes and it has a high G+C content at 65.5% (Cole et al., 1998). Genome analysis indicated that it has an efficient DNA repair system, as 45 genes related to DNA repair mechanisms are present.
1.3 Epidemiology

TB is a major global public health problem and has a severe economic impact on countries where it is endemic. In 2014, South-East Asia and Western Pacific regions reported the greatest share of TB cases (58%), followed by Africa (28%), where the highest incidence rate of 281 cases per 100,000 individuals was observed (WHO, 2015). The largest number of cases by country was reported was India (23%), Indonesia (10%) and China (10%). TB has high morbidity rate and the World Health Organization (WHO) in their Global Tuberculosis Report in 2015 estimated that there are approximately 2-3 billion people infected worldwide, although only 5-15% of the total infected population progress to active symptomatic disease. The probability of developing the symptomatic disease is much higher about 10% per year (Narasimhan et al., 2013) among the HIV positive population. According to the WHO, there were 9.6 million new TB cases in 2014, 5.4 million in men, 3.2 million in women and 1 million in children. TB has a very high mortality rate and the WHO reported that in 2014 there were 1.5 million deaths caused by TB, with 1.1 million among HIV negative individuals and 400,000 among HIV positive individuals (WHO, 2015). The estimation of the TB incident rate is a very expensive process and requires long term studies; therefore, it might not reflect the actual rate as there may be underreporting or misdiagnosis. The WHO stated in their Global Tuberculosis Report (WHO, 2015) that different methods were used to estimate the TB incidence: (1) case notification data combined with expert opinion about case detection gaps; (2) results from national TB prevalence surveys; (3) notifications in high-income countries adjusted by a standard factor to account for under-reporting and under-diagnosis; and (4) results from inventory/capture-recapture studies.
1.4 Immunopathogenesis of *M. tuberculosis*

TB spreads via the inhalation of aerosols produced by an infected individual as a result of coughing. When inhaled, bacilli are phagocytosed by cells, including alveolar macrophages and tissue dendritic cells (DCs). Post-phagocytosis, these cells produce various pro-inflammatory cytokines, responsible for the migration of other immune cells from the bloodstream to the site of infection, and hence lead to the recruitment of more neutrophils and monocytes as well as more DC. The presence of *Mycobacteria* within DCs results in their activation and migration to local lymph nodes, where they will present processed *Mycobacterium* antigens to T lymphocytes. This ability to survive intracellularly enables *M. tuberculosis* to avoid the potent immune response and persist (Suhail, 2010), leading to chronic antigen stimulation and the accumulation of T cells around macrophages (Grace and Ernst, 2015). Chronic cytokine stimulation results in further differentiation of macrophages into different forms, including epithelioid, foamy macrophages loaded with lipid droplets, and multinucleated giant cells (Russell *et al.*, 2009).

Infected DCs produce cytokines, such as IL-12 and IL-18, which are important for the induction of natural killer (NK) T cells. Activation of NK cells results in the production of IFN-γ, which activates macrophages to produce TNF-α. TNF-α plays a critical role in the immune response to *M. tuberculosis* infection, as it causes further macrophage activation, and the production of various cytokines and chemokines, as well as apoptosis (Fallahi-Sichani *et al.*, 2012). The recruitment of more immune cells leads to increased cytokine production and further cell migration and recruitment, leading to the formation of an immune cell aggregation, known as a granuloma.
To control mycobacterial infection, the formation and maintenance of granulomas is vital. A granuloma provides a microenvironment in which different immune cells are present and can communicate via different cytokines. Bacteria are trapped within a granuloma and prevented from spreading further throughout a host. However, *M. tuberculosis* is able to disrupt the organised structure of a granuloma, a typical feature of TB reactivation. Infected DCs release IL-12 and IL-23, which are cytokines that act on the receptors primarily expressed by T lymphocytes, such as CD4 and CD8T, leading to their stimulation (Robert *et al.*, 2008). There are various subsets of CD4$^+$ cells, including T helper 1 (Th1) and T helper 2 (Th2) cells, T-regulatory cells, and Th17 cells. Different subsets of CD4$^+$ cells produce different cytokines, for example Th1 cells produce IFN-γ, mycobacteria-specific interferon, and TNF-α (Prezzemolo *et al.*, 2014), Th2 cells produce IL-4, IL-10 and TGF-β (Tsuyuguchi, 1995), while Th17 cells produce IL-17 and IL-22 (Janelle and Skokos, 2012). CD4$^+$ lymphocytes play a very important role in the fight against TB, and any defect in their function can lead to disease progression and the reactivation of latent TB. HIV results in the loss of CD4$^+$ T cells and hence there is a surge in TB reactivation (Chetty *et al.*, 2015). Animal models that are deficient in CD4$^+$ T cells have shown an impaired ability to fight and control TB, resulting in death (Caruso *et al.*, 1999; Lin *et al.*, 2012).

The presence of CD8$^+$ T cells within a granuloma highlights their important role in the control and containment of *M. tuberculosis* (Dhruv *et al.*, 2006). Unlike CD4$^+$ T cells which are more active during the early stage of *M. tuberculosis* infection, CD8$^+$ T cells are more active in the later stage of *M. tuberculosis* infection (Lazarevic *et al.*, 2005). *M. tuberculosis* infects various cells in addition to phagocytic cells, such as type II epithelial cells which express MHC class II but not MHC class I, and CD8 cells have the ability to recognise these...
sub-classes of infected cells, thereby emphasising the unique role of CD8 cells in the different stages of the immune response to *M. tuberculosis* infection (Hariff *et al.*, 2014).

### 1.5 *M. tuberculosis* Cell Wall Interaction with the Immune System

The protein Acr1 is known to be associated with the cell wall, and its interaction is covered in details in section 1.9.2. However, in this section a brief description of examples of cell wall component interactions with the immune system will be presented.

The mycobacterial cell wall plays a major role in the survival and pathogenesis of *M. tuberculosis*. Unlike Gram-negative bacteria, the *M. tuberculosis* cell wall is largely composed of fatty acids, known as mycolic acids, which are linked to arabinogalactan and attached to a small layer of peptidoglycan. The cell wall also contains many lipoglycans, such as lipoarabinomannan (LAM), lipomannan (LM) and phosphatidyl-mylo-inositol mannosides (PIM). PIM is the precursor of LAM and it forms approximately 56% of all phospholipids found in the mycobacterial cell wall (Nigou *et al.*, 2001). LAM can bind to Toll receptors and can insert itself into membranes, leading to the induction of many signalling events (Kleinnijenhuis *et al.*, 2011). In addition, the capping of LAM with short mannose oligosaccharides has been found to be required for the persistence of *M. tuberculosis* and BCG within macrophages through suppression of the IL-12 response (Nigou *et al.*, 2001) and it also inhibits apoptosis (Dao *et al.*, 2004). IL-12 is a key cytokine in the host defences against *M. tuberculosis* infection, and is important for eliciting the Th1 immune response, with some studies showing that individuals with a mutation in their IL-12 gene or its receptors have an increased susceptibility to *M. tuberculosis* (Altare *et al.*, 1998).
Mycolic acid is recognised by CD1-restricted T-Cells and mycolyltransferase (Ag85) is a powerful protective antigen against *M. tuberculosis* (Brennan, 2003). Phosphatidylinositols and different phosphorylated forms are important regulatory molecules within eukaryotic cells (Vergne *et al.*, 2004), and they have also been shown to play vital roles in signal transduction, cytoskeletal organisation, and regulation of discrete membrane trafficking events during organelle biogenesis (Corvera *et al.*, 1999; Di Paolo and De Camilli, 2006; Gillooly *et al.*, 2001; Simonsen *et al.*, 1999).

1.6 Dormancy

Humans become infected by inhaling microscopic aerosol droplets containing TB bacilli (Kaufmann, 2001), and invading *M. tuberculosis* after entering the terminal alveoli are engulfed by macrophages. The environment inside these macrophages is hostile, consequently *M. tuberculosis* has to adapt to this stressful environment by: (1) re-programming the macrophage to prevent its own destruction; (2) creating a confined environment where it can safely isolate itself from the host by initiating the formation of a granuloma which is composed of various immune cells; (3) slowing its metabolism, ceasing replication and entering dormancy, where it is resistant to host defences and anti-bacterial agents (Gengenbacher and Kaufmann, 2012); and (4) re-activation from the dormant status after a long latent infection and proliferation, which causes symptomatic infection. Approximately 10% of latently infected individuals experience reaction of their TB (Fattorini *et al.*, 2013), and switching to dormancy it not only a means of survival but also a strategy to modulate the immune response during different stages of TB infection (Mariotti *et al.*, 2013).
Understanding dormancy and the phenotypical resistance of dormant bacilli will help in eliminating dormant bacilli and in the development of drugs that have high penetration into the granuloma, and therefore is essential for the eradication of *M. tuberculosis* from humans (Evangelopoulos *et al.*, 2015; Fattorini *et al.*, 2013; Lenaerts *et al.*, 2015; Prideaux *et al.*, 2015).

It is very difficult to simulate the exact granuloma environment in order to properly study dormancy under conditions such as low pH, oxygen depletion, iron limitation, nitrosative stress and nutrient starvation (Timm *et al.*, 2003; Voskuil *et al.*, 2004) which are appropriate and truly representative of human latent TB infection (LTBI). However, there are many *in vivo* and *in vitro* models that can be used to study *M. tuberculosis* host interactions and to investigate the adaptive response of *M. tuberculosis* which enables it to become non-proliferating or dormant.

*In vivo* models are probably the best models used to study dormancy, as they provide the ideal environment for the multiple stresses surrounding *M. tuberculosis*. Different animals have been used for *in vivo* models, including non-human primate macaque monkey, guinea pigs, mice, marmosets, and rabbits; however, they all have limitations. The most commonly used model, due to availability and cost, is the mouse model but this is not the best representation of *M. tuberculosis* infection in humans, due to the high bacterial burden in susceptible mice and poor clearance, and the different murine immune response compared to humans in terms of granuloma formation (Sugawara *et al.*, 2004). In contrast, rats have
been reported to be a better model for understanding host-TB interactions (Amit et al., 2011). Whilst it has been reported that non-replicating TB bacilli could be detected in lung lesions of infected guinea pigs (Lenaerts et al., 2007), they are not a good model for studying LTBI, as they are highly susceptible to a low dose inoculum of M. tuberculosis (Ordway et al., 2008). Rabbits are another good model system, as they have high resistance to M. tuberculosis resulting in LTBI (Patel et al., 2011) but due to cost and the non-availability of immunological reagents for rabbits, they are not often used (Amit et al., 2011). The best model which can more accurately mimic human TB disease is Cynomologus macaques (Kaushal et al., 2012) however, this has the disadvantages of high cost and complex bio-containment issues.

In vitro models use one or a group of stresses to simulate the conditions that M. tuberculosis faces inside the body, such as hypoxia, oxidative and nitrosative stresses, exposure to nitric oxide and carbon monoxide, starvation of essential nutrients including carbon, nitrogen and phosphorus, as well as iron limitation. Different methods to induce dormancy include: (1) non-replicative oxygen depletion (NRP) model, in which a bacterial culture is placed in sealed flasks and is gently stirred to allow access to oxygen in the medium until it is gradually depleted (Voskuil et al., 2004; Wayne and Hayes, 1996); (2) static culture oxygen depletion model (Kendall et al., 2004), in which a bacterial culture is left to settle and oxygen levels are gradually depleted; (3) short course of complete starvation (Betts et al., 2002); (4) addition of nitric oxide (Voskuil et al., 2003), human macrophages (THP1) activated with vitaminD3 and retinoic acid model (Estrella et al., 2011); (5) multiple stresses model using low carbon and nitrogen nutrients, an acidic pH, low oxygen and high carbon dioxide levels (Deb et al., 2009); (6) controlled batch culture model (Brian et al., 2002); and (7) the gradual
depletion of oxygen and nutrients within a controlled environment (Bacon et al., 2014). The static culture oxygen depletion model has the limitation that it is difficult to reproduce, as a slight change in the settling and growth rates may upset the equilibrium between replication and settling that is required to maintain the oxygen gradient, and hence orderly shift to dormancy. In addition, it is not possible to isolate and study the sequential events that characterise the process of the shift down to dormancy in the heterogeneous population of settling bacilli (Wayne and Hayes, 1996)

1.7 Reactivation and Resuscitation

LTBI is caused by *M. tuberculosis* persisting in the dormant stage that is normally phenotypically, but not genetically, resistant to antibiotics (Connolly et al., 2007; Sarathy et al., 2013; Wayne and Hayes, 1996). This phenotypical resistance comes from the fact that most, if not all, of the antibiotics that are used to treat *M. tuberculosis* are targeted against actively growing or metabolically active bacilli and are not active against viable non-replicating (dormant) cells. Reactivation of dormant actinobacteria, such as *Micrococcus luteus*, is initiated by resuscitation–promoting factor (Rpf) (Koltunov et al., 2010; Mukamolova et al., 2002) and *M. tuberculosis* has five homologues of resuscitation-promoting factors, RpfA-E (Kana et al., 2008; Romano et al., 2012). The possession of a number of *rpf* genes means that some redundancy may exist, as only multiple gene knockouts and not single knockouts result in the impairment of resuscitation *in vitro* and attenuation in mice (Kana et al., 2008). Although there is a heterogenic population during both LTBI and active TB, during LTBI there are more non-replicating, metabolically inactive (dormant)
bacilli than metabolically active cells, and the opposite situation is found during active TB infection (Gengenbacher and Kaufmann, 2012).

1.8 Heat Shock Proteins

All living cells respond to different metabolic, environmental and pathophysiological stresses by up-regulating the expression of a particular group of proteins known as heat shock proteins (Hsps). The heat shock response (HSR) is a fundamental cytoprotective pathway which confers resistance to heat stress, and it has been proposed that the HSR was evolutionarily selected to prevent anticipated future stress damage, rather than to facilitate recovery from an existing stress effect (Verghese et al., 2012). Hsps are generally diverse and ubiquitous, and are induced under various stress conditions; they are classified into six major families, known as small heat-shock proteins (sHsps), Hsp40, Hsp60, Hsp70, Hsp90 and Hsp100 (Bakthisaran et al., 2015; Buchner, 1996; Sun and MacRae, 2005). Each major heat shock family is composed of members that are either constitutively produced or are regulatory induced, targeting different subcellular compartments. Protein members from the same family show significant sequence homology and are structurally, as well as functionally, related, but homology and similarity is not present between different chaperone families (Walter and Buchner, 2002).

Hsps are also known as chaperones and play a vital role in the transport of proteins into the correct cellular compartments, the assembly of multiprotein complexes, the folding and unfolding of proteins, the regulation of protein degradation within the proteasome pathway, the protection of cells against stress and apoptosis, as well as cell-cycle control and signalling (Schmitt et al., 2007; Takayama et al., 2003). They also stimulate antigen presenting cells
(APCs), such as macrophages and DCs, and play a role in antigen presentation via chaperoning antigenic peptides to class I and class II molecules of the major histocompatibility complexes (Li and Srivastava, 2004). Most chaperones have a broad range of protein clients because they promiscuously bind to unfolded proteins (Tyedmers et al., 2010). Hsps are numerous and providing a full account of them is out of the scope of this study; therefore, only those relevant to this study, which is the sHsp α-crystallin 1 and α-crystallin 2 will be further expanded on.

1.9 Small Heat Shock Proteins

sHsps have molecular masses ranging from 12-43kDa and are characterised by the presence of a highly conserved region of 80-100 amino acids within their C-terminal domain, known as the α-crystallin domain. The N-terminal region exhibits variability both in length and amino acid sequence, and is responsible for much of the variability between different sHSps and hence their diversity (Figure 1). The role of the α-crystallin domain is to facilitate subunit interactions, while the role of the N-terminal domain is in multimerisation (Ganea, 2001). α-crystallins function primarily as molecular chaperones preventing improper protein association (Abgar et al., 2001) through their ability to bind to non-native proteins under various stress situations, leading to inhibiting their irreversible aggregation and the prevention of cell damage (Lindner et al., 2000).

![Image of domain organisation of sHsp](image)

**Figure 1** Domain organisation of sHsp

The α-crystallin domain is flanked by the N-terminal domain (NTD) in green and the C-terminal domain (CTD) in yellow.
Based on their main function, chaperones are divided into two classes; foldases and holdases. The Hsp 70/40 system and Hsp90 are ATP dependent and belong to foldases class, whereas chaperone like sHsps are holdases. Holdases are ATP-independent and bind aggregation-prone proteins often under stress conditions and protect them from aggregation. When the stress condition is lifted then the bound proteins are released for refolding with the help foldases (Basha et al., 2012; Haslbeck et al., 2005)

1.9.1 Mycobacteria α-crystallins

Previous studies have shown that *M. tuberculosis* responds to the stress it faces within macrophages by producing many proteins (Lee and Horwitz, 1995). Among these stress induced proteins are sHSP members of the α-crystallin protein family.

Mycobacteria possess three classes of α-crystallin genes; *acr1*, *acr2* and *acr3*, which share 25-30% homology with each other. Not all mycobacteria species have the same copy number or class of genes. For example, *M. leprae* has only one copy of the *acr3* gene, while *M. avium* has four copies of the *acr3* gene (one of which may not be functional as it has an IS1245 insertion sequence within it) and a single gene of the *acr2* gene, and *M. marinum* and *M. smegmatis* each have one copy of each of the three classes. *M. tuberculosis*, however, has only one copy of *acr1* and *acr2*, and both influence the persistence of infection (Stewart et al., 2005). The *acr1* and *acr2* genes have an overall similarity of 43% and 55% within the α-crystallin domain (Kennaway et al., 2005).
Acr1 is encoded by \textit{Rv2031c}, which is also known as Hsp16.3, HspX or the 16kDa antigen (Kennaway et al., 2005). Kennaway et al. (2005) reported that Acr1 is a dodecamer as shown by nanelectrospray mass spectrometry, and using 3-dimentional analysis of negative stain electron microscopy forms a tetrahedral assembly with 12 polypeptide chains. Acr1 is associated with bacterial dormancy and its expression is induced by hypoxia (Stewart et al., 2005), in fact Acr1 is synthesised in larger quantities than any other protein during the early stages of hypoxia (Sherman et al., 2001).

Acr2 was originally identified by Ohara et al. (1997) as a new ribosome binding protein and named as heat-stress-induced ribosome binding protein (HrpA). This protein is 18kDa and is only induced at temperatures higher than 37\degree C (Tabira et al., 2000), where it was found to be mainly associated with the ribosome 30S subunit. Tabira et al. (2000) reported that HrpA was a sHsp as it has the following features: (i) a low molecular mass of 12-30kDa; (ii) the ability to form large oligomeric complexes (9-50 subunit); (iii) involvement in non-ATP dependent chaperoning; and (iv) has an \(\alpha\)-crystallin domain (conserved carboxyl-terminal domain). Furthermore, Tabira et al. (2000) reported that HrpA has 30.2\% identity and 43.4\% homology at the amino acid level to HSP16.3, also known as HspX. Unlike HrpA, HspX is induced by hypoxia (Tabira et al., 2000).

Stewart, Wernisch et al (2002) have studied HrpA, also known as an hsp protein encoded by \textit{Rv0251c}, and due to its similarity (41\% identity) to the \(\alpha\)-crystallin 14kDa antigen (Acr1) it was renamed Acr2 as it was the second \(\alpha\)-crystallin protein to be identified in \textit{M. tuberculosis}. It has been reported that during heat shock, Acr2 aids translation initiation due to its role in stabilising the 30S ribosome subunit (Ohara et al., 1997). Acr2 was shown by
nanoelectrospray mass spectrometry to form a range of oligomers, composed of dimers and tetramers, while electron microscopy showed that a variety of particles sizes are present (Kennaway et al., 2005).

Acr2 expression is induced by different stress conditions, including heat shock, oxidative stress and phagocytosis by human monocytes and monocyte-derived macrophages (Schnappinger et al., 2003; Stewart et al., 2002a; Wilkinson et al., 2005). It also has been reported that it is highly expressed during both chronic and acute infection in mice, but its deletion does not impair *M. tuberculosis* growth in either murine BMDM or in mouse organs, although it delays disease progression (Stewart et al., 2005).

Eukaryotic sHSPs, such as Hsp90 and α-crystallins have been reported to protect some peptidase activities of the active form of the 20S proteasome against oxidative inactivation (Conconi et al., 1998). As there are similarities in both structure and function between sHSPs it is possible that *M. tuberculosis* Acr1 and Acr2 proteins may play a role in protection against nitrosative and oxidative stress, possibly by interacting with the mycobacterial proteasome (Forrellad et al., 2013). The fact that *acr2* along, with other proteases, is positively up-regulated by ClgR, the *clp* gene regulator, suggests it may play a role in protein homeostasis (Estorninho et al., 2010).
### 1.9.2 Role of Acr1 and Acr2 in Infection

Acr1 and Acr2 are members of the crystallin family of molecular chaperones in *M. tuberculosis*, and both are known to contribute to *M. tuberculosis* infection and virulence, although the studies on their roles are inconclusive. Studies investigating the role of Acr1 have yielded conflicting findings, as one has reported that deleting *acr1* significantly impaired *M. tuberculosis* growth in both murine bone marrow-derived macrophages (BMDM) and THP-1 cells (Zhang *et al.*, 1998), and another showed the exact opposite, reporting that the deletion increased growth in BALB/c mice and in both resting and activated BMDM (Hu *et al.*, 2006). A third study proposed that Acr1 plays a role in slowing *M. tuberculosis* growth, as its overexpression resulted in reduced growth of BCG within murine lungs and liver (Spratt *et al.*, 2010).

Acr1 plays an important role in granuloma formation, and exposing macrophages to Acr1 resulted in the secretion of CXCL16, which is a CXC type chemokine expressed by many APCs, including macrophages (Kim *et al.*, 2001). This chemokine is important for the recruitment of different immune cells that are vital for the development of a granuloma, including Tc1 CD8⁺, NK T cells and Th1 type CD4⁺ (Bafica *et al.*, 2005; Cooper, 2009). CXCL16 is a large trans-membrane protein that is attached to the membrane by a mucin-like ‘stalk’. The α-crystallin protein activates two A - disintegrin and metalloproteinase (ADAM) proteases (ADAM10 and/or ADAM17) that cleave this stalk and release the bioactive extracellular domain of CXCL16 (Healy and King, 2012). Healy and King (2012) concluded that Acr1 induces the release of CXCL16 via activation of ADAM10/ADAM17, and as a consequence recruits more cells to the granuloma facilitating the conditions favourable for dormancy. Acr1 induces the tolerogenic phenotype by modulating the expression of PD-L1,
Tim-3, indoleamine 2,3 dioxygenase (IDO) and IL-10, leading to the inhibition of DC maturation and differentiation via targeting of the STAT-6 and STAT-3 pathways. *M. tuberculosis* has a better survival rate in Acr1 treated DCs as this results in the reduction of their antigen uptake and hence a reduction in their ability to activate T cells (Siddiqui et al., 2014).

Other stresses, such as oxidative stress, phagocytosis and heat shock, can all lead to the induction of the *acr2* gene (Stewart et al., 2005). Stewart et al. (2005) reported that the deletion of *acr2* (∆acr2) rendered *M. tuberculosis* to be sensitive to oxidative stress, although it did not impair its growth in mouse bone marrow macrophages. They also added that ∆acr2 did not affect the bacterial load during mouse infection; however, disease progression was less severe and more prolonged. Stewart et al. (2005) have related this to a reduction in immune-related pathology resulting from the reduction of immune cell (T-cells and macrophages) recruitment to the lungs of the mice infected with ∆acr2. The study observed that although ∆acr2 does not affect the growth of *M. tuberculosis*, shown by a ‘normal’ bacterial load for ∆acr2 in the infected mice, it impairs pathogenesis, thus causing less severe disease. Stewart et al. (2005) proposed two reasons for this observation: (1) the loss of the Acr2 chaperone activity function introduced a significant reduction in the mycobacterial peptides available for immune cell activation; and (2) the difference in the pathogenesis between the ∆acr2 and the wild type could be due to the physiological characteristics of ∆acr2. From the observation that the ∆acr2 mutants elicited fewer T cells and CD 11b+ cell recruitment to the lungs during the early stages of infection, the authors suggested that there should be a shift in the balance within the granuloma from actively replicating immune
controlled bacteria to the dormant state, as this would reduce the progressive accumulation of immune mediated damage and hence reduce disease severity.

It has been reported that the isolated multidrugacr1deficient (Δacr1) clinical strain of M. tuberculosis isolated from HIV negative patients with chronic pulmonary TB shows no difference in replication in murine and human macrophages compared to strain H37rv (Timm et al., 2006). The authors concluded that Acr1 is dispensable for bacterial growth within the human lung. Therefore, it is possible that theacr1 andacr2 genes are partially interchangeable as far as their chaperone functions are concerned, and as the deletion of theacr2 gene has a negative impact on the severity of the disease progression, this study will further investigate the effect of the deletion of both genes on the fitness for survival in both in vitro and in vivo conditions, as well as disease severity and progression. This will be helpful in determining if there is a redundancy in the function of Acr1 and Acr2.

1.10 Regulation of Gene Expression

Bacteria, including mycobacteria, normally live in harsh, stressful and constantly changing environments. Therefore, in order to survive they must quickly adapt to the various stresses exerted by the medium in which they are in. This adaption requires dynamic gene expression that responds to the stimuli the bacteria sense. Transcription regulation, controlled by DNA binding proteins, mediates the expression of relevant genes in response to extra or intra cellular stimuli that result in either the up or down regulation of specific genes. In addition to transcriptional repression or activation, post-transcriptional control at the mRNA level is also required for switching specific genes on and off in response to corresponding stimuli.
A detailed understanding of the mechanisms of gene expression and regulation is directly relevant to this study. Examining the roles of Acr1, Acr2 and SsrA in response to stress requires a comprehensive review of the current understanding regarding gene regulation in mycobacteria, as it is these processes that Acr1, Acr2 and SsrA impact upon most heavily.

1.10.1 Transcriptional Regulation of Gene Expression in the *M. tuberculosis* Complex

Adaptation of *M. tuberculosis* to environmental changes during infection requires an efficient regulatory system that mediates gene expression in response to particular changes. Therefore, *M. tuberculosis* has a host of a very complex regulatory systems with redundancies and overlapping functions. The genome encodes more than 140 transcription regulators (Bishai, 1998; Timm et al., 2003), 13 polymerase sigma (σ) factors (Manganelli et al., 2004), 11 paired two-component systems, two unpaired (orphan) six response regulators (Haydel and Clark-Curtiss, 2004), and 11 protein kinases (Prisic and Husson, 2014). Studying regulatory systems is very important in order to understand how a successful pathogen, such as *M. tuberculosis*, may overcome the various stresses exerted by a surrounding environment. The few regulatory systems that are involved in controlling gene expression in response to cold shock, heat shock (Shires and Steyn, 2001; Stewart et al., 2002b), oxidative stress (Raman et al., 2001), and hypoxia (Roberts et al., 2004; Sherman et al., 2001) are examined.
1.10.2 Sigma Factors of the *M. tuberculosis* Complex

The success of a pathogen to establish infection is determined by its ability to establish itself and overcome the harsh and often competitive environment present within the host. *M. tuberculosis* is extremely successful because it is able to adapt to the changing environments it finds itself through employing various mechanisms that enable it to re-programme its transcriptional machinery to slow down unnecessary metabolic processes and up-regulate virulence and stress response pathways needed for survival. Therefore, it adjusts its protein synthesis at the transcriptional level based upon the stress signals it senses within the surrounding environment. Gene expression in bacteria is regulated at the level of transcription initiation, which is mediated by the RNA polymerase (RNAP) holoenzyme which consists of five subunits: $\alpha 2\beta\beta'$ω (the core subunit) and a dissociable subunit $\sigma$ (sigma). *M. tuberculosis* has 13 sigma factors; SigA, SigB, SigC, SigD, SigE, SigF, SigG, SigH, SigI, SigJ, SigK, SigL and SigM. Sigma factors are divided into two main families, known as sigma 70 ($\sigma^{70}$) and sigma 54 ($\sigma^{54}$). SigA and SigB are the principle sigma factors, are essential in *M. smegmatis*, and *M. smegmatis* mutants are more sensitive to oxidative stress. SigC, SigD, SigE, SigG, SigH, SigI, SigJ, SigK, SigL, and SigM (ECF; Extracytoplasmic function) form a subfamily of sigma factors, and SigF (Manganelli et al., 1999). SigF is a stress response sporulation sigma factor in *Bacillus subtilis* (Clarkson et al., 2004). For the purposes of this study, only a few selected $\sigma^{70}$ factors will be elaborated on.

SigA is an essential housekeeping and is involved in enhancing growth within human macrophages and during the early stages of lung infection, where it modulates the expression of genes that contribute to virulence (Wu et al., 1997; Wu et al., 2004). In the hypervirulent strain *M. tuberculosis* W-Beijing, gene expression of *eis*, which encodes the enhanced
intracellular survival protein, is regulated by SigA and its expression directly correlates to the expression of eis (Shiping et al., 2009). The Eis protein is involved in the suppression of the host immune response (Kim et al., 2012).

SigB shares similarity in terms of its amino acid sequence with the primary sigma factor, SigA (Gomez et al., 1998), and it is positively up-regulated by SigE, SigH and SigL, which can transcribe it using its transcription site (Dainese et al., 2006; Fontán et al., 2009). *M. tuberculosis* σ mutants are more sensitive to thioridzine, which modulates the expression of genes encoding membrane proteins, efflux pumps oxido-reductases and enzymes involved in fatty acid metabolism and aerobic respiration, indicating the need of σ to resist the effects of thioridzine (Dutta et al., 2010). A *M. tuberculosis* sigB mutant showed a defective survival response when exposed to cell envelope, oxidative and hypoxic stresses, but attenuation was observed in THP1 cells and during mouse and guinea pig infections. Because SigB is regulated by many regulatory pathways, it may play a central role in the *M. tuberculosis* stress response (Fontán et al., 2009).

SigE regulates the response to cell envelope damage in *M. tuberculosis*, as it plays a role in the regulation of genes involved in the maintenance of cell envelope integrity and function during macrophage infection, and hence the modulation of the inflammatory response (Fontan et al., 2008a). SigE is dispensable for the replication of *M. tuberculosis* in human pneumocytes but is essential for the arrest of phagosome maturation in THP-1 derived macrophages (Casonato et al., 2014).
SigH is similar to SigB and regulates the response of thiol oxidative stress and heat shock and phagocytosis, leading to the induction of *sigB*, *sigE* and the thioredoxin regulon. A long induction of SigH resulted in the induction of the ATP-dependent clp proteolysis regulon, several members of the mce1 virulence regulon, and the sulfate acquisition/transport network (Mehra and Kaushal, 2009).

SigF regulates a family of proteins that play a role in the immunopathology of *M. tuberculosis*, regulates Sig^B^ and Sig^C^ protein expression, and also genes that are essential for cell wall synthesis and the survival of *M. tuberculosis* (Mustyala *et al.*, 2015). It is directly regulated by phoY1 (Ernest *et al.*, 2007).
1.10.3 Two Component Systems

Two component systems (TCSs) are signal transduction mechanisms which are present in all prokaryotes except Mycoplasma genitalium (Barrett and Hoch, 1998; Heermann and Fuchs, 2008; Stock et al., 2000) and enable them to sense, respond and adapt to various stressors surrounding them. More than three hundred TCSs have been identified across approximately one hundred different bacterial species (Barakat et al., 2011). TCSs are composed of a sensor histidine kinase (HK), which is a transmembrane protein that senses an external stress and transmits a signal into the cell through ATP phosphorylation of a corresponding response regulator (RR), which then binds to its cognate promoter DNA leading to transcription regulation (Figure 2). In this manner a TCS allows an organism to sense, transduce and initiate a response. The Saccharomyces cerevisiae genome has only HK and two RRs and none are found within the mammalian genome (Watanabe et al., 2008).

Figure 2 A Two Component System (TCS) signalling pathway

Illustration showing the sensor histidine kinase (HK) and its cognate response regulator (RR) of the TCS. In the event of the HK senses intracellular signals or environmental such as starvation, cold/heat shock as well as antimicrobial compound, it undergoes conformational changes resulting in the autophosphorylation (using ATP as phosphate source) of the conversed histidine residue in the receiver domain (C-terminal domain). In a magnesium-dependent manner the phosphoryl group is transferred to an aspartate residue in the amino-terminal (N-terminal) domain of the RR. The phosphorylated RR induces structural changes of its output domain leading to its involvement in transcription regulation (Marta et al., 2014).
The sensor kinase component interacts directly with the signal leading to phosphorylation via ATP, and the resulting phosphate is transferred to the HK which then passes the signal to the RR leading to the required gene activation in response to the signal (Barrett and Hoch, 1998). HK/RR signal transduction systems are different to serine/threonine and tyrosine phosphorylation in higher eukaryotes (Watanabe et al., 2008).

Unlike *Escherichia coli* which has over 30 complete TCSs (Marta et al., 2014), *M. tuberculosis* possesses only 11 complete TCSs, and 8 unlinked sensor kinases and response regulators (Parish et al., 2003; Prisic and Husson, 2014; Zahrt and Deretic, 2000). As there are so many TCSs, a full account of them all is out of the scope of this study (Table 1). Therefore, only relevant selected examples will be elaborated on.

<table>
<thead>
<tr>
<th>TCS</th>
<th>Encoded by</th>
<th>Regulation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SenX3/Regx3</td>
<td>Rv0490/Rv091</td>
<td>Regulates phosphate dependent gene expression</td>
<td>(Glover et al., 2007)</td>
</tr>
<tr>
<td>NarL/NarS</td>
<td>Rv0844c/Rv0845</td>
<td>Regulation of nitrate metabolism</td>
<td>(Malhotra et al., 2015)</td>
</tr>
<tr>
<td>U/U/TCrA</td>
<td>Rv0600c/Rv0601c/Rv0602c</td>
<td>A unique three proteins two-component system unknown function</td>
<td>(Haydel and Clark-Curtiss, 2004); (Shrivastava et al., 2009)</td>
</tr>
<tr>
<td>KdpE/KdpD</td>
<td>Rv1027/Rv1028c</td>
<td>Regulates the response to virulence-related conditions</td>
<td>(Freeman et al., 2013)</td>
</tr>
<tr>
<td>TrcS/TrcR</td>
<td>Rv1032c/Rv1033c</td>
<td></td>
<td>(Fontan et al., 2004)</td>
</tr>
<tr>
<td>TerY/TcrX</td>
<td>Rv3764c/Rv3765c</td>
<td>Involved in virulence</td>
<td>(Parish et al., 2003)</td>
</tr>
<tr>
<td>PrrB/PrrA</td>
<td>Rv0902c/Rv0903c</td>
<td>Involved in early intracellular multiplication during macrophage infection</td>
<td>(Haydel et al., 2012)</td>
</tr>
<tr>
<td>PhoP/PhoR</td>
<td>Rv0902c/Rv0903c</td>
<td>Involved in cell wall lipids complex production</td>
<td>(Pérez et al., 2001)</td>
</tr>
<tr>
<td>MprA/MprB</td>
<td>Rv0981/Rv0982</td>
<td>Regulation of different genes engaged in physiology and pathogenesis</td>
<td>(Curcic et al., 1994)</td>
</tr>
<tr>
<td>DosS-DosT/DosR</td>
<td>Rv3132c/Rv3133c</td>
<td>Involvement in hypoxic adaptation</td>
<td>(Kinger and Tyagi, 1993; Ohno et al., 2003)</td>
</tr>
<tr>
<td>MtrB/MtrA</td>
<td>Rv3245c/Rv3246c</td>
<td>Proliferation in macrophages; essential for <em>M. tuberculosis</em> viability</td>
<td>(Parish et al., 2003)</td>
</tr>
</tbody>
</table>

**Table 1. List of the complete two-component systems of *M. tuberculosis***
1.10.3.1 PhoP/PhoR (Rv0757/Rv0758)

PhoP/PhoR is involved in cell wall complex lipids production and it is required for intracellular growth as observed in the mouse model infection (Pérez et al., 2001). A dramatic change in colony morphology and reactivity to the basic dye neutral red has been observed in *M. tuberculosis* ΔPhoP (Gonzalo Asensio et al., 2006). Deleting PhoP also resulted in the production of safe, live-attenuated *M. tuberculosis* ΔPhoP, which showed better safety and protection than BCG (Martin et al., 2006). PhoP/PhoR is activated in response to environmental stimuli and modulates the mycomembrane via the regulation of the biosynthesis of lipids and is also involved in the regulation of the ESXI secretion system (Frigui et al., 2008; Gonzalo-Asensio et al., 2008).

1.10.3.2 MprA/MprB

The mycobacterium persistence regulator is encoded by Rv0981/Rv0982, and MprA is the RR and MprB the sensor HK. This was the first TCS characterised in *M. tuberculosis* (Curcic et al., 1994) and is only induced during macrophage infection and not in broth cultures, thereby suggesting it is important for the survival of *M. tuberculosis* within macrophages (Zahrt and Deretic, 2000), although recent data suggest otherwise (Bretl et al., 2012; He et al., 2006; He and Zahrt, 2005). MprA/MprB regulates different genes involved in physiology and pathogenesis in *M. tuberculosis* (Bretl et al., 2014; Zahrt et al., 2003) that are required for persistent infection (White et al., 2010). It is conserved in all mycobacterium species, including *M. leprae* (Zahrt and Deretic, 2001) and is found upstream within an operon with *pepD* (encodes an HtrA-like serine protease) and *moaB2* (encodes a predicted molybdopterin biosynthetic protein) (He and Zahrt, 2005; White et al., 2010). Recently,
MprA/MprB has been identified as being part of the conserved cell envelope stress response network and it is negatively regulated by the MprAB extracytoplasmic domain (ECD) (Bretl et al., 2014). Bretl et al. proposed that signalling through the MprA/MprB system is inhibited by the MprAB ECD under normal stress conditions or under conditions where the proteins within the extracytoplasmic space are properly folded. This inhibition is removed when exposed to cell envelope stress because the proteins of the extracytoplasmic space become either unfolded or misfolded. MprA directly regulates the expression acr2 (Pang and Howard, 2007). Pang and Howard concluded that MprA has a direct but complex interaction with the acr2 promoter, which contains multiple binding sites and has indirect interactions with SigE, SigH and HspR. For instance, MprA controls a wide variety of genes and its function may overlap with other regulatory systems.

MprA expression regulates the expression of sigB and sigE (He et al., 2006) which in turn have large regulons. SigE is involved in positive feedback for loop cell wall/membrane damaging agents, such as SDS and controls genes that are involved in the modulation of the inflammatory response and hence is essential for the virulence of M. tuberculosis (Fontan et al., 2008b). MrpA and SigE indirectly regulate one another (Prisic and Husson, 2014; Sureka et al., 2007). MrpA/MprB and DosRS-DosT have been reported to co-regulate genes that may be involved in the general stress response (Bretl et al., 2012). This shows how complex, redundant and overlapping the response to environmental stress is and the degree of overlapping responses to environmental stress that is characteristic of M. tuberculosis.
1.10.3.3 DosS-DosT/DosR

DosS-DosT/DosR or devR-devS- Rv2027c is encoded by \textit{Rv3132c/Rv3133c} and is involved in adaptation to hypoxia (Kinger and Tyagi, 1993; Ohno \textit{et al.}, 2003). \textit{DosR} was originally called \textit{devR} (differentially expressed in the virulent strain) (Dasgupta \textit{et al.}, 2000; Kinger and Tyagi, 1993), and is composed of a RR \textit{DosR} (dormancy survival regulator encoded by \textit{Rv3133c}) and two sensor kinases, \textit{DosS} (\textit{Rv3132c}), a redox sensor and \textit{DosT} (\textit{Rv2027c}), a hypoxia sensor. The ability of \textit{DosT} to phosphorylate \textit{DosR} was first described by Roberts \textit{et al.} (2004). \textit{M. smegmatis} has \textit{DosR} and \textit{DosT} homologues but lacks a \textit{DosS} homologue (Bretl \textit{et al.}, 2011; Lee \textit{et al.}, 2008), whilst \textit{M. leprae} lacks the \textit{DosR-DosS} two component system entirely (Gerasimova \textit{et al.}, 2011). \textit{dosR} and \textit{dosS} are genetically linked and are transcriptionally coupled with each other together with the downstream gene \textit{Rv3134c} (a putative alanine-alanine-rich protein) (Dasgupta \textit{et al.}, 2000). In contrast, \textit{dosT} is encoded at a different location within the genome. \textit{DosR} is the best characterised \textit{M. tuberculosis} RR (He and Zahrt, 2005), modulating its own expression and a 48 gene regulon that responds to hypoxia and nitric oxide (Ohno \textit{et al.}, 2003; Voskuil \textit{et al.}, 2003), and is the primary mediator of a hypoxic signal within \textit{M. tuberculosis} (Park \textit{et al.}, 2003). The 48 genes that are up-regulated by \textit{DosR} are termed the ‘dormancy regulon’ and are clustered into a small number of discrete transcriptional units scattered around the chromosome (Park \textit{et al.}, 2003; Zhang \textit{et al.}, 2010), which may facilitate an efficient, rapid and coordinated response to environmental stimuli (Bretl \textit{et al.}, 2011). \textit{DosR} recognises a 18/20-bp palindromic sequence that is present upstream of almost all hypoxia and nitric oxide regulated genes (Florczyk \textit{et al.}, 2003), including the hypoxic response gene \textit{hspX} (Park \textit{et al.}, 2003).
The promoters of the genes in the dormancy regulon often contain two or more recognition sequences, and cooperative binding of these sites is necessary for their induction. Phosphorylated DosR binds to three copies of the motif at \textit{hspX} (Chauhan and Tyagi, 2008). Exposure to high levels of nitric oxide and carbon monoxide induces the expression of \textit{dosR-dosS} and the genes in the dormancy regulon (Figure 3) (Kumar \textit{et al.}, 2007; Santhosh and Paul, 2013).

**Figure 3. \textit{M. tuberculosis} dormancy response to hypoxic conditions.**

Schematic diagram illustrating the sensor Histidine Kinase (HK) and its cognate Response Regulator (RR) of the Two Component System (TCS). The sensor kinase senses specific environmental signal such as hypoxia, nitric oxide (NO), carbon monoxide (CO) and ascorbic acid leading to its autophosphorylation using ATP as phosphate source. The phosphoryl group is then transferred in a magnesium-dependent manner to an aspartate residue in the RR protein (DosR). Phosphorylation of the DosR leads to the induction and up-regulation of the DosR regulon. The DosR regulon control survival of the bacilli in anaerobically-induced state of dormancy (Marta \textit{et al.}, 2014).
Many studies have described the role of DosR-DoS-DosT system in *M. tuberculosis* pathogenesis. It has been reported that that *dosR* and other members of it regulon are up-regulated in *M. tuberculosis* following infection of human monocytes-derived macrophages, murine BMDM and human monocytes (Dasgupta *et al.*, 2000; Haydel and Clark-Curtiss, 2004; Schnappinger *et al.*, 2003). The clinical highly virulent *M. tuberculosis* W-Beijing strain has been reported to have even higher levels of *dosR* expression (Reed *et al.*, 2007). The immune response to DosR regulon antigens, includes anti-HspX antibodies present in the cerebrospinal fluid (CSF) of human TB meningitis patients and the induction of IFN and the production of other cytokines in T-cell lines and other immune cells of *M. tuberculosis* infected individuals (Haldar *et al.*, 2012; Tai, 2013). Although proteins from the DosR regulon are produced during infection and are immunogenic, its exact role in in *M. tuberculosis* virulence and dormancy remains incompletely understood. This is because studies in which *dosR*, *dosS* and or *dosT* have been mutated have variable and provide inconclusive results (Converse *et al.*, 2009; Pang and Howard, 2007; Parish *et al.*, 2003). Although the expression of the *dosR* regulon is quickly up-regulated following activation by hypoxia, expression decreases over time (Rustad *et al.*, 2009).

### 1.11 Transcriptional Repressors/Activators

From the information available from other microorganisms and based on homology at the sequence level, there are 31 transcriptional repressors which have been identified within the *M. tuberculosis* genome (Fontan *et al.*, 2004).
1.11.1 HspR and HrCA

Two heat shock regulators have been identified in M. tuberculosis; (i) HspR, a transcriptional repressor for Hsp70 regulon members, homologous to Streptomyces HspR; and (ii) HrcA, a RR for Hsp60 (GroE) which is homologous to Bacillus subtilis HrcA. These negatively control the HSR. HspR is a DNA-binding protein related to the MerR family (group of transcription activators) and it recognizes inverted repeat sequences (HAIR; heat inverted repeat) in the promoter region of the hsp70 operon, resulting in a reduction in transcription under normal conditions. In vivo, there is a strong interaction between Hsp70 and HspR (Stewart et al., 2001). The Hsp Acr2, encoded by Rv0251c, has a HAIR like domain 71bp upstream of its start codon. Acr2, along with Hsp70 and Rv0250, were up-regulated in a M. tuberculosis ΔhspR mutant during heat shock (Stewart et al., 2002b). Over expression of Hsp70 proteins reduce the survival of M. tuberculosis and impairs its ability to persist (Stewart et al., 2001).

The Streptomyces coelicolor dnaK operon encodes DnaK (Hsp70; chaperone machine) and HspR (repressor of the operon). HspR represses the dnaK operon by binding to several inverted repeat sequences present in the promoter region (dnaKp). DnaK functions as a transcriptional co-repressor by binding to HspR at its operator sites. DnaK is involved in gene regulation by directly activating the repressor and not by activator inactivation. Similar systems, include DnaK-sigma32 in which DnaK is involved in the regulation of sigma32 synthesis in bacteria (Chattopadhyay and Roy, 2002; Grossman et al., 1987) and Hsp70-HSF in humans where Hsp70 may negatively regulate HSF (heat shock transcription factor) (Abravaya et al., 1992).
The *M. tuberculosis* HSR is under both negative (via HspR and HrcA) and positive regulation (some sigma factors). Three sigma factors (SigH, SigE and SigB) have been reported to be involved in the positive regulation of Hsps. Under heat stress, SigB partially controls the expression of the *hsp70* operon, *acr2* and the chaperons *groEL/groES*. In addition, *acr2* expression seems to be controlled by SigE when bacteria are treated with diamide (Fontan *et al.*, 2004). In addition to controlling the expression of *sigE* and *sigB* under heat shock stress conditions, SigH is also the main regulator controlling the expression of the *hsp70* operon and *clpB* (Manganelli *et al.*, 1999).

### 1.11.2 ClgR the *clp* Gene Regulator

ClgR, encoded by the *clp* gene (Rv2745c), is a transcriptional regulator that regulates the transcription of more than ten genes, including four protease systems (ClpP1/C, ClpP2/C, PtrB and HtrA-like protease Rv1043) and three genes encoding chaperones (Acr2, ClpB and chaperonin Rv3269). ClgR is highly up-regulated in *M. tuberculosis* under conditions such as heat stress and during macrophage infection. The regulation of these systems by ClgR is essential for *M. tuberculosis* replication in macrophages during early infection (Estorninho *et al.*, 2010). ClgR is highly induced by redox, and SigH and SigE overexpression was found to be down-regulated in *M. tuberculosis* ΔclgR; therefore it may play a role in the management of the intra-phagosomal environment (McGillivray *et al.*, 2014). McGillivray et al. also observed that *ClgR* expression correlates with the expression of genes involved in sulphate assimilation, hypoxia and reaeration. It was concluded that ClgR has many different stress-related functions and is important for *M. tuberculosis* pathogenicity regardless of its role in the induction of the Clp proteolytic pathway. As there are similarities in the structure and function between sHsps, it is possible that *M. tuberculosis* Acr1 and Acr2 proteins may
play a role in protection against nitrosative stress, possibly by interacting with the mycobacterial proteasome (Forrellad et al., 2013). The fact that Acr2, along with other proteases, is positively up-regulated by ClgR (clp gene regulator) suggests that it may play a role in protein homeostasis (Estorninho et al., 2010).

1.12 Proteases

Proteolysis in bacteria plays an important role in cellular function, and there are five classes of ATP dependent proteases, including ClpAP, ClpXP, Lon, HsIUV (also known as ClpYQ), HflB (FtsH) (Koodathingal et al., 2009). These proteases play a key role in the protein quality control system via degrading truncated, misfolded, aggregated or denatured proteins. Clp also plays a role in the regulation of the function of certain regulatory proteins within the cell (Russo et al., 2009). In B. subtilis the proteolytic subunit ClpP, along with the regulatory ATPase subunit ClpC or ClpX, is required for the normal response to stress, the development of genetic competence, and sporulation (Nakano et al., 2002). M. tuberculosis has two ClpP proteolytic subunits (ClpP1 and ClpP2) and both are essential for in vitro growth, but ClpP1 and ClpP2 are co-expressed in normal conditions and their promoter is not induced by heat shock or oxidative stress; both are positively regulated by ClgR. ClpP1 and ClpP2 recognise different substrates, as ClpP1 degrades ssrA-tagged proteins whereas ClpP2 does not (Personne et al., 2013). ClgR became accumulated in a Corynebacterium glutamicum ClpC mutant, indicating that the transcription regulator ClgR is also a substrate for both ClpP1 and ClpC (Engels et al., 2005).
1.13 Translational Regulation of Gene Expression in Bacteria

Small non-coding RNA (sRNA) are the most available class of translational regulators present within bacteria (Lorraine and George, 2013; Papenfort and Vogel, 2009), and another sRNA is the 5′-UTR. Recent studies have revealed the role of sRNA, 5′-UTR, riboswitches, antisense transcripts and tmRNA in translational gene regulation. For the purpose of this study only tmRNA will be expanded on in section 1.19.

1.14 Regulation of Acr1 and Acr2 Gene Expression

In order for *M. tuberculosis* to adapt and survive within the hostile environment of a host the ability to sense the surrounding and respond appropriately and effectively is required. *M. tuberculosis* has a host of signal transduction systems, including TCSs, Ser/Thr protein kinases, and orphaned RRss (Bretl et al., 2011; Cole et al., 1998; Fernandez et al., 2006). There are more than 200 *M. tuberculosis* genes that show rapid changes in expression following phagocytosis and a rapid reprogramming occurs involving 400 transcriptional changes within 24h of infection (Rohde et al., 2007; Schnappinger et al., 2003). One of many responses *M. tuberculosis* utilises to overcome the environmental stress experienced within a macrophage is to produce stress proteins (Schnappinger et al., 2003) including Hsps.

There is experimental evidence that hypoxia may signal the induction of latency (Wayne and Sohaskey, 2001). In addition, nitric oxide has been identified as an environmental trigger for *M. tuberculosis* dormancy/persistence (Park et al., 2003; Voskuil et al., 2003). Acr1 is
known to be associated with LTBI and its role in the survival of mycobacteria in the non-replicating phase is well established (Cunningham and Spreadbury, 1998). The acr1 gene is under the control of the TCS DosS/DosT-DosR. DosT and DosS are sensors kinases that respond to low oxygen tension and nitric oxide, while DosR is the transcriptional regulator (Kumar et al., 2007). This two component regulatory system (dosRS dormancy survival regulator and sensor) controls the response to hypoxia in M. tuberculosis and regulates the transcription of approximately 50 genes, most of which are predicted to protect against hypoxia (Hu et al., 2006; Rohde et al., 2007) and nitric oxide (Converse et al., 2009; Kumar et al., 2007).

The expression of acr2 is under the negative control of HspR and positive control of SigH and SigE (Stewart et al., 2002b), as well as the MprA/MprB TCS (Pang and Howard, 2007). Pang and Howard (2007) studied the effect of heat shock and SDS stress on ∆mprAB mutant by analysing mRNA and comparing it to the parental stain. Their study revealed that the level of acr2 gene expression was higher under heat shock but lower when subjected to SDS stress compared to the parental strain. They also reported that MprA has four binding sites, three of which (one overlaps with the house-keeping SigE, SigH and HspR predicted binding site) are downstream of the acr2 gene transcriptional start point (TSP) and one of which is upstream. PhoP has been reported to be a repressor of acr2 expression under normal growth conditions (Singh et al., 2014a), and acr2 is among the genes that have been identified to be regulated by the protease Clp regulator ClgR (Estorninho et al., 2010).
Table 2. Summary of the different regulatory systems of *M. tuberculosis* α-crystalline 1 and 2

### 1.15 Stress Response of *M. tuberculosis*

The ability of *M. tuberculosis* to survive inside a macrophage by evading its killing mechanisms gives it a huge advantage. *M. tuberculosis* is a very successful pathogen, employing a wide variety of tools in order to overcome all the stresses that it encounters inside the human body. *M. tuberculosis* has many mechanisms to overcome the microbiocidal ability of macrophages, including resistance to toxic compounds, preventing phagosome maturation, and preventing the induction of apoptosis (Forrellad *et al.*, 2013). The intercellular environment subjects *M. tuberculosis* to many stresses, examples of which include nutrient limitation, hypoxia, oxidative stress, nitric oxide and low pH (Rohde *et al.*, 2007; Schnappinger *et al.*, 2003; Voskuil *et al.*, 2003). The response of *M. tuberculosis* to
stress involves a very complex network of gene regulation and a comprehensive review of the literature to give a full account of this is beyond the scope of this study. Therefore, only what is relevant to the genes under study will be examined.

1.15.1 Heat Shock

Fever and night sweats are symptoms associated with TB infection, and a high body temperature is one of defence mechanisms that the body uses to fight the invading microbe. Raising the temperature beyond the optimum for *M. tuberculosis* growth subjects it to stress and leads to the mis-folding of proteins, rendering them non-functional and subject to degradation. To overcome this issue, *M. tuberculosis* up-regulates the expression of chaperonins, such as Hsp Acr2 and Hsp20.

1.15.2 Response to Acid pH

Inside a non-activated macrophage *M. tuberculosis* is located within phagosomes where it has the ability to block phagosomal maturation, lysosomal fusion, and acidification (Clemens and Horwitz, 1995; de Chastellier, 2009; Sakamoto, 2012), but IFN-γ activation restores acidification and confers anti-mycobacterial activity (Herbst et al., 2011; Vandal et al., 2008). Depending upon the level of activation, the pH of the macrophage compartment ranges from 4.5-6.2 (Schaible et al., 1998; Vandal et al., 2009). *M. tuberculosis* has been reported to survive at pH 4.5 *in vitro* (Vandal et al., 2008). However, the interpretation of Mycobacteria resistance to acid in *in vitro* experiments is complicated because the survival of bacteria in acid is dependent upon the culture conditions, including bacterial density and composition of the media (Bodmer et al., 2000; Sung and Collins, 2003).
However, studies have shown that the cell wall forms a physical barrier that prevents toxic molecules from entering into the cell (Hett and Rubin, 2008). This plays an important role in resistance to acid, and many of the genes that are up-regulated during exposure to acid stress are related to the cell wall (Richter et al., 2007; Singh et al., 2005; Singh et al., 2003; Tran et al., 2005). OmpA is a pore forming protein which is encoded by OmpATb and induced at pH 5.5 (Raynaud et al., 2002). It is involved in generating a rapid ammonia burst leading to neutralisation of the medium pH before exponential growth of *M. tuberculosis* *in vitro* (Song et al., 2011). In their study, Song et al. (2011) observed the acceleration of *M. tuberculosis ompATb* mutant growth after ammonia was added, suggesting that ammonia is one of the mechanisms that *M. tuberculosis* uses to neutralise an acid environment. However *in vivo* *ompATb* is not the only mechanism, as it was observed in a mouse infection model that there were multiple mechanisms for resisting phagosomal acidification (Song et al., 2011). This may be because the response to acid stress can be associated with protection against other forms of stress, such as SDS and heat shock (Vandal et al., 2009). The TCS PhoP/PhoR regulon may also play a role in enabling *M. tuberculosis* to resist an acidic environment, as it contains many genes that are up-regulated during acidic stress, and this up-regulation was lost in a *phoP* mutant (Gonzalo-Asensio et al., 2008).

### 1.15.3 Response to Oxidative Stress

The ability of *M. tuberculosis* to overcome and resist killing by macrophages is vital for its virulence. Bactericidal compounds, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced by phagocytes after phagocytosis. Nitric oxide and related nitrogen intermediates (RNI) are effective anti-mycobacterial agents and signal-transducing molecules. RNI play a role in *M. tuberculosis* gene regulation of expression *in*
vivo, as they up-regulate genes that may be conducive to the survival of TB bacilli in the infected host, and along with hypoxia may regulate mycobacterial gene expression via overlapping signal transduction pathways (Ohno et al., 2003). Their bactericidal properties result from their ability to react with wide range of molecules, including nucleic acids, proteins, carbohydrates and lipids. Intracellular pathogens, such as *M. tuberculosis*, have evolved to have counter mechanisms to overcome the damaging effects of oxygen and nitrogen intermediates (ROI and RNI, respectively).

*M. tuberculosis* uses alternative defence mechanisms for oxidative and nitrosative stresses other than OxyR mechanism. This is because OxyR; ahpC (alkyl hydroperoxide reductase C) regulator, is not functional in all *M. tuberculosis* complex (MTBC) including *M. bovis*, *BCG*, *M. africanum* and *M. microti* (Deretic et al., 1995). *M. tuberculosis* uses wide range of molecules to either detoxify ROI and RNI before they cause stress or by repairing damage they have caused (Sandgren et al., 2009). There are various proteins, including AhpC, superoxide dismutase (SOD), KatG, Mel2, Rv2136c, Rv224c, PonA2, Acr1 and Acr2 that are directly or indirectly necessary for *M. tuberculosis* complex species to survive the oxidative and nitrosative stresses, and at the same time all also important for virulence. Rv2136c, Rv224c, PonA2 are thought to contribute to cell wall integrity, which may suggest that the mycobacterial lipid-rich cell envelope can act as an effective barrier against the entry of RNI and ROI (Forrellad et al., 2013).
1.15.4 Hypoxia

Low oxygen is one of many stress conditions that *M. tuberculosis* encounters in the central part of the granuloma (Cunningham and Spreadbury, 1998). The response to low oxygen tension is initially controlled by the TCS DosS/DosT-DosR was describe in section 1.10.3.3. The DosR regulon was not found to be essential for virulence in chronic murine infection (Bartek *et al.*, 2009), and it was suggested that *M. tuberculosis* has evolved to encode DosR in order to deal with survival in an anaerobic environment rather than for pathogenesis or antibiotic resistance. It has also been suggested that there may be genes other than the DosR regulon which contribute to *in vitro* and *in vivo* dormancy (Rustad *et al.*, 2008). However, the DosR regulon has been reported to be required for metabolic processes from the point of entry into the dormancy state and throughout, as it is required for the quick transition between hypoxic and aerated environment without damaging or killing the bacterial cell (Leistikow *et al.*, 2010). Generally, all studies however agree that DosR is essential for the survival of *M. tuberculosis* in the hypoxic environment, and deletion of dosR blocks the initial hypoxic response (Rustad *et al.*, 2008). The DosR regulon includes many genes, and prominent amongst them is *acr1*. Acr1 is highly expressed during hypoxia (Rosenkrands *et al.*, 2002) and has chaperonin activity protecting proteins from misfolding during stress conditions. It is important for the survival of a phagocytosed *M. tuberculosis* and it accumulates during the latent stationary phase of TB infection (Yuan *et al.*, 1996), consequently it is a marker for LTBI (Dubaniewicz *et al.*, 2012). Other regulators also play a role in the hypoxic response, including SigE and SigC.
1.15.5 Role of Toxin AntiToxin Systems

The toxin-antitoxin (TA) system is composed of stress-responsive genetic elements (Ramage et al., 2009) that are ubiquitous in prokaryotic genomes and provide mechanisms by which bacteria can quickly alter their growth rate in response to environmental stress. TA loci were first identified in E. coli (Ogura and Hiraga, 1983). The toxin is not a protein secreted to target the human host but instead targets the bacterial cellular components. The TA system is composed of two parts; the toxin and its antagonist anti-toxin. The toxin is a stable protein while the anti-toxin (either a protein or sRNA) is less stable (Unterholzner et al., 2013). Under appropriate growth conditions, the anti-toxin binds to its conjugate toxin and inhibits its toxin effect. However, when cells are exposed to environmental stressors, such as high temperature or starvation, stress-induced proteases degrade the anti-toxin leading to the release of the free toxin, which then is free to interact with the target cellular component and eventually results in cell death (Lee et al., 2015). TA systems have been reported to be important for many cellular functions, such as multidrug tolerance (Korch and Hill, 2006), biofilm formation (Wang and Wood, 2011), and cellular growth arrest under stress conditions (Yamaguchi and Inouye, 2011). To date there are five classes of TA systems (I-V) based on their genetic structure and regulation (Unterholzner et al., 2013). Based on how an antitoxin (as a protein or sRNA) interacts with the toxin, TA systems are grouped into three major groups: (i) in type I systems the antitoxin RNA acts as anti-sense RNA for the toxin mRNA, thereby inhibiting its translation; (ii) in type II systems the anti-toxin protein neutralises the toxin protein; and (iii) in type III systems an RNA antitoxin inhibits toxicity by binding to the protein toxin to form an RNA-protein complex (Lee et al., 2015; Unterholzner et al., 2013), (iv) in type IV, the toxin CbtA interfere with the cell division by inhibiting the cytoskeletal proteins MreB and FtsZ polymerisation (Masuda et al., 2012),
(v) in type V the antitoxin GhoS (identified as endoribonuclease) specifically cleaves the toxin GhoT which encodes a membrane-lytic peptide (Wang et al., 2012).

**Figure 4. Different toxin-antitoxin systems based on their mode of action**

Schematic diagrams illustrating the functions of the different Toxin-antitoxin systems; (a) The antitoxin in type I acts as anti-sense inhibiting the translation of the toxin; (b) the antitoxin in type II inhibits the toxin toxicity by neutralising it in a protein to protein interaction; (c) the antitoxin RNA in type III inhibits the toxicity of the toxin by binding to the toxin protein and form RNA-protein complex; (d) the antitoxin in type IV inhibits the polymerisation of the cytoskeletal proteins MreB and FtsZ necessary for the cell division; (e) The antitoxin in type V acts as a endoribonuclease cleaving the toxin mRNA preventing its transcription (Wen et al., 2014)
TA systems are highly conserved within the *M. tuberculosis* complex (Tiwari *et al.*, 2015) and there are 79 TA systems present in the chromosome, 68 of which are well known and 11 which are novel TA systems. Some are strongly induced in drug-tolerant persisters (Sala *et al.*, 2014), while some have been shown to be activated by phagocytosis of TB bacilli by macrophages or hypoxia. The *M. tuberculosis* TA system VapBC30 has been reported to regulate cellular growth (Lee *et al.*, 2015), HigB toxin expression, where in the absence of its anti-toxin HigA resulted in growth arrest and cell death (Schuessler *et al.*, 2013), while over expression of MazF toxin induced bacteriostasis and enabled *M. tuberculosis* to adapt to oxidative stress, nutrient depletion and exposure to antibiotics (Tiwari *et al.*, 2015) and cell cycle regulation (Ramirez *et al.*, 2013).

### 1.15.6 Role of tmRNA

Protein synthesis, a description of the structure of tmRNA and its function are provided in details in section 0 and 0. In this section selected examples are described to highlight its role in the stress response.

The role of transfer-messenger RNA (tmRNA) in gene regulation and its expression in *M. tuberculosis* is not yet fully elucidated and further research is required to have a clear understanding of the role of tmRNA in *M. tuberculosis*. However, tmRNA is ubiquitous, highly conserved, and there have been studies to understand its role in other microorganisms. In addition to its role as a rescuer for ribosomes, tmRNA is a regulatory RNA which means its production and removal must be controlled to ensure appropriate physiological responses. It is cell cycle regulated and is important for the control of cell cycle progression in
Caulobacter crescentus (Hong et al., 2005), where the proteolysis of tmRNA substrates is required for the correct timing of cell cycle regulated events (Hong et al., 2007). Studies of the soil-dwelling, antibiotic-producing bacterium Streptomyces coelicolor (Barends et al., 2010) reveal that tmRNA mediated trans-translation plays a role in stress management. Barends et al. (2010) analysed proteins that were His8-tagged using recombinant tmRNA and identified ten target transcripts whose translation was dependent upon tmRNA: DnaK, Hsp70, thiostrepton-induced protein A, universal stress protein A, elongation factor Tu3, and the cell-cycle control proteins DasR, SsgA, SsgF, and SsgR. Phenotypically, deletion of tmRNA from S. coelicolor resulted in inefficient sporulation, and this phenotype may relate to the tmRNA-tagging of sporulation regulators, SsgR, SsgA and SsgR proteins (Barends et al., 2010).

An in vitro study of C. crescentus identified 73 tmRNA tagged proteins, including proteins involved in DNA replication, recombination and repair (Hong et al., 2007). Deletion of the tmRNA gene affects the timing of loss of motility, the initiation of DNA replication, cell division, and the production and removal of cell-cycle-regulated proteins. Although this function of tmRNA has not been observed in other bacteria, it may be a feature of actinomycetes such as Mycobacterium.

1.16 Protein Synthesis

Protein synthesis is very important for the survival of all living cells, and synthesising protein is very expensive in terms of the energy needed, which requires approximately 50% of the energy of growing cells. About 20-40% of the total protein produced by E. coli is used to
synthesize ribosomes and other translation factors (Keiler, 2015). Ribosomes are large complexes composed of proteins and RNA, and they are the production machinery of proteins. Protein synthesis starts with copying of the genetic code of a gene (coding for a particular protein) present in the genome through a process known as transcription to produce mRNA.

**Figure 5. Protein synthesis**

Illustration of the proteins synthesis phases of initiation, elongation and termination. The bacterial Ribosome 70S composed of two subunit 50S and 30S. The initiation complex composed the initiator tRNA; fMet-tRNA (Formylmethionine tRNA) at the P-site (production site) interact with start codon of the mRNA by forming codon-anticodon duple. Following the binding of the ternary complex of aa-tRNnA-EF-Tu-GTP (aminoacyl-tRNA-elongation facto Tu-GTP) the initiation complex enters the elongation phase. The GTP is hydrolysed and the EF-Tu-GDP and the phosphate (P) detached from the ribosome following successful decoding. Then the aa-tRNA moves into the A-site (attachment site). The elongation phase occurs through a process known as peptidyl transfer when the nascent peptide chain is transferred from the peptidyl-tRNA in the P-site to the aa-tRNA in the A-site extending the peptide chain by one amino acid. The translocation of the tRNA-mRNA complex is facilitated by the EF-G-GTP from A-and P sites to the P-and E- sites (Exit site). A reversal translocation step (dash arrows) can be carried out by EF4-GTP to mobilise stalled ribosomes. The termination phase of protein synthesis occurs when the stop codon enters the A- site assisted by release factors. The free ribosome now be recycled and the process starts again (Yamamoto et al., 2014).
Ribosomes receive mRNA for a specific protein and decode (translate) the DNA code into peptides to produce a polypeptide chain, i.e. proteins. The translation process takes place in three steps, termed initiation, elongation and termination. The process of protein synthesis is a very highly regulated process and is optimised for both correctness of the translation (fidelity) and turnover time (Ramakrishnan, 2002). Bacteria ribosomes (70S) are composed of two different non-additive sizes subunits, termed 30S and 50S. Each subunit has three binding sites for tRNA, termed aminoacyl sites, also known as attachment sites, to which aminoacylated tRNA attaches, peptidyl sites, also known as production sites, which hold the aminoacylated tRNA transfer the amino acid to the nascent peptide chain and the exit site which holds the deacylated tRNA before leaving the ribosome. Therefore, both subunits are involved in the translocation of the mRNA from aminoacyl site to the peptidyl site and exit site, translating a single codon at a time and adding an amino acid to the nascent polypeptide chain (Figure 5).

Whilst the process of synthesising proteins is very energy expensive, it is vital to produce the correct protein in order to serve its biological function within the cell. Therefore, it is very important to check the fidelity, a process that takes place in the 30S subunit that binds to the mRNA and the anticodon stem-loop of the tRNA, where incoming aminoacylated tRNA is checked for binding to the correct mRNA codon. The acceptor arm of the aminoacylated tRNA binds to the 50S subunit that catalyses the peptide bond formation between the incoming amino acid at the aminoacyl site and the nascent peptide chain at the peptidyl site.
Because a bacterial cell consumes a lot of energy in making ribosomes and because ribosomes are the decoding/translation apparatus, if production stalls then it is more sensible for the cell to rescue, and free a stalled ribosome and then recycle it into the protein production process. There are multiple reasons and situations for ribosome stalling on an mRNA to occur: (i) the ribosome has reached a stable secondary structure on the translated mRNA (Tholstrup et al., 2011); (ii) a lack of a charged tRNA for the specific amino acid codon; (iii) a stop codon (Sunohara et al., 2004); (iv) a lack of or low level of ribosomal release factors (Garcia-Pineres et al., 2007); (v) a ribosome reaches a cluster of rare codons (Li et al., 2006); (vi) as a part of the cell cycle or metabolic regulations (Garza-Sánchez et al., 2006); and (vii) the mRNA lacks a stop/termination codon (Klauer and van Hoof, 2012)

1.17 Transfer Messenger RNA

Stalled ribosomes are rescued by a process known as trans-translation, which is mediated by tmRNA or SsrA. When a ribosome become stalled because of defective mRNA, tmRNA recognises and binds to it and its peptide tag sequence is translated to tag the incomplete protein for degradation. Therefore, tmRNA serves as a global quality control system and increases the fidelity of gene expression via promoting the synthesis of full length proteins, and it plays an important role in bacterial development, pathogenesis and environmental stress responses (Janssen and Hayes, 2012; Keiler and Ramadoss, 2011). Ribosomes are targeted by tmRNA not only because of truncated mRNA, as it has been observed that tmRNA tagging occurs even on full length proteins and incomplete proteins can be synthesised from full length mRNA. This observation suggests that ribosome stalling, rather than mRNA truncation, triggers tmRNA tagging.
This type of messenger RNA, also known as 10Sa and SsrA (small stable RNA A) was first identified in *E. coli* by Ray and Apirion (1979). It is conserved in all bacteria, is cell cycle controlled (Hong *et al.*, 2005) and its activity is required for normal cellular physiology (Hong *et al.*, 2007). It is essential for some bacteria but not all (Huang *et al.*, 2000; Julio *et al.*, 2000), and is ubiquitous, as it is found in chloroplasts, eubacteria, and mitochondria of some eukaryotes (Andersen *et al.*, 2006; Gueneau de Novoa and Williams, 2004). It combines the properties of mRNA and tRNA, therefore it was renamed tmRNA (Atkins and Gesteland, 1996). It was first identified in *M. tuberculosis* in 1992 (Tyagi and Kinger, 1992).

Normally, tRNA does not encode any protein and therefore it is not translated; however, tmRNA carries a peptide tag sequence of 10-13 amino acids in its mRNA domain. Therefore, tmRNA has a dual function: (1) to free a stalled ribosome at the 3’ end of truncated mRNA; and (2) to tag the nascent incomplete protein for degradation (Hayes *et al.*, 2002; Keiler, 2011) by different protease complexes, including ClpXP, ClpAP, FtsH (Ahlawat and Morrison, 2009; Baker and Sauer, 2012; Dziedzic *et al.*, 2010), Tsp and Lon (Choy *et al.*, 2007). tmRNA binds only to 70S ribosomes and not 30S or 50S subunits or polysomes (Komine *et al.*, 1996; Tadaki *et al.*, 1996). Small protein B (SmpB) and tmRNA (Figure 6) are a very important protein quality control mechanism within bacterial cells (Keiler, 2007). Control via trans-translation and tagging ensures that incomplete proteins and damaged RNA are degraded, prevents the accumulation of non-functional and potentially harmful products, and at the same time frees stalled ribosomes to be recycled for more productive translation.
1.17.1 Structure of tmRNA

tmRNA size ranges from 230 to 400 nucleotides (Janssen and Hayes, 2012), and in *M. tuberculosis* it is 368 nucleotides; previously it was thought to be 607 nucleotides but recently it has been re-annotated and the size reduced by 239 nucleotides. Structural variations are seen in some cyanobacteria and proteobacteria, where the functional tmRNAs are composed of two non-covalently linked RNA chain k (Keiler et al., 2000). The two domains, the tRNA like domain (TLD) and mRNA domain (MLD) are the most conserved features of tmRNA.

tmRNA has four pseudoknots (pK1-4) that are present in the TLD and MLD of most bacterial species. The role of pk1 in tmRNA function is purely structural, and it can be replaced by a variety of hairpin structures which help to stabilise the region enclosed by the TLD and MLD, preventing tmRNA misfiling, however deleting pk1 entirely is damaging for protein tagging (Tanner et al., 2006; Wower et al., 2009). tmRNA folding and maturation seem to be the primary role of pk2, pk3 and pK4, not its trans-translational activity (Wower et al., 2004). A reduction in the number of pseudoknots does not decrease the tagging efficiency of two-piece tmRNA (Gaudin et al., 2002) in α-proteobacteria, cyanobacteria and β-proteobacteria (Keiler et al., 2000).

The TLD interacts with the ribosome in the same way as canonical tRNA, and in tRNA the stem is extended by a 3′-terminal codon CCA (cytidine-cytidine-adenosine) but can only be aminoacylated with alanine (Komine et al., 1994; Ushida et al., 1994). The pk2, pk3 and
pk4 form an arc around the head of the 30S subunit and during translocation from the aminoacyl to the peptidyl site, the region composed of hp2a to hp2d, and pk1 undergoes significant conformational changes, while tmRNA maintains its overall structure (Wower et al., 2014). pK1 partially unfolds as the TLD is translocated to the exit site (Fu et al., 2011).

MLD is constructed of a short single strand open reading frame (ORF) for the proteolytic tag, and the ORF can contain 8-35 residues (Guidice et al., 2014), although there are 10 residues (A)ANDENYALAA in E. coli (Moore and Sauer, 2007) and 12 residues (A)ADSHQRDYLAA in M. tuberculosis, with the first (A) carried by the tmRNA. In addition, the MLD has a conserved helix (H5) carrying a termination codon (Guidice et al., 2014). The MLD differs from canonical mRNA in three aspects: (i) it does not carry any start sites upstream to the AUG initiation codon, such as a Shine-Dalgarno sequence; (ii) it uses an alanine and sometimes a glycine codon as a resume codon; and (iii) the frame selection is determined by the five nucleotides immediately upstream of the resume codon (Karzai et al., 2000).

**Figure 6. Structure of tmRNA**

The diagram on the left shows the secondary structure of tmRNA highlighting the various domains, such as the TLD, pseudoknots (PK1, 2, 3 and 4) and the proteolytic tag ORF. On the right is the atomic model for the tmRNA-SmpB complex. The tag sequence presented in this diagram is for E. coli not M. tuberculosis. (Andini and Nash, 2011)
1.17.2 SmpB Function

For tmRNA to function it needs protein cofactors, such as SmpB and EF-Tu (elongation factor) and ribosomal protein S1, which bind to either free tmRNA or the tmRNA:SmpB complex by connecting the MLD, pk2 and pK3 (Figure 7) (Shi et al., 2011). SmpB is a protein which has high affinity to tmRNA and is encoded immediately upstream of ssrA in E. coli (Moore and Sauer, 2005); however, in M. tuberculosis it is separated by Rv3099c, a hypothetical protein which may have a related function to the smpB-tmRNA complex. SmpB plays an important role in tmRNA structure, stability and activity (such as trans-translation). It has been suggested that the interaction of SmpB with tmRNA is divided into two parts: its interaction with TLD to facilitate processing, aminoacylation, binding to the ribosome and peptidyl-transfer, and the interaction with the upstream region of the peptide tag sequence to assist in the mRNA function (Kurita et al., 2007). It has also been found that SmpB protects tmRNA from RNaseR degradation (which degrades tmRNA from a non-tRNA-like 3’ end), as it has been observed in C. crescentus that the level of SmpB during the cell cycle correlates with ssrA RNA stability, which means SmpB binding controls the time of ssrA mRNA degradation (Hong et al., 2005). The level of ssrA mRNA has been reported to be significantly reduced in M. tuberculosis ΔsmpB compared to wild type (Personne and Parish, 2014).

Kurita et al. (2007) in their study stated that ‘SmpB serves to bridge two separate domains of tmRNA to determine the initial codon for tag-translation.’ It has also been reported (Kurita et al., 2010) that the C-terminal tail of SmpB has a role in placing the alanyl-tmRNA-SmpB complex into the aminoacyl site of the ribosome following hydrolysis of GTP by EF-Tu. Kurita et al. also reported that mutation of the C-terminal tail prevented peptidyl-transfer or
analy-tmRNA and aminoacyl-site binding of SmpB. tmRNA also binds to EF-Tu, which facilitates a productive interaction with a stalled ribosome. SmpB has also been suggested to be involved in the survival of *M. tuberculosis* within macrophages (Tyagi *et al.*, 1996).

### 1.17.3 Trans-Translation Process

Ribosome stalling on truncated mRNA is required for tmRNA recruitment, and tmRNA tagging is the fate of the majority of the proteins that are synthesised from non-stop mRNA. The trans-translation process happens very quickly when there is no codon or an complete codon is present at the aminoacyl site, but transfer still can take place when a transcript extends up to six nucleotides down-stream the of aminoacyl site codon (Janssen and Hayes, 2012). The efficiency of tmRNA action decreases rapidly with the increasing down-stream length of mRNA, nearly reaching zero if it exceeds 15 bases (Ivanova *et al.*, 2004). Ivanova *et al.* (2004) also reported that *in vivo*, tmRNA action does not occur on ribosomes containing full length mRNAs.

As it is seen in Figure 7, before binding to the protein cofactors, tmRNA is first aminoacylated with alanine by alanyl-tRNA synthetase. Alanyl-tmRNA binds to SmpB and EF-Tu, and enters the aminoacyl site of the stalled ribosome without a codon anticodon interaction unlike tRNA (SmpB occupies the position of the anticodon). The truncated polypeptide is transferred to the alany-tmRNA and the complex (nascent polypeptide, alanyl-tmRNA complex) is translocated to the peptidyl site and the mRNA is released and degraded by RNases. Following the translocation and release of the mRNA, the tmRNA peptide tag code sequence is translated. Translation is terminated when it reaches the
termination codon at the end of the tmRNA tag ORF, leading to the release of the tagged protein and freeing the ribosome. The tmRNA tag at the 3’ end of the tagged polypeptide can be recognised by many proteases, including FtsH, ClpAP, ClpXP, Tsp and Lon protease (Ahlawat and Morrison, 2009; Choy et al., 2007; Gur and Sauer, 2008; Karzai et al., 2000).

Figure 7. tmRNA function

Schematic diagram describing the processes involved in freeing the stalled ribosome by the tmRNA. Obtained from http://www.scitopics.com/tmRNA_SsrA_and_trans_translation.html (Keiler, 2011)

Trans-translation is part of the regulatory network or example the action of RelE and MazF (TA systems) which generate vast numbers of truncated mRNAs leading to non-stop translation complexes induces stasis, permitting a cell to conserve resources during severe stress (Keiler and Feaga, 2014). The ssrA gene encoding tmRNA has also been suggested to function as a cell cycle progression controller (Hong et al., 2005), to help survive during stress conditions, such as exposure to antibiotics (Andini and Nash, 2011; Braud et al., 2006;
Palečková et al., 2006; Yang and Glover, 2009) heat shock and oxidative stress (Paget et al., 2001).

1.17.4 Trans-Translation Independent Ribosomal Rescue

The release of a stalled ribosome at the 3’ end of an mRNA lacking a stop codon is essential for a bacterium to continue protein synthesis. The tmRNA based trans-translation pathway to release such stalled ribosomes is found in almost all bacteria, and is essential in some but in others can be inactivated or disabled. Deleting ssrA in bacteria where it is non-essential produces a mildly defective phenotype but its function can be complemented by the ssrA_{DD} variant (Keiler, 2008; Moore and Sauer, 2007). The non-essentiality of tmRNA trans-translation in E. coli is due to the presence of an alternative rescue mechanism mediated by the protein YhdL, which has been renamed the alternative rescue factor A (ArfA). ArfA is a small protein that allows RF2 (a class-1 release factor that recognises U(A/G)A) to hydrolise peptidyl-tRNA on a nonstop translation complex (Figure 8). ArfA is a trans-translation backup, and is itself subject to the trans-translation process as it is constructed of a truncated mRNA; therefore, in the presence of a functional tmRNA it is tagged and degraded.
**Figure 8. Alternative pathway for ribosome rescue ArfA and ArfB**

ArfA and ArfB are a backup system for the rescue of stalled ribosomes when the trans-translation process is impaired. (a) ArfA recognises the non-stop complex and binds in or near the empty mRNA channel. The binding of the ArfA facilitate the binding of the peptide chain release factor 2 (RF2) to the aminoacyl-tRNA binding site. RF2 catalyses hydrolysis the hydrolysis of peptidyl-tRNA bond resulting in the release of the ribosome, the nascent peptide and the mRNA (there is no tagging for degradation). The arfB does not require RF2 to function therefore, it binds directly and catalyses the release of the ribosome along with the nascent peptide and the mRNA.

(Keiler, 2015)

Therefore, ArfA is only needed when the tmRNA trans-translation is disabled or overwhelmed, and only then is it produced and it takes over the function or helps tmRNA to rescue the stalled ribosomes. Deletion of both ssrA and arfA is lethal for *E. coli*, and ssrA is essential in ΔarfA cells (Chadani et al., 2010). *Shigella flexneri* does not possess a gene
encoding ArfA and therefore ssrA is essential for its survival, although ssrA can be deleted in S. flexneri which express the E. coli arfA gene (Ramadoss et al., 2013). ArfB (YaeJ) is another protein paralogue for the release factors (RFs) and directly catalyses peptidyl-tRNA hydrolysis within non-productive translation complexes (NTCs) (Chadani et al., 2012) in a manner similar to that for tmRN-smpB recognition (Keiler and Feaga, 2014) and is non-essential in either ssrA⁺ or ΔssrA E. coli (Handa et al., 2011). ArfB can function alone to rescue stalled ribosomes but ArfA requires the presence of RF2 (Shimizu, 2012). ArfB is widely distributed across α, β, γ and δ proteobacteria, bacteriodetes, actinobacteria and cyanobacteria, but is absent from firmicutes (Janssen and Hayes, 2012). Its homologue is also found in eukaryotes, including human ICT1, which is an essential protein and an integral component of the mitochondrial ribosome (Richter et al., 2010). PrfH is another E. coli putative stop-codon independent RF although its function may be redundant with ArfB (Baranov et al., 2006) and it is not essential, as E. coli ΔssrA ΔyaeJ ΔprfH exhibit no growth impairment or phenotype compared to E.coli ΔssrA cells under normal laboratory conditions (Janssen and Hayes, 2012). C. crescentus ssrA is not essential for its survival and it does possess an arfA homologue. Instead, it has been reported to have a protein known as CC1214 that has homology to E. coli ArfB, and this can hydrolyse peptidyl-tRNA in nonstop translation complexes in vitro (Feaga et al., 2014).
1.18 Study Rationale

*M. tuberculosis* is such a successful pathogen due to the plethora of mechanisms it possesses to establish itself and overcome the various stresses it encounters in the harsh environment of the human body. The high risk to public health, which has become even greater with the occurrence of multidrug resistant strains, necessitates the discovery of new antimycobacterial drug treatments. The ability of *M. tuberculosis* to persist in the body in a dormant state adds to the difficulty in treatment. Therefore, in depth understanding of its behaviour is important. Acr1 and Acr2 are both employed by *M. tuberculosis* to resist different stresses inside the body, as well as to modulate the host response to its benefit and to help it persist. Therefore, understanding their role is relevant and important to the identification of new targets that can be used to attack this bacterium. Although these two proteins are regulated by different regulation systems and induction of their expression is caused by different conditions, they are both known to contribute to *M. tuberculosis* infection and virulence. The ability of *M. tuberculosis* to remain virulent without Acr1 (Timm *et al.*, 2006) may suggest possible redundancy in the functions of Acr1 and Acr2. Therefore, investigating the existence of such redundancy may shed more light on their role and the possibility of them or their effects being drug targets.

Another mechanism that is vital for the survival of *M. tuberculosis* is protein synthesis regulation. Rapid adaptation of a pathogen to the environmental changes that it senses and responds to within a host is of paramount importance. This includes the regulation of protein synthesis, which is a complex and expensive process, and errors in it may be fatal. The trans-translation process carried out by tmRNA is the primary quality control mechanism utilised by bacteria, regardless of whether they are pathogens. However, in some pathogens tmRNA
is essential for their survival and pathogenicity, but it is unknown if tmRNA is essential for 
*M. tuberculosis*. Therefore, understanding the role of tmRNA in *M. tuberculosis* may 
establish the potential for this molecule as a mycobacterial drug target (Shi *et al.*, 2011). To 
date, there is no published data regarding the exact role of tmRNA in the survival of *M. 
tuberculosis* and how it affects the growth of *M. tuberculosis*. However, during the course 
of this study an investigation published by Personne and Parish (Personne and Parish, 2014) 
using mutagenesis to try and differentiate between the importance of the different of the 
tmRNA in TB. *M. tuberculosis* Acr1, Acr2 and tmRNA have all been identified to be up-
regulated during certain environmental stress conditions, these represent attractive targets 
for investigation. Therefore, this study was designed to investigate the possibility of 
functional overlap and redundancy between Acr1 and Acr2, and to investigate the role of 
tmRNA in the survival of *M. tuberculosis* by testing the following hypotheses:

**Hypothesis A:**
There is some functional redundancy between the two α-crystallins and that one can 
compensate to some extent for the loss of the other.

**Hypothesis B:**
Transfer messenger RNA (tmRNA) is involved in the translation of stress proteins during 
the stress response
1.19 Aims and Objectives

**Aim A:**
To understand the roles of Acr1 and Acr2 in the stress response of *M. tuberculosis*, and to reveal the degree of functional redundancy.

Objectives for Aim A:
1. Make a single mutant of the *acr2* gene.
2. Make a double mutant of the *acr1* and *acr2* genes.
3. Compare the phenotypes of the mutants in different environments (heat shock, NRP, macrophage infection). Assess if there is a ‘synthetic phenotype’ in the double mutant.
4. Examine the intracellular localisation of Acr2 by immune-electron microscopy to elucidate its location in the cell wall.

**Aim B:**
To examine the role of tmRNA in the translational control of stress responses.

Objectives for Aim B:
1. Attempt to knockout tmRNA in wildtype and tmRNA-Hist *M. tuberculosis*.
2. Make a tmRNA mutant and examine its phenotype.
3. Attempt to express WT tmRNA and tmRNA-Hist in *M. tuberculosis*.
4. Attempt to identify the target(s) of the tmRNA degradation/translation process by examining proteins using His pull-down and LC-MS.
Chapter 2

Methods and Materials
2.1 Bacterial strains and growth conditions

Bacterial strains used:

- *E. coli* DH5-α strain for routine cloning applications
- *M. bovis* BCG (Bacillus Calmette–Guérin)
- *M. tuberculosis* H37Rv
- *M. smegmatis* GroEL ΔC

All DNA vector construction was carried out in *Escherichia coli* (DH5α) strain. *E. coli* were grown at 37°C in Luria-Bertani (LB) broth and agar containing 50µg kanamycin ml⁻¹ or 100µg hygromycin ml⁻¹. Wild type (WT) *M. tuberculosis* H37Rv, Δacr1, Δacr1Δacr2, ssrA-ΔC His merodiploid and ssrA merodiploid strains were grown at 37°C with aeration (slow shaking 150 RPM) in Middlebrook 7H9 Broth (Difco) containing 0.1% Tween80 and 10% albumin-dextrose-catalase (ADC) or Middlebrook 7H11 (Difco) medium containing 5% Oleic acid-albumin dextrose-catalase (OADC) enrichment. Two per cent (2%) sucrose was added to media for counter selection of *sacB*, kanamycin at 15µgml⁻¹ or hygromycin at 50µgml⁻¹ were added where appropriate.

To grow *M. bovis* Bacillus Calmette–Guérin (BCG) str. Pasteur, *M. smegmatis* groELΔC and *M. smegmatis* groELΔCΔlon merodiploid harbouring *M. tuberculosis* H37rv ssrAΔHis variant, starter cultures were grown in Middlebrook 7H9 broth (Difco) containing 0.1% Tween80 and 10% ADC until mid-log. 500ml of Roisin’s broth media containing 0.1% Tween 80 was inoculated from the starter culture and incubated shaking at 37°C until OD600 of 1.0. For different stress condition two hours before the extraction the respective culture was either treated with a final concentration of 16µgml⁻¹ Erythromycin, 0.5mM H₂O₂ or heat shocked at 42°C. These stresses were done either individually or in combination.
2.2 **PCR**

All the PCR products were amplified by Phusion® High-Fidelity DNA polymerase (Thermo Scientific) with a denaturing temperature of 98°C for 10 seconds, an annealing temperature varied between 58°C to 70°C for different primers pairs, extension at 72°C with varied time (15sec per kb) according to the length of the amplified fragment and final extension at 72°C for 7 minutes. A negative control was routinely used. All PCR were run in 1% agarose in TAE buffer. The different PCR products for cloning were amplified with primers (Table 3) containing relevant restriction sites.

2.3 **Ligation**

Before the ligation all the linearised (with the relevant restriction enzymes) vectors were treated with Calf intestine alkaline phosphatase (CIAP) and purified using Qiagen PCR clean up kit. Also all the PCR products were purified with the same kit. All ligation reactions contained 100ng of the vector while insert concentration varied depending on the size of the vector and the insert for each reaction. All the ligations reactions were done using T4 DNA ligase (Promega) and incubated over night at 4°C.

2.4 **E. coli Transformation**

In 1.5ml Eppendorf tube, 50 µl of E. coli (DH5α) strain competent cells were mixed with 2µl of the (overnight incubated) ligation mixture and incubated on ice for 30min before they were heat shocked at 42°C for 45 seconds and put back on ice for 2 minutes. 450µl of warm SOC medium were added to the cells, gently mixed and incubated at 37°C for 1hr. Volumes
of 20ul, 50ul and 100ul were spread on LB agar containing 100µg hygromycin ml⁻¹ or 50µg Kanamycin ml⁻¹ where appropriate and incubated at 37°C overnight. The resulting transformant colonies were sub-cultured onto LB agar containing the relevant antibiotics. Finally, the different transformants were PCR screened using the relevant primers Table 3.

Table 3. Primers list

<table>
<thead>
<tr>
<th>Primer no</th>
<th>Primer sequence</th>
<th>Forward</th>
<th>Reverse</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>⁵GCAGGATCCCGAAGGCAAGTCAAGGTT³</td>
<td>F</td>
<td>R</td>
<td>amplify ssrA gene flank1 with BamH1 restriction site</td>
</tr>
<tr>
<td>P2</td>
<td>⁶CGAACTAGTCCGATGACAGTGAAAGTT³</td>
<td>F</td>
<td>R</td>
<td>amplify ssrA gene flank1 with SpeI restriction site</td>
</tr>
<tr>
<td>P3</td>
<td>⁵CAGAGATCTCCAGGAACCGTGTCGTTG³</td>
<td>F</td>
<td>R</td>
<td>amplify ssrA gene flank2 with BglII restriction site</td>
</tr>
<tr>
<td>P4</td>
<td>⁶CAGATCTAGTGGTTGGATTGCGAGAT³</td>
<td>F</td>
<td>R</td>
<td>amplify ssrA gene flank2 with XbaI restriction site</td>
</tr>
<tr>
<td>P5</td>
<td>⁵TGGTGCAACTGATCTCAAC³</td>
<td>F</td>
<td>R</td>
<td>used for sequencing PG5::hyg/ssrA from hyg toward Flank1</td>
</tr>
<tr>
<td>P6</td>
<td>⁶GTACGGGGTTGGTGCTCT³</td>
<td>F</td>
<td>R</td>
<td>used for sequencing PG5::hyg/ssrA from hyg toward Flank2</td>
</tr>
<tr>
<td>P7</td>
<td>⁵GCTGAGCTCTTTTCCGATGACAGTGAAAT³</td>
<td>F</td>
<td>R</td>
<td>amplify ssrA gene with its putative promoter for pK-ssrA</td>
</tr>
<tr>
<td>P8</td>
<td>⁶ACTGAGCTGCAGCGCGGTGGAAGT³</td>
<td>F</td>
<td>R</td>
<td>sequencing pK-ssrA from within ssrA gene</td>
</tr>
<tr>
<td>P9</td>
<td>⁵TCTGACCGGGAAGTAAATG³</td>
<td>F</td>
<td>R</td>
<td>detecting Kanamycin gene in pK-ssrA and pK-hist-ssrA also used to screen pK-ssrA and pK-hist-ssrA merodiploid</td>
</tr>
<tr>
<td>P10</td>
<td>⁶CAGACTAGCCGTCCTTAA³</td>
<td>F</td>
<td>R</td>
<td>screening for the deletion of ssrA gene</td>
</tr>
<tr>
<td>P11</td>
<td>⁵TTCCATAGGATGGCAAGATCC³</td>
<td>F</td>
<td>R</td>
<td>sequencing pK-hist-ssrA</td>
</tr>
<tr>
<td>P12</td>
<td>⁶GGCAATCAGGTCGAACATC³</td>
<td>F</td>
<td>R</td>
<td>screening for hist tag on pK-hist-ssrA also used to screen pK-hist-ssrA merodiploid</td>
</tr>
<tr>
<td>P13</td>
<td>⁵CACCACACCACCAACCC³</td>
<td>F</td>
<td>R</td>
<td>screening for the deletion of ssrA gene</td>
</tr>
<tr>
<td>P14</td>
<td>⁶CGATGCCGTTGGAATGTA³</td>
<td>F</td>
<td>R</td>
<td>screening for hist tag on pK-hist-ssrA also used to screen pK-hist-ssrA merodiploid</td>
</tr>
<tr>
<td>P15</td>
<td>⁵GCCGATACGGTCTGTGACT³</td>
<td>F</td>
<td>R</td>
<td>amplifying acr1 gene with its putative promoter with sacl restriction site for pK-acr1 also used to check for the deletion of acr1 gene</td>
</tr>
<tr>
<td>P16</td>
<td>⁶GACCCATACGGCGGACTGC³</td>
<td>F</td>
<td>R</td>
<td>amplifying acr2 gene with its putative promoter with NotI restriction site for pK-acr2</td>
</tr>
<tr>
<td>P17</td>
<td>⁵CACCACACACCGCAGTGA³</td>
<td>F</td>
<td>R</td>
<td>screening for the deletion of acr2 gene</td>
</tr>
<tr>
<td>P18</td>
<td>⁶CGATGCCGTTGGAATGTA³</td>
<td>F</td>
<td>R</td>
<td>screening for the deletion of acr2 gene</td>
</tr>
<tr>
<td>P19</td>
<td>⁵ACTGAGCTCTGCGACAGCAAACAGGTT³</td>
<td>F</td>
<td>R</td>
<td>amplifying acr1 gene with its putative promoter with sacl restriction site for pK-acr1 also used to check for the deletion of acr1 gene</td>
</tr>
<tr>
<td>P20</td>
<td>⁶GCTGAGCTCCACCAAGTGCTGATG³</td>
<td>F</td>
<td>R</td>
<td>amplifying acr2 gene with its putative promoter with NotI restriction site for pK-acr2</td>
</tr>
<tr>
<td>P21</td>
<td>⁵ATCGACACCAGCAATGC³</td>
<td>F</td>
<td>R</td>
<td>screening for the deletion of acr2 gene</td>
</tr>
<tr>
<td>P22</td>
<td>⁶GCCTGACACCAAGCAGCTG³</td>
<td>F</td>
<td>R</td>
<td>screening for the deletion of acr2 gene</td>
</tr>
<tr>
<td>P23</td>
<td>⁷GTAGGCGCGCGCAAGACCG³</td>
<td>F</td>
<td>R</td>
<td>screening for the deletion of acr2 gene</td>
</tr>
<tr>
<td>P24</td>
<td>⁸GCCGGCGCGCGGACCG³</td>
<td>F</td>
<td>R</td>
<td>screening for the deletion of acr2 gene</td>
</tr>
<tr>
<td>Primer no</td>
<td>Primer sequence</td>
<td>Forward</td>
<td>Reverse</td>
<td>Purpose</td>
</tr>
<tr>
<td>----------</td>
<td>----------------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>P25, P26</td>
<td>5’ GCG TAG GCG TCT GAA AGA GA 3’&lt;br&gt;5’ GAT CTT GTC GCC TCT TGT CC 3’</td>
<td>F</td>
<td>R</td>
<td>To amplify GroEL1 from <em>M. smegmatis</em> groEL1ΔC</td>
</tr>
<tr>
<td>P27, P28</td>
<td>5’ GAC GAC GAG GGT CGA CAG 3’&lt;br&gt;5’ ATC CGC AAG ACG CTC AAG 3’</td>
<td>F</td>
<td>R</td>
<td>To sequence <em>M. smegmatis</em> groEL1ΔC</td>
</tr>
<tr>
<td>P29, P30</td>
<td>5’ GTC GGA TCC CGA TGG TCA ACA TCA ACC AG 3’&lt;br&gt;5’ CGT ACT AGT GCC ATG CCT CCA AAC TT 3’</td>
<td>F</td>
<td>R</td>
<td>To amplify <em>lon</em> upstream flank with BamH1 restriction site&lt;br&gt;To amplify <em>lon</em> upstream flank SpeI restriction site</td>
</tr>
<tr>
<td>P31, P32</td>
<td>5’ CGT AGA TCT GCC TCT CAA CGG TGT TCA TC 3’&lt;br&gt;5’ GTC TCT AGA CCT CTT CGT CGA ACG TGA AT 3’</td>
<td>F</td>
<td>R</td>
<td>To amplify <em>lon</em> downstream flank with <em>Bgl</em>II restriction site&lt;br&gt;To amplify <em>lon</em> downstream flank with <em>Xba</em>I restriction site</td>
</tr>
<tr>
<td>P33, P34</td>
<td>5’ GACTACAAGGCTGGGACAT 3’&lt;br&gt;5’ GGTCATCAGCATCACACAC 3’</td>
<td>F</td>
<td>R</td>
<td>To check for the deletion of <em>lon</em> gen from <em>M. smegmatis</em> groEL1ΔC</td>
</tr>
<tr>
<td>P35, P36</td>
<td>5’ AGG CAA GAG ACC ACC GTA AG 3’&lt;br&gt;5’ TTA GTG ATG GTG GTG GTG GT 3’</td>
<td>F</td>
<td>R</td>
<td>To detect the His tag for checking ssrA&lt;sup&gt;9His&lt;/sup&gt; expression</td>
</tr>
<tr>
<td>P37, P38</td>
<td>5’ CAT GAG CTC GAA TTC GAA ATC GGC ATG CGC TT 3’&lt;br&gt;5’ TGC GAG CTC AAG CTT TGG CAC GAT ACC TTG GTG 3’</td>
<td>F</td>
<td>R</td>
<td>To amplify <em>smpB-Rv</em>3099c-ssrA from <em>M. tuberculosis</em> H37Rv to be cloned into pKinta</td>
</tr>
<tr>
<td>P39, P40</td>
<td>5’ GCTTCTAGAGAATTCCTTTCCGATGACGAATGGAAT 3’&lt;br&gt;5’ ACGTCTAGAAAGCTTTGATGCCGCTGGAGTGTAG 3’</td>
<td>F</td>
<td>R</td>
<td>To clone ssrA&lt;sup&gt;9His&lt;/sup&gt; into pMV306 expression vector</td>
</tr>
<tr>
<td>P41, P42</td>
<td>5’ GATTCCATCACGCACCACCACCAC 3’&lt;br&gt;5’ GTGGTGGTGGTGGCGCTGATGTAATC 3’</td>
<td>F</td>
<td>R</td>
<td>Use for site directed mutagenesis</td>
</tr>
</tbody>
</table>
2.5 Construct screening

The resulting transformants of various constructs were screened with PCR using relevant primers (Table 3). From each transformation, two successful PCR positive transformants were sub-cultured in LB broth with the relevant antibiotics and incubated overnight at 37°C with slow shaking. Cells were harvested the following day to extract the plasmid DNA. Extracted plasmid DNA was further screened by digestion with the relevant restriction enzyme and then sent to "Eurofins (MWG)" for sequencing using the relevant primers (Table 3).

2.6 Gene replacement of ssrA and acr2

All manipulations of *M. tuberculosis* H37rv were done within the class I safety cabinet in CL3 laboratory

2.6.1 Preparing H37rv competent cells

50ml of 7H9 broth (in 75ml sterile conical plastic flask) was inoculated with a loop full of *M. tuberculosis* H37rv (WT, knock out or merodiploid strain) and incubated for 5 to 7 days at 37°C with shaking at low speed. This culture was used for preparation of the competent cells when the OD reached between 0.5-1.0. The culture was transferred to a 50ml Falcon tube and spun at 4000RPM for 10min at 37°C. The pellet was re-suspended in 50ml of 37°C pre-warmed sterile 10% glycerol and spun again at 4000RPM for 10min. This processes were repeated resuspending in decreasing amounts of 10% glycerol, first 12.5ml, then 5ml, and finally 1ml.
2.6.2 Electroporation for knockout and plating

The replacement of the \textit{acr2} gene with the \textit{hyg} gene and the replacement of the \textit{ssrA} gene with the \textit{aph} gene was done by introducing pSMT183 or PG5\textit{ssrA::hyg} (Stewart \textit{et al.}, 2001) suicide plasmids (plasmids that cannot replicate in mycobacteria) for the \textit{acr2} gene or the \textit{ssrA} gene replacement respectively. Four hundred microliters (400ul) of the competent cells \textit{WT M. tuberculosis H37Rv}, \textit{M. tuberculosis H37rv merodiploid ssrA} or \textit{M. tuberculosis H37rv merodiploid his-ssrA} strains) were mixed with 1µg of the relevant UV light treated plasmid DNA. PG5\textit{ssrA::hyg} was electroporated into \textit{WT M. tuberculosis H37rv} and the two merodiploid \textit{M. tuberculosis H37rv} with a second copy of wild-type \textit{ssrA} and with \textit{ssrA}^{9\text{His}} variant strains. The mixture was pipetted into a 2mm electroporation cuvette, and the cuvette placed into the electroporation pod (in the safety cabinet) and pulsed with 2500 volts. The content of the cuvette was transferred to a fresh 10ml 7H9 broth (containing 0.05% Tween80 and 10% albumin-dextrose-catalase and free of antibiotics) and incubated at 37°C for 24hrs with gentle shaking at 150RPM. A negative control using water instead of DNA was also routinely carried out. The overnight incubated 10ml 7H9 broth was spun down and the pellet of the cells was resuspended in 1ml of fresh 7H9. A volume of 500ul of the resuspended cells was evenly spread over the surface of two 7H11 agar plates containing 5\% OADC, 50µg hygromycin ml$^{-1}$ and 2\% sucrose. The plates were incubated at 37°C until transformants became visible.
2.6.3 Complementation of ssrA gene and plating
For complementation, pKinta (Stewart et al., 2001) plasmid was used which is a ColE1-based E.coli plasmid that carries aph kanamycin resistance gene, int gene, encoding an integrase, and an attP site from the L5 mycobacteriophage. This plasmid integrates into the chromosome in a single copy at the attB site by specific recombination. The ssrA gene was introduced into WT M. tuberculosis H37rv using pK.ssrA plasmid construct to generate a strain with two copies of ssrA genes (merodiploid strains). This was also performed with the pK.ssrA-9His. The WT M. tuberculosis H37rv competent cells were prepared as described in section 2.6.1, electroporated and plated in the same manner as described in section 2.6.2.

2.6.4 Complementation of acr1 and acr2 genes and plating
The pKinta plasmid was also used to complement the single acr1 and acr2 as well as the double genes acr1&acr2 in Δacr1, Δacr2 and Δacr1Δacr2 strains respectively. The respective M. tuberculosis H37rv knockout (KO) strains competent cells were prepared as described in section 2.6.1, electroporated with the respective gene/s and plated in the same manner as described in section 2.6.2.

2.6.5 M. tuberculosis H37rv transformants screening
Colonies from transformations were individually sub-cultured onto another 7H11 plate (divided into 6 or 8 sections). Two to three weeks later DNA was extracted as described in section 2.8. The transformants from the complementation plates were PCR screened (primers P11&P12) for the presence of kanamycin resistance gene. PCR screening was also performed to confirm the deletion using the relevant primers (Table 3) The PCR products were digested using a relevant restriction enzyme to demonstrate that the amplified products identity and finally they were sequenced.
2.7 Producing BCG str. Pasteur merodiploid harbouring *M. tuberculosis* H37rv *ssrA*-His variant

2.7.1 Preparing BCG str. Pasteur competent cells
The BCG competent cells were prepared as described in section 2.6.1.

2.7.2 Preparing *M. smegmatis* groEL1ΔC competent cells
From solid 7H11, WT *M. smegmatis* groEL1ΔC or *M. smegmatis* groEL1ΔCΔlon strain, a 50ml of broth culture 7H9 (in 75ml sterile conical plastic flask) was inoculated and incubated for 48 hours at 37°C shaking at low speed. This culture was used for preparation of the competent cells when the OD reached between 0.5-1.0. The flask containing the culture was incubated on ice for 1.5 hours and the culture was transferred to a 50ml Falcon tube and made competent as described for *M. tuberculosis* H37Rv, section 2.6.1, but using ice-cold 10% glycerol rather than warmed glycerol.

2.7.3 Electroporation of BCG str. Pasteur with *ssrA*<sup>His</sup> variant
A pKinta plasmid carrying either *ssrA*<sup>9His</sup> or *ssrA*-<sup>6His</sup> gene was introduced into BCG Pasteur wild type to generate a strain harbouring either *M. tuberculosis* H37Rv *ssrA*<sup>9His</sup> or *ssrA*<sup>6His</sup> variant in addition to its native *ssrA*. The BCG str. Pasteur wild type was electroporated and plated in the same manner as described in section 2.6.4.

2.7.4 Electroporating of *M. smegmatis* groEL1ΔC with *ssrA*<sup>His</sup> variant
The pKinta plasmid carrying either *ssrA*<sup>9His</sup> or *ssrA*<sup>6His</sup> gene was introduced into *M. smegmatis* groEL1ΔC wild type or *M. smegmatis* groEL1ΔCΔlon to generate a strain harbouring either *M. tuberculosis* H37Rv *ssrA*<sup>9His</sup> or *ssrA*<sup>6His</sup> variant in addition to its native *ssrA*. The respective *M. smegmatis* groEL1ΔC was electroporated and plated in the same
manner as described in section 2.6.3, but, the mixture of competent cells and respective plasmid were incubated in ice for 1 minute prior to the electroporation.

### 2.8 *M. tuberculosis* H37rv genomic DNA (gDNA) preparation

A 20ml cultures of *M. tuberculosis* H37rv were setup and grown to late log phase. The cultures were spun at 3500RPM for 15 min in Falcon tubes. The supernatants were carefully poured off and the cell pellets suspended in 1ml GTE solution (25mM Tris-HCl, pH.8.0, 10mM EDTA, 50mM glucose). The suspended cell pellets were transferred to a 2ml micro centrifuge tube and spun for 5min at 13,000RPM. The supernatants were aspirated out and the cell pellets resuspended in 450µl of GTE solution. Finally, 50µl of lysozyme (10mg/ml) was added to each and gently mixed prior to incubation overnight at 37°C without shaking. Following the overnight incubation, the suspensions were heated to 80°C for 2hrs. A volume of 100µl of 10% SDS was added to the different suspensions and gently mixed, followed by the addition of 50µl of proteinase K (10mg/ml) which was mixed and incubated at 55°C for 40min. A volume of 200µl of 5M NaCl was added and gently mixed followed by the addition of 160µl of preheated cetyltrimethylammonium bromide (CTAB) solution (in 65°C oven), this was gently mixed and incubated for 30min at 65°C in water bath. An equal volume of chloroform-isoamyl alcohol (24:1 ration) was added, gently mixed and spun for 5min at 13000RPM. The aqueous upper layer was aspirated and transferred to a new micro centrifuge tube to which another equal volume of preheated chloroform-isoamyl alcohol (24:1) was added, gently mixed and spun at 13000RPM for 5min. A volume of 800µl of the aqueous upper layer was transferred to a new micro centrifuge tube to which a volume of 560µl of isopropanol was added, gently inverted until the DNA precipitate was seen. The DNA was incubated at room temperature for 5min and spun for 10min at 13000RPM.
supernatant was discarded and 1ml of 70% ethanol was added to the pellet, gently inverted a few times and spun for 5min at 13000RPM. The supernatant was removed and the pellet left to air dry for about 10min. The dried pellets were resuspended with 50μl of DNAse free water and stored at 4°C to allow the pellet to resuspend overnight. The concentration of the DNA was determined by Nanodrop. For longer storage period the gDNA preps were stored at -20°C

2.9 BCG str. Pasteur cytosolic and membrane bound protein extraction

This method was adapted from the method described in Sinha et al., 1995. A volume of 500ml Roisin’s medium was inoculated 1 in 10 with a BCG str. Pasteur starter culture and incubated until it reached an OD of 1.0. The culture was split into Falcon tubes and spun for 10min at 4000RPM at 4°C. The supernatant was decanted into a clean container and filter sterilised. Then a volume of 100ml (20ml at a time) were concentrated using corning® Spin-X® UF20 concentrator (MW cut-off of 10,000). The spin -X UF6® concentrator was spun at 4000RPM for 35minutes in 20ml aliquots. The 100ml of supernatant were concentrated to 0.5ml to which the appropriate volume of sample buffer was added and the mix boiled for 5min.

The pellet was washed three times with ice cold Phosphate-Buffered Saline (PBS; 10mM phosphate, 150mM NaCl, pH7.4)and resuspended in sonication buffer (50mM Tris-HCl, 10mM MgCl₂, 0.02% Sodium Azide, pH 7.4) containing proteases inhibitors (1mM EGTA and 1mM PMSF). As no sonicator was available, the cells were disrupted using a Lysing
Matrix system (MP Biomedicals FastProtein™ Blue). A volume of 1ml of the resuspended pellet was transferred to the Lysing Matrix tubes, incubated in ice for 5min, transferred to the ribolyser and run for 30sec at speed of 6.5 followed by incubation in ice for 5min. Three cycles were run with 5min incubation on ice in between. The tubes were spun for 10min at 13000RPM at 4°C. The supernatants were transferred to new 2ml micro centrifuge tubes and spun for 10min at 13000RPM at 4°C and pooled. The cell pellets were washed three times with sonication buffer and resuspended in 10ml of Tris-Buffered Saline (TBS; 10mM Tris, 150mM NaCl, pH7.4). The twice spun pooled supernatant was split into two 5ml ultracentrifuge tubes and spun at 150,000g (35000RPM) for 90min at 4°C. The supernatants (Cytosolic) were aspirated and stored at -20°C until needed. The plasma membrane pellets were resuspended in 5ml TBS and spun again at 150000g for 90min (this step was done twice). After two further washes with TBS the pellets were resuspended in TBS, pooled and stored at -20°C.

2.10 ssrA-His variant tagged protein extraction and purification

2.10.1 Protein extraction
The BCG str. Pasteur or M. smegmatis groEL1ΔC or M. smegmatis groEL1ΔCΔlon merodiploid harbouring either pk.ssra9His or pK.ssra6His transformants were grown in a volume of 400ml Roisin’s medium containing 30µg⁻¹ Kanamycin to an OD₆₀₀ of 1.0. The cultures were transferred to 50ml Falcon tubes and spun for 10min at 4000RPM. The supernatants were discarded and the pellets were washed three times with ice cold PBS. Finally, the pellets were resuspended in ice cold lysis buffer (70mM Hepes pH8, 20mM Imidazole, 650mM NaCl) containing 1mM PMSF protease inhibitor, 1mM EGDTA (sigma) and 0.02% Sodium Azide (sigma). A volume of 1ml of the resuspended pellet was
transferred to the Lysing Matrix tubes, incubated on ice for 5min, transferred to the ribolyser and run for 30sec at speed of 6.5 followed by incubation on ice for 5min. Three cycles were run with 5min incubation on ice in between. The tubes were spun for 10min at 13000RPM at 4°C and the supernatants transferred to a new 2ml micro centrifuge tubes and spun for 10min at 13000RPM at 4°C and pooled.

**2.10.2 ssrA-His variant tagged protein extraction purification**
The 1ml His Trap™ HP (GE Healthcare Life Sciences) column was first equilibrated by washing with 5 ml (five fold volume) with binding buffer (70mM Hepes pH8, 20mM Imidazole, 500mM NaCl, 0.1mM β-mercaptoethanol). The culture supernatant (described in section 2.10.1) was passed through the column with a flow rate of 1ml per min. Then washed with five times the sample volume of binding buffer. The protein was eluted with 5ml of elution buffer (60mM Hepes pH8.0, 400mM Imidazole, 150mM NaCl, 3% Glycerol) at flow rate of 1ml min⁻¹. The eluate was collected into a series of 0.5 ml aliquots. The amount of the protein in each eluate aliquot was checked by NanoDrop™ and by visualising on a 12% acrylamide gel. The protein band from aliquot with the most protein was selected and sent for identification by mass spectrometry.

**2.11 Western Blot**

**2.11.1 Protein Sample Preparation**
An appropriate volume lysate or His Trap™ HP column elute was mixed with an equal volume of sample buffer (50mM Tris HCl pH6.8, 2% w/v SDS, 5% v/v 2-mercaptoethanol, 10% v/v glycerol, 0.05% w/v bromophenol blue) and boiled for 5minutes.
2.11.2 Protein Gel Preparation

All the protein electrophoresis was performed using 12% acrylamide pH8.8 (resolution part) and 3% acrylamide pH6.8 (stacking part).

2.11.2.1 Resolving gel (12%)

For two 1mm gels (7mmx8mm) a volume of 10ml resolving gel was prepared containing 4ml of 30% acrylamide gel solution, 2.5ml of 1.5 M Tris HCl buffer pH8.8, 3.3ml sterile protease free RO water, 100µl of 10% ammonium persulfate (APS, freshly prepared) and 100µl of 10% SDS. Prior to pouring the gel into the mould a 10µl of Tetramethylethylenediamine (TEMED (Thermo Scientific™) was added and thoroughly mixed. 1ml of water saturated n-butanol was added to prevent it from drying. The resolving gel was poured first and left to polymerise.

2.11.2.2 Stacking gel (3%)

For two 1mm gels (7mmx8mm) a volume of 3 ml stacking gel was prepared containing 0.5ml of 30% acrylamide gel solution, 0.38 ml of 0.5M Tris HCl buffer pH6.8, 2.1ml sterile protease free RO water, 30µl of 10% APS, freshly prepared), 30µl of 10% SDS. Prior to pouring the gel into the mould a 3ul of TEMED was added and thoroughly mixed. Before pouring the stacking gel mixture the water saturated n-Butanol was removed by inverting the gel mould rack on a paper towel. After pouring the stacking gel on top of the resolving gel the combe was placed and the gel left to polymerise. Finally, the gel cassette was assembled into the electrophoresis tank.
2.11.3 Protein sample Loading and Electrophoresis
Before loading the samples, the wells were washed with the running buffer (0.05M Tris, 0.055M 0.1% SDS) and up to 20µl of pre-mixed and boiled protein sample were pipetted into the corresponding well and run at 150V for 1hr.

2.11.4 Protein Gel staining
After the electrophoresis was complete the gels were removed from the cases stained for 30 minutes in Coomassie Blue stain while shaking. Then the stain was removed and the gel was destained with 10 % glacial acetic acid, 30 % methanol until protein bands became visible.

2.11.5 Protein Gel blotting
The second gel was transferred to a PVDF membrane using semi-dry blotting unit. The gel was removed from the cassette and placed into transfer buffer (0.303% Tris HCl, 1.44% Glycine, 20% Methanol). The PVDF was activated first by dipping it into absolute Methanol for 30seconds then placed into transfer buffer. Two blotting papers were cut to the size of the gel and left soaked into the transfer buffer. The first blotting paper was put on the surface of the blotting unit, the wet PVDF membrane was layered on top of it followed by the gel then the second wet blotting paper. Finally, the unit was closed and run for 30minutes to 45minutes at 20 V.
2.11.6 Membrane Blocking
The PVD membrane was blocked in 10% fat free milk solution in TBS (0.08% NaCl, 0.03% Tris HCl pH7.6) containing 0.1% Tween20 for 1hr at room temperature while shaking.

2.11.7 Primary Antibodies Treatment
The blocked membrane was transferred to a 50ml Falcon tube containing 5ml of primary antibody diluted to an appropriate concentration (1 in 1000 or 1 in 5000) in 3% fat free milk solution in TBS (0.08% NaCl, 0.03% Tris HCL pH7.6) containing 0.1% Tween20 and rolled for 1hr followed by rinsing three times (10 minutes each) in approximately 10ml TBS containing 0.1% Tween 20 buffer.

2.11.8 Secondary Antibodies (HRP conjugate) Treatment
Secondary antibodies were diluted to 1:5000 or 1:10000 in 5 ml 3% fat free milk solution in TBS (NaCl 0.08%, 0.03% Tris HCL pH7.6) containing 0.1% Tween 20 and added to the Western blot and left rolling for 1hr before washing as with the primary antibody.

2.11.9 Western Blotting Development
The blot was developed using Clarity™ Western ECL (Bio Rad) substrate. The signal was detected either by using photo sensitive (X-ray) film or using a FluoroChemQ imaging system.
2.12 *In vitro* Stress Assays

The *M. tuberculosis* H37Rv strains were grown in 7H9 until mid-log phase. The OD was adjusted to a standard OD.

2.12.1 Hypoxia

One millilitre of culture at OD 0.47 was dispensed in triplicate into 2ml tubes. 400ml of sterile liquid paraffin was layered on top of the 1ml of culture and the tube was closed tight. The tubes were incubated at 37°C without shaking. At each time point (10 days, 20 days and 30 days) the respective tubes were removed and the paraffin layer was carefully removed. The tube content was carefully and slowly mixed by pipetting up and down and a sample of 50ul taken and serially diluted. Three 20ul from each dilution were cultured.

2.12.2 Heat shock

One millilitre of culture at OD 0.17 was dispensed into 2ml O-ringed screw capped tubes in duplicate and transferred to 53°C water bath. At each time point (15min, 30min, 45min, 60min, 90min and 120 min) a tube was removed from the water bath and gently mixed and a 20ul sample serially diluted out to 10^-6. Three 20ul from each dilution was spotted separately on 7H11 agar plates. The plates were sealed by tape, bagged and incubated at 37°C for up to 8 weeks. A time 0 sample was also done by taking a sample of 20ul from the adjusted OD culture, diluted and cultured the same way as described above.

2.12.3 pH

One millilitre of OD 0.4 culture was dispensed into 2ml O-ringed screw capped tube in duplicate, the tubes spun down and the supernatant was discarded. The cells pellets were
washed with sterile PBS once to remove the 7H9 residual and the cell pellets resuspended in 1ml of sterile water. A 100µl aliquot of the washed cells was added in triplicate to Roisin’s minimal medium with pH adjusted to pH 6.6 and pH 4.4 and incubated without shaking. After 7 days of incubation the tube content was gently mixed and a 20µl aliquot taken and serially diluted to $10^{-6}$ and cultured on 7H11 agar for up to 8 weeks. For T₀, 100µl of the washed pellets was added to 1ml of sterile water which was diluted and cultured as described for the different pH samples.

2.12.4 H₂O₂
A cell pellet from 1 ml of culture, OD 0.4, was washed with sterile PBS to remove the residual 7H9 and resuspended in 1ml of sterile water. Aliquots of 100µl of cells were added, in triplicate, to 1ml Roisin’s containing H₂O₂ at 0mM, 10mM and 20mM and incubated at 37°C without shaking. After 2 hours of incubation the content of the tube was gently mixed and a sample of 20ul was taken and serially diluted out to $10^{-6}$ and plated onto 7H11 plates and sealed with tape. For T₀ 100µl of the washed pellets was added to 1ml of sterile water, diluted and cultured as described for the different H₂O₂ concentrations samples.

2.13 THP-1 Cell infections

2.13.1 THP-1 cell preparation
THP-1 cells, a human monocyte-like cell line (ATCC® TIB-202™), was obtained from in-house stocks. THP-1 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 200mM L-glutamine, and 10% Foetal Bovine Serum (FBS) and incubated in 5% CO₂ at 37°C. Cells were routinely propagated into 75 cm² flasks (BD
falcon). For assays, THP-1 cells were seeded at a density of between $1 \times 10^5$ cells/ml and $1 \times 10^6$ cells/ml in 24-well tissue culture plates. To promote differentiation, cells were centrifuged and re-suspended in fresh media to a concentration of $1 \times 10^6$ cells per ml and treated with final concentration of 100nM Phorbol 12-myristate 13-acetate (PMA) (P8139, Sigma). After treatment with PMA, 200µl of cell suspension was immediately added 96-well plates. The cells were dispensed in an array of 6 wells per strain to be tested (3 wells to be lysed for colony forming unit (CFU) counts and 3 wells for infected cells viability control), with 6 separate wells for the non-infected cells viability controls. Cells were incubated for 3 days to allow them to differentiate and stick to the well surface. Before infection, cells were washed three times with warm PBS then 100µl of warm RPMI with 10% FCS, 10mM L-glutamine and 10mM pyruvate) medium was added.

### 2.13.2 Infection with *M. tuberculosis*

The different *M. tuberculosis* H37Rv strains were grown to mid log phase and ODs adjusted and diluted in warm RPMI (with 10% FBS) medium. An appropriate volume of the diluted culture was added to the respective wells to give a multiplicity of infection (MOI) of 0.1. At each time point (2hours, 24hrs, 48hrs and 7 days) a plate was taken from the incubator, all the wells were washed with warm PBS and 50µl of 1% triton added to three of the wells and the lysate serially diluted and plated onto 7H11 and incubated for up to 8 weeks for the viable *M. tuberculosis* count. The other wells and the 6 wells for the non-infected control were fixed with 4% Formaldehyde, placed overnight in formalin chamber before being stained with 0.5% Crystal Violet to determine the number of THP-1 cells present post infection at each time point.
2.14 RNA extraction

*M. smegmatis groEL1ΔC* and merodiploid variants were grown in 10ml of 7H9 Broth overnight. Two hours prior to harvesting the cultures were treated with either 16ug ml\(^{-1}\) erythromycin or 0.5mM H\(_2\)O\(_2\). The RNA was extracted using ThermoFisher Scientific Trizol®Plus RNA purification system. All the steps carried out according to the kit protocol. Trizol reagent is a monophasic solution of phenol guanidine isothiocyanate which maintains the RNA integrity but, at the same time disrupt cells and dissolve its components and acts as highly efficient RNase inhibitor during cell lyses.

2.15 Reverse Transcriptase Polymerase Chain reaction (RT-PCR)

2.15.1 First-strand complimentary DNA (cDNA) Synthesis

The cDNA was generated using Invitrogen SuperScript\textsuperscript{TM}III reverse transcriptase and Invitrogen random hexamer primers. The RNA samples were treated with DNase prior to the RT. The first-strand cDNA synthesis was done in two steps.

**Step 1**

In a total volume of 13ul, 1 μg of DNase treated RNA, 200ng of random primers, 1μl of 10mM dNTPs mix (dATP, dGTP, dCTP and dTTP) were mixed and heated to 65°C for 5minutes. The tubes were briefly spun and incubated on ice for 1 minute.

**Step 2**

The mixture from step1, 4μl of 5X First-strand buffer, 1 μl of 0.1M DTT, 1μl (40units) of Invitrogen RNase OUTTM and 1μl (200units) of Invitrogen SuperScript\textsuperscript{TM}III RT were
added and gently mixed. The mixture was incubated for 5 minutes at 25°C. Then incubated at 50°C for 30 minutes and 55°C for another 30 minutes. Finally, the mixture was incubated at 75°C for 15 minutes to inactivate the enzyme.

2.15.2 PCR

The cDNA was amplified using the Phusion® High-Fidelity DNA polymerase.

<table>
<thead>
<tr>
<th>Initial denaturation</th>
<th>98°C for 2.5min</th>
<th>1 cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>98°C for 10sec</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>60°C for 10sec</td>
<td>25 cycles</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C for 10sec</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C for 2min</td>
<td>1 cycle</td>
</tr>
</tbody>
</table>

2.16 Site directed mutagenesis

To modify the \( ssrA^{9\text{His}} \) to \( ssrA^{6\text{His}} \) primers P41&P42 (Table 3) containing the bases require to be restored and its complementary were designed using PrimerX (http://www.bioinformatics.org/primerx/cgi-bin/DNA_1.cgi). Two separate PCR reactions for each primer were carried out using Phusion® High-Fidelity DNA polymerase (Thermo Scientific). The pK.\( ssrA^{9\text{His}} \) was the template using the following protocol:

<table>
<thead>
<tr>
<th>Initial denaturation</th>
<th>98°C for 2.5min</th>
<th>1 cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>98°C for 10sec</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>67°C for 10sec</td>
<td>25 cycles</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C for 2.5min</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C for 7min</td>
<td>1 cycle</td>
</tr>
</tbody>
</table>

The PCR products from both tubes were pooled and denatured at 95°C for 30 seconds and left to cool. The mixture was treated for 2 hours at 37°C with \( DpnI \) restriction enzyme to degrade the parental strands. This enzyme degrades only the methylated template DNA leaving the newly synthesised PCR product. A volume of 5 ul of \( DpnI \) treated DNA was used to transform \( E.coli \) DH5α and transformants were screened as described in section 2.4.
2.17 Electron microscopy

2.17.1 Fixation
The WT *M. tuberculosis* H37rv was grown shaking for 90 days to allow slow oxygen depletion. A volume of 1ml was spun down in polypropylene O-ringed 2ml tubes at 13000RPM for 5minutes. The supernatant was removed and the cell pellet re-suspended in 1ml of 4% formaldehyde, 0.2% glutaraldehyde. The tubes were tightly closed and kept in a formalin chamber overnight.

2.17.2 Blocking
The fixed WT *M. tuberculosis* H37rv were spun at 13,000RPM for 5minutes, the fixative removed and the cell pellet was washed three times in PBS pH7.4. After the last PBS wash the pellet was resuspended in 1ml 1% agar prepared in water (at just above gelling point) and immediately spun at 13000RPM for 30second before cooling at 0°C. Small blocks of agar containing embedded bacteria were cut and incubated in 3ml of 2% uranyl acetate for 30 minutes at room temperature. The gel blocks were then rinsed 3 times in 5ml of water.

2.17.3 Dehydration and Embedding
The rinsed uranyl acetate treated agar blocks were dehydrated in an ethanol series of concentrations 50% and 70% in 5ml with three changes for each. The dehydrated agar blocks were transferred to London Resin (LR) at 2:1 ratio (LR white to 70% Ethanol) and incubated for one hour at room temperature, before transferring to pure LR white and incubating for 1 hour. Finally, the samples were moved to fresh LR and incubated at room temperature overnight with gentle shaking. Blocks were incubated twice in LR white for one hour each
before transferring to gelatine capsules, and placing in the centre, filling with LR white and securely closing. Carefully the capsules were transferred to an oven and baked for 24 hours at 50°C.

2.17.4 Sectioning
Ultrathin sections were cut at the University of Sussex by Dr Julian Thorpe

2.17.5 Immunogold Staining of LR white sections for EM
The block was blocked with goat serum diluted 1 in 10 with 0.05M Tris-buffered saline (TBS), pH 7.6 for 30 minutes at room temperature. The grids were drained and transferred to the primary antiserum, anti-Acr2 or Anti-Acr1 rabbit polyclonal or in control rabbit diluted 1 in 10 and 1 in 1000 in TBS containing 1% (W.V) Bovine Serum Albumin (BSA) and 0.01% (w/v) sodium azide for one hour at room temperature. The grids were rinsed three times in TBS containing 1% (w/v) BSA followed by incubation for one hour at room temperature in 1:15 goat anti-rabbit IgG gold conjugate (10nm) (Biocell) diluted in the TBS/BSA/sodium azide mix. The grids were then rinsed four times for 10 minutes each in TBS/BSA and then four times for 5 minutes each in distilled water. Finally, the sections were counter stained with lead citrate.

2.17.6 Electron Microscope Imaging.
The immunogold stained ultrathin sections were examined by Dr Robert Francis
2.2 Materials

2.2.1 Media

**LB (Luria-Bertani) Agar**

15g  Tryptone  
5g  Yeast extract  
5g  NaCl  
1%  agar  
1L  Dist. H$_2$O$_2$

**LB (Luria-Bertani) Broth**

15g  Tryptone  
5g  Yeast extract  
5g  NaCl  
1L  Dist. H$_2$O$_2$

**SOC Medium Super optimal broth with catabolic repressor (250 mL Total Volume)**

1.  25g  Yeast Extract  
5 g  Tryptone  
0.15g  NaCl  
0.00 g  KCl  
0.51 g  MgCl$_2$  
2.5 mL  MgSO4

Autoclaved the solution

Added 10 mL of 20 % Glucose

Adjust to pH7 and Add water to 250ml
**7H9 Broth**

Prepared as the manufacturer instruction. Before used ad final concentration of 10% ADC (Albumin Dextrose Catalse), 0.1% Tween80 were aseptically added.

**7H11 Agar**

The agar was prepared as the manufacturer instruction. Before pouring a final concentration of 5% ODAC was aseptically added.

**7H11 with 2% Sucrose and 50 µg/mL Hygromycin**

The agar was prepared as the manufacturer instruction. Before pouring a final concentration of 5% ODAC (Oleic Acid Albumin Dextrose Catalse), final concentration of 2% sucrose and the appropriate antibiotic concentration were aseptically added.
Roisin’s medium

1.0 g KH$_2$PO$_4$
2.5 g Na$_2$HPO$_4$
5.9 g NH$_4$Cl
2.0 g K$_2$SO$_4$
1 ml Trace element Solution
0.5 ml 1M CaCl$_2$
0.5 ml 1M MgCl$_2$
5 ml Glycerol
2ml Tween 80

After adjusting the pH to 6.6 it was filter sterilised and stored at 4°C

1000X Trace elements Solution amount per litre

80 mg ZnCl$_2$
400 mg FeCl$_3$-6H$_2$O
20 mg CuCl$_2$-2H$_2$O
20 mg MnCl$_2$-4H$_2$O
20 mg Na$_2$B$_4$O$_7$-10H$_2$O
20 mg (NH$_4$)$_6$Mo$_7$O$_{24}$-4H$_2$O
2.3.2 Buffers

**TAE Buffer:**
Tris Acetate EDTA Buffer with 2.0 M Tris acetate, 0.05 M EDTA, pH 8.2 - 8.4 (at 25°C)

**Transfer Buffer**
25 mM Tris
190 mM glycine
20% methanol

**Tris buffered Saline (TBS) + 0.1% Tween 20**
20 mM Tris pH 7.5
150 mM NaCl
0.1% Tween 20

**TE Buffer**
Tris 1 M
EDTA 0.5 M
Water to 100 mL
Protein Extraction and purification Buffers

**Lysis Buffer**

- 70mM Hepes pH8
- 20mM Imidazol
- 650mM NaCl
- 10% Glycerol
- 0.5mM β-mercaptoethanol

**Binding Buffer for protein purification by Nickel column**

- 70mM Hepes
- 20mM Imidazole
- 500mM NaCl
- 0.1mM β-mercaptoethanol
- pH 8.0

**Elution Buffer for protein purification by Nickel column**

- 60mM Hepes
- 400mM Imidazole
- 150mM NaCl
- 3% Glycerol
SDS PAGE Staking Gel Buffer

0.5M Tris HCL pH6.8
0.4% w/v SDS

SDS PAGE Resolving Gel Buffer

1.5M Tris HCl pH8.8
0.4%w/v SDS

SDS PAGE Sample Buffer 1X

50mM Tris HCl pH6.8
2% w/v SDS
5% v/v β - mercaptoethanol or 41.6 mM DTT
10% w/v Glycerol
0.05% Bromophenol blue

SDS PAGE Running/Tank buffer 10X

0.5M Tris
0.55M Glycine
1% SDS

Blocking Buffer

10% w/v fat free milk in TBS 0.1% Tween20
2.3.3 Stains

Ponceau stain

0.2% (w/v) Ponceau S
5% glacial acetic acid

Coomassie Brilliant Blue staining solution

1% g/v of Coomassie Brilliant
Methanol (50% [v/v])
Glacial acetic acid (10% [v/v])
H₂O (40%)

Stir the solution for 3-4 hours and then filter through Whatman filter paper.
Store at room temperature.

2.3.4. Gels

Agarose (Sigma-aldrich)

Acrylamide gel

30% Acrylamide gel BioRad
3.3.4 Restriction Enzymes Used

*EcoRI* (Promega)

*HindIII* (Promega)

*BamHI* (Promega)

*SpeI* (Promega)

*SacI* (Promega)

*BgalII* (Promega)

*XmnI* (New England Biolabs; NEB)

PpuMI (New England Biolabs; NEB)

3.3.5 Media and supplements Source

7H11 Agar (Difco)

7H9 Agar (Difco)

ADC (Sigma)

OADC Difco
Chapter 3

Genetic manipulation of the ssrA gene to understand the function of the mycobacterial tmRNA
3.0 Introduction

In this chapter, it was attempted to investigate the role of the mycobacterial transfer messenger RNA (tmRNA), encoded by the ssrA gene. tmRNA, also known as the 10Sa RNA, is a highly conserved gene across bacterial species, which is up-regulated in stress conditions and possesses features of both tRNA and mRNA. The similarity between tRNA and ssrA based on the gene sequence in M. tuberculosis H37rv was first described by Tyagi and Kinger in 1992 (Tyagi and Kinger, 1992). The mRNA function of the ssrA was first demonstrated in E. coli by Tu et al., in 1995. However, tmRNA has dual functions: (1) to free the stalled ribosome at the 3’ ends of truncated mRNA, and (2) to tag the nascent incomplete protein for degradation (Hayes et al., 2002; Keiler, 2011) by different protease complexes including ClpXP, ClpAP, FtsH (Ahlawat and Morrison, 2009; Baker and Sauer, 2012; Dziedzic et al., 2010), Tsp and Lon (Choy et al., 2007). In E.coli, it has been shown that an accessory protein, SmpB, is also required for tmRNA to fully function (Karzai et al., 2000; Shimizu and Ueda, 2002) and the deletion of SmpB produced a similar phenotype to that observed in cells lacking ssrA (Karzai et al., 1999).

Deletion of the ssrA gene can affect viability, response to environmental stress and virulence in various bacteria (Keiler and Ramadoss, 2011). For example, disruption of the ssrA gene in E.coli can lead to a slower growth rate and reduction in motility on semi-solid agar (Komine et al., 1994), inhibition of λ-P22 Hybrid phage growth (Retallack et al., 1994), and negative regulation of some protease activity (Kirby et al., 1994; Retallack et al., 1994). ssrA RNA can also function as a cell cycle progression controller (Hong et al., 2005), to help
survive exposure to antibiotics, (Andini and Nash, 2011; Braud et al., 2006; Palečková et al., 2006; Yang and Glover, 2009) heat shock and oxidative stress (Paget et al., 2001). The ssrA gene also plays a role in Salmonella (enterica) typhimurium pathogenicity (Julio et al., 2000) and is essential for the growth of Shigella flexneri (Keiler, 2007) and Neisseria gonorrhoeae (Huang et al., 2000).

Studying the antibiotic producing actinomycete, Streptomyces coelicolor, Barens et al (Barends et al., 2010) revealed that tmRNA mediated trans-translation plays a role in stress management. Barens et al, analysed proteins that were histidine-tagged using recombinant tmRNA and identified 10 target genes whose translation is dependent on tmRNA: “Dnak heat shock protein 70, thiostrepton-induced protein A, universal stress protein A, elongation factor Tu3, and the cell-cycle control proteins DasR, SsgA, SsgF, and SsgR”. Although this function of tmRNA has not been observed in other bacteria, we hypothesised that it may be a feature of actinomycetes that may thus also occur in mycobacteria.

At the beginning of this project, there were few studies on the function of tmRNA in mycobacteria (Andini and Nash, 2011; Shi et al., 2011) but none reporting the gene replacement of ssrA. This chapter describes work to elucidate the function of the tmRNA in M. tuberculosis H37Rv. The specific objectives were to:

1. Attempt to knockout tmRNA in wild-type M. tuberculosis H37Rv

2. Attempt to knockout tmRNA in M. tuberculosis H37rv which was made merodiploid by the introduction of a second ectopic copy of the wild-type ssrA or by a recombinant ssrA in which the codons encoding the protease recognition tag had been replaced with codons encoding histidines (tmRNA-His)
3. Express tmRNA-His in knockout or merodiploid strains of *M. tuberculosis* and identify his-tagged proteins by LC-MS. This would:
   a. Define the protein substrate repertoire of the tmRNA tagging system for protease degradation.
   b. Identify if tmRNA is involved in the expression of chaperone and other stress proteins as demonstrated in the actinomycete, *Streptomyces*
3.1 Construction of a suicide vector to knockout ssrA (PG5ssrA::hyg)

The suicide vector PG5 was used as the parent vector for the ssrA gene knockout construct. PG5 contains the hyg gene and sacB gene as well as an aph kanamycin resistance gene and does not replicate in mycobacteria. The ssrA gene’s upstream and downstream flanks were PCR amplified using primers 3&4 (1027bp) and 1&2 (1254bp) respectively (Table 3). The flanks were directionally cloned (downstream flank at BgIII/XbaI and upstream flank at BamHI/Spel restriction sites) into PG5, flanking the hyg gene in the same orientation as in the mycobacterium genome flanking the ssrA gene. The resulting plasmid was PG5ssrA::hyg (Figure 9)

Figure 9. PG5ssrA::hyg construct

PG5ssrA::hyg map showing the hygromycin gene sandwiched by the ssrA flanking DNA. Hygromycin is the selection marker and SacB enables the counter selection against the plasmid backbone.

To confirm that the right fragments were cloned in the final construct, it was digested using the respective restriction enzyme used to clone that particular fragment. Further confirmation
of the orientation for the flanking DNA sequence and for the integrity of the plasmid sequence was provided by sequencing (data not shown).

Figure 10. pG5ssra::Hyg plasmid screening

The gel (1% agarose, TAE) picture shows the result of the PG5ssra::Hyg digestion that confirms the cloning of the respective flanks. The lanes are as follows: The two flanks released from pG5ssra::Hyg digested with the BamHI, SpeI, BglII and XbaI at same time (lane 1), linearised pG5ssra::Hyg with BglII (lane 2), linearised pG5 (lane 3) and undigested pg5ssra::Hyg (lane 4).
3.2 Deletion of the *ssrA* gene from WT *M. tuberculosis* H37rv

To delete the *ssrA* gene, the WT *M. tuberculosis* H37rv was electroporated with PG5*ssrA::*hyg. The transformants were recovered on 7H11 containing 50µg hygromycin ml$^{-1}$ and 2% sucrose and supplemented with 5% Oleic Acid Albumin Dextrose Complex (OADC). Only the recombinants where double cross-over gene replacement has taken place should grow on this selection. This is because the single cross over integration of the plasmid would include the *sacB* gene and its product will convert sucrose to levans which is toxic to bacterial cell. Five transformant colonies were produced from two independent transformations which were performed on cells with good transformation efficiency. These transformants were screened by PCR to amplify the region starting from outside of the *ssrA* gene flanks (cloned into PG5*ssrA::*hyg) and travelling inward across the *ssrA* gene, using primers P15&P16 (Table 3) All the colonies produced a PCR product of 3805bp, indicative of the fact that the *ssrA* gene was not deleted. If a deletion/gene replacement had occurred a product of 4315bp would have been produced (Figure 11&Figure 12). Further confirmation of the absence of gene replacement was provided by the digestion of the PCR fragment with *BgII*. The digestion did not show the expected bands that should be shown in the event that the gene replacement has taken place (Figure 15).
Figure 11. The ssrA deletion PCR product BglII digest schematic diagram

This diagram visualises the restriction digestion of the regions amplified from the outside flanks of the ssrA gene and across it in order to screen for its replacement with the hyg gene in the ssrA merodiploid. BglII cleaves the ssrA gene downstream flank cloned into PG5ssrA::hyg vector just few bases downstream of the hgy gene to produce two fragments of 1833bp (extended downstream flank) and 2501bp (containing extended upstream flank sequence plus hyg gene sequence.

Figure 12. PCR to screen the transformants for ssrA gene replacement.

The gel (1% agarose, TAE) picture shows the PCR products amplified from M. tuberculosis H37rv transformants, transformed with PG5ssrA::hyg, by using the primers (P15&P16) that anneal at sites outside of the flanking sequence and travel inward across the ssrA to give a 3805bp product for wild-type ssrA and a 4315bp product. All the screened isolated clones in lane 1- lane 7 showed 3805bp band indicating that the ssrA gene was not replace by hyg.
3.3 Generation of \textit{ssrA} merodiploid \textit{M. tuberculosis} H37Rv.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure13.png}
\caption{\textbf{pK.ssrA} construct}
\end{figure}

\textbf{pK.ssrA} construct map showing the \textit{ssrA} gene cloned at the \textit{SacI} site. This construct was introduced into \textit{M. tuberculosis} H37Rv wild type to produce the \textit{ssrA} merodiploid strain. The map also shows the location of the peptide tag within the \textit{ssrA} gene sequence as well as the location of primers P11&P12 (used to detect \textit{aph} gene) annealing sites.

It was suspected that the \textit{ssrA} may be essential for viability. One way to test this was to attempt \textit{ssrA} deletion in a merodiploid strain with a second copy of the \textit{ssrA} gene. The second copy of the gene was introduced into the wild type \textit{M. tuberculosis} H37Rv genome using the integrating plasmid pKINTA (Stewart \textit{et al.}, 2001). This plasmid integrates at the \textit{attB} integration site far from the native site of the \textit{ssrA} gene within the \textit{M. tuberculosis} genome. The construct, \textbf{pK.ssrA} (Figure 13) was generated by cloning the PCR amplified
sstrA gene with its putative promoter (using primers P7&P8 (Table 3) to amplify a fragment of 1192bp) into the SacI site of pKINTA. M. tuberculosis H37rv was transformed with pK_ssrA by electroporation and plated onto 7H11 containing kanamycin. The integration of the plasmid was confirmed by PCR to check for the presence of the aph gene sequence using primers P11&P12 (Table 3) to amplify 581bp fragment. PCR products from a successful pK_ssrA transformant screening, using primers 11&12, is shown in Figure 14.

Figure 14. Amplification of the aph gene to demonstrate integration of pK_ssrA construct to make srrA merodiploid M. tuberculosis

The gel (1% agarose, TAE) picture shows the result of the PCR products from M. tuberculosis H37rv wild type transformants electroporated with pK-ssrA. Lanes 1 – 4 show a positive PCR amplification of a 581bp fragment using the primers 11&12 that indicate the presence of the aph gene in the 4 selected transformants. Lane 5 is a positive control using the pk-ssrA construct and lane 6 is the negative control using wild type M. tuberculosis H37rv genomic DNA.
3.4 Deleting the in situ ssrA gene from the ssrA merodiploid M. tuberculosis H37rv strain

The ssrA merodiploid M. tuberculosis H37rv strain was used in the third attempt to delete the ssrA gene by transformation with the PG5ssrA::hyg suicide plasmid. Twenty individual colonies were picked and sub-cultured into 7H11 plate (containing 50µgml⁻¹ hygromycin and 15µgml⁻¹ Kanamycin) out of which four were PCR screened using primers P15&P16 to amplify the region from the outside of the flanking sequences (that were cloned into PG5 ssrA::hyg vector) and inward across the ssrA gene. These primers were designed to produce a fragment of 3805bp if no gene replacement took place or 4334bp if the gene was replaced with the hyg gene. The PCR products were digested with BglII and as it is shown in the schematic diagram (Figure 11) the digest should produce two fragments of 2501bp and 1833bp.

Three of the four transformants produced 4334bp PCR fragments indicative of gene replacement and one produced 3805bp (refer to lane 4 in Figure 12). All the four PCR products in lane 1 to 4 (for transformants no 2, 6, 8 and 3 respectively) were validated by cutting with BglII. The digest as explained in the schematic diagram in Figure 11 should produce two fragments with sizes of 2501bp and 1833bp if gene replacement has occurred. From the gel picture in Figure 15 it can be seen that transformants 2, 6 and 8 (Lane 1, 2 &3) had PCR fragments that were cut into two fragments (2501bp and 1833kb) indicative of the ssrA gene replacement with the hyg gene. However, the PCR product from transformant 3 (lane 4, Figure 15 was not cut indicative of the presence of the ssrA gene.
Figure 15. PCR screening of PG5ssrA::hyg

The gel (1% agarose TAE) picture shows the BglII digest of the PCR products amplified from *M. tuberculosis* H37rv transformants that were electroporated with PG5ssrA::hyg using primers (P15&P16) that anneal at sites outside of the flanking sequences cloned into PG5ssrA::hyg and inward across the ssrA gene. The PCR product in lane 5 is an example of the 4334bp PCR product amplified from transformant 2, 6 and 8 before digestion. The PCR product in lane 4 is from transformant 12 which is 3805bp and its size remained unchanged after treating it with BglII which is indicative of the wild-type ssrA and no ssrA gene replacement. The two bands seen in lanes 1, 2 and 3 are the result of BglII digestion of 4334bp amplified (from transformant 2, 6 and 8 respectively) indicating that the ssrA gene was replaced by the hyg gene.

3.5 Construction of a ssrA<sup>9His</sup> variant gene and integrating construct

To test whether the protease degradation tagging function of tmRNA was essential and also to define the tmRNA substrate repertoire, it was necessary to build a construct that would express a variant ssrA in which the sequence encoding the protease tag (QRDYALAA) was replaced with sequence encoding histidines. The peptide tag sequence was replaced with codons encoding 9 histidines and compensatory mutations were made in the surrounding sequence to maintain the structure of the tmRNA molecule (sequence 1&2, Figure 16). To achieve this, the 343bp DNA sequence including the *XmnI* and *PpuMI* restriction sites of
the ssrA gene was synthesized by “Eurofins MGW” (the sequence was received cloned into a plasmid named as “pEXA-ssrA-HIS tag”) and sub-cloned from the pEXA-ssrA HIS tag plasmid to the pK-ssrA construct similarly digested with XmnI and PpuMI in order to release the wild type ssrA fragment containing the peptide tag sequence. The final construct, pK\textsubscript{ssrA}\textsuperscript{9His} (Figure 17), was first screened by PCR using primers P17&P18 (Table 3. Primers list) and then confirmed by sequencing. The integrative pK\textsubscript{ssrA}\textsuperscript{9His} was electroporated into \textit{M. tuberculosis} H37rv and transformants were screened for both the existence of the \textit{aph} gene (as described earlier) and the histidine tag sequence using P11 &P12 (Table 3. Primers list)

**Sequence (1) from wild type ssrA (ADSHQRDYALAA)**

\begin{verbatim}
GGATGCGAGGGGCT (XmnI restriction site) GAACCGGTTTGCACCTCGCGCATCGAATCAAGGGGAAGCGTGCCGGTGCAAGGCAAGGACCACC GCCGATTACATCAAGCGAAGACTACGCTCTCGTGCC GTAAGCGACGGCTAGTCTGTCAAGAAGGGGAACCGCCCTCGGCCCAGACCTCGACATCGACTAG AGGATCCACCGATGATCGTGGCAGGACAACCACAGGACTGGGATCGTC ATCTCGGCTAGTCCCGTGACCGAGATCCGAGAGCATAGCGAATCGC ACAGGAGAGAAGCC TT (PpuMI restriction site) GAGGGAATGCGGT
\end{verbatim}

**Sequence (2) from his-ssrA (ADSHHHHHHHHHH)**

\begin{verbatim}
GGATGCGAGGGGCT (XmnI restriction site) GAACCGGTTTGCACCTCGCGCATCGAATCAAGGGGAAGCGTGCCGGTGCAAGGCAAGGACCACC GCCGATTACATCAAGCGAAGACTACGCTCTCGTGCC GTAAGCGACGGCTAGTCTGTCAAGAAGGGGAACCGCCCTCGGCCCAGACCTCGACATCGACTAG AGGATCCACCGATGATCGTGGCAGGACAACCACAGGACTGGGATCGTC ATCTCGGCTAGTCCCGTGACCGAGATCCGAGAGCATAGCGAATCGC ACAGGAGAGAAGCC TT (PpuMI restriction site) GAGGGAATGCGGT
\end{verbatim}

**Figure 16. ssrA and ssrA\textsuperscript{9His} variant sequence**

Comparison of the sequence of the native \textit{M. tuberculosis} ssrA and its variant ssrA\textsuperscript{9His}. The sequences shown are not the complete sequence of the ssrA but, just the segment 102bp upstream and 202bp downstream form the peptide tag stop codon TAA. The 16bp region downstream of the stop codon in the ssrA variant was modified in the ssrA\textsuperscript{9His} variant to maintain the RNA structure to as near to the native ssrA. The codons for QRDYALAA were replaced with codons for 9 histidines. The sequence in red is the wild type ssrA tag sequence (sequence 1) and the ssrA\textsuperscript{9His} variant tag sequence (sequence 2). The underlined sequence is the 9 Histidines sequence.
**Figure 17. pK.ssrA<sup>H</sup>His construct**

pK.ssrA<sup>H</sup>His construct map showing the ssrA<sup>H</sup>His gene cloned at SacI site. This construct was introduced into *M. tuberculosis* H37rv wild type to produce the Hist-ssrA merodiploid strain. The map also shows the location of the His-tag within the ssrA gene sequence as well as the location of primers P11&P12 (to detect *aph* gene) and primers P17&P18 (to detect the His-tag sequence) annealing sites.
Figure 18. Screening for pK.ssrA<sup>9His</sup> M. tuberculosis H37rv merodiploid

The gel (1% agarose, TAE) picture shows the result of the PCR product from M. tuberculosis H37rv wild type transformants electroporated with pK.ssrA<sup>9His</sup>. The left hand side of the gel picture shows a positive PCR amplification of 581bp fragment from transformants 1-5 using primers P11&P12 indicating the presence of the aph gene. The right hand side of the gel shows a positive PCR amplification of 526bp fragment from the same transformants respectively indicating the presence of the histidine tag sequence.
3.6 Attempt to delete the ssrA gene from the ssrA\textsuperscript{9His} expressing \textit{M. tuberculosis} H37rv strain

Having demonstrated that it was possible to delete the \textit{in situ} ssrA gene from a merodiploid \textit{M. tuberculosis} H37Rv, we attempted to delete ssrA from a strain complemented with the ssrA\textsuperscript{9His} variant, which is intended to confer the ribosome recycling function of the tmRNA but replace the protease tagging function with a His-tagging function.

Electroporation of the ssrA\textsuperscript{9His} H37rv strain with the PG5 ssrA::hyg vector and selection on hygromycin/sucrose 7H11 resulted in only 7 colonies all of which showed the presence of the wild-type ssrA gene. All of them showed a <4kb ssrA gene region PCR fragment upon screening as before and further, the product did not cut with BglII restriction enzyme (data not shown). This indicated that the protease tagging function of the tmRNA may be essential to \textit{M. tuberculosis} H37rv viability and alternatively the alterations to the ssrA to introduce the histidine codons may have altered the other functions of the tmRNA in addition to the protease tagging role.
3.7 Experiments to detect ssrA tagged substrate proteins using mycobacteria expressing a ssrA\textsuperscript{His}

The fact that the deletion of ssrA from \textit{M. tuberculosis} H37rv was only achieved in a merodiploid strain carrying a second wild-type copy of ssrA was strongly indicative that it is an essential gene and also that the two main functions of tmRNA may both be essential for viability. Without the ability to study the phenotype of ssrA mutant strains, further study in this project to understand tmRNA function in mycobacteria focused on the detection of His-tagged proteins produced in a merodiploid carrying the wild-type ssrA in addition to a His-tagging variant ssrA. Several different expression constructs of the ssrA\textsuperscript{His} variant were made which included variations of the ssrA sequence alongside expression with and without the upstream genes that include the \textit{smpB} gene. These expression constructs were transformed into two host strains, \textit{M. bovis} BCG and \textit{Mycobacterium smegmatis} groEL1\DeltaC (Noens \textit{et al.}, 2011) and the resultant recombinant strains are listed in Table 4. The \textit{M. smegmatis} groEL1\DeltaC strain lacks the natural histidine rich C terminal of its GroEL which can hamper His purification in mycobacteria and was obtained from the European Molecular Biology Laboratory (EMBL) Hamburg. Some of the ssrA\textsuperscript{His} constructs were also transformed into \textit{M. smegmatis} groEL1\DeltaC\Deltalon which lacks the Lon protease. The following sections describe the attempts to purify His-tagged ssrA substrate proteins using these different mycobacterial recombinant strains.
<table>
<thead>
<tr>
<th>Merodiploid</th>
<th>The construct harboured</th>
<th>Integrative/Replicative</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCG&lt;sup&gt;9HisA&lt;/sup&gt;</td>
<td>pK-ssrA&lt;sup&gt;HIS&lt;/sup&gt;</td>
<td>Integ/Native promoter</td>
</tr>
<tr>
<td>BCG&lt;sup&gt;9HisB&lt;/sup&gt;</td>
<td>pMV306-ssrA&lt;sup&gt;HIS&lt;/sup&gt;</td>
<td>Integ/hsp promoter</td>
</tr>
<tr>
<td>BCG&lt;sup&gt;9HisC&lt;/sup&gt;</td>
<td>pK-smpB-Rv3099c-ssrA&lt;sup&gt;HIS&lt;/sup&gt;</td>
<td>Integ/native promoter</td>
</tr>
<tr>
<td>BCG&lt;sup&gt;9HisD&lt;/sup&gt;</td>
<td>pMV261-smpB-Rv3099c.ssrA&lt;sup&gt;HIS&lt;/sup&gt;</td>
<td>Rep/hsp promoter</td>
</tr>
<tr>
<td>M. smegmatis groEL1Δ&lt;sup&gt;C9HisA&lt;/sup&gt;</td>
<td>pK-ssrA&lt;sup&gt;HIS&lt;/sup&gt;</td>
<td>Integ/Native promoter</td>
</tr>
<tr>
<td>M. smegmatis groEL1Δ&lt;sup&gt;C9HisB&lt;/sup&gt;</td>
<td>pMV306-ssrA&lt;sup&gt;HIS&lt;/sup&gt;</td>
<td>Integ/hsp promoter</td>
</tr>
<tr>
<td>M. smegmatis groEL1Δ&lt;sup&gt;C9HisC&lt;/sup&gt;</td>
<td>pK-smpB-Rv3099c-ssrA&lt;sup&gt;HIS&lt;/sup&gt;</td>
<td>Integ/hsp</td>
</tr>
<tr>
<td>M. smegmatis groEL1Δ&lt;sup&gt;C9HisD&lt;/sup&gt;</td>
<td>pMV261-smpB-Rv3099c.ssrA&lt;sup&gt;HIS&lt;/sup&gt;</td>
<td>Integ/native promoter</td>
</tr>
<tr>
<td>M. smegmatis groEL1Δ&lt;sup&gt;C9HisE&lt;/sup&gt;</td>
<td>pMV361- smpB-Rv3099c.ssrA&lt;sup&gt;HIS&lt;/sup&gt;</td>
<td>Integ/hsp promoter</td>
</tr>
<tr>
<td>M. smegmatis groEL1Δ&lt;sup&gt;C9HisF&lt;/sup&gt;</td>
<td>pK-smpB-Rv3099c-ssrA&lt;sup&gt;HIS&lt;/sup&gt;</td>
<td>Integ/ native promoter</td>
</tr>
<tr>
<td>M. smegmatis groEL1Δ&lt;sup&gt;C9HisG&lt;/sup&gt;</td>
<td>pMV261-smpB-Rv3099c.ssrA&lt;sup&gt;HIS&lt;/sup&gt;</td>
<td>Rep/native promoter</td>
</tr>
<tr>
<td>M. smegmatis groEL1Δ&lt;sup&gt;C9HisH&lt;/sup&gt;</td>
<td>pK-smpB-Rv3099c-ssrA&lt;sup&gt;HIS&lt;/sup&gt;</td>
<td>Integ/native promoter</td>
</tr>
<tr>
<td>M. smegmatis groEL1Δ&lt;sup&gt;C9HisI&lt;/sup&gt;</td>
<td>pMV261-smpB-Rv3099c.ssrA&lt;sup&gt;HIS&lt;/sup&gt;</td>
<td>Rep/native promoter</td>
</tr>
<tr>
<td>M. smegmatis groEL1Δ&lt;sup&gt;C9HisJ&lt;/sup&gt;</td>
<td>pK-smpB-Rv3099c-ssrA&lt;sup&gt;HIS&lt;/sup&gt;</td>
<td>Integ/native promoter</td>
</tr>
<tr>
<td>M. smegmatis groEL1Δ&lt;sup&gt;C9HisK&lt;/sup&gt;</td>
<td>pMV261-smpB-Rv3099c.ssrA&lt;sup&gt;HIS&lt;/sup&gt;</td>
<td>Integ/native promoter</td>
</tr>
<tr>
<td>M. smegmatis groEL1Δ&lt;sup&gt;C9HisL&lt;/sup&gt;</td>
<td>pK-smpB-Rv3099c-ssrA&lt;sup&gt;HIS&lt;/sup&gt;</td>
<td>Integ/native promoter</td>
</tr>
<tr>
<td>M. smegmatis groEL1Δ&lt;sup&gt;C9HisM&lt;/sup&gt;</td>
<td>pMV261-smpB-Rv3099c.ssrA&lt;sup&gt;HIS&lt;/sup&gt;</td>
<td>Integ/native promoter</td>
</tr>
</tbody>
</table>

**Table 4.** *M. bovis* BCG and *Mycobacterium smegmatis* groEL1AC (*M. smegmatis* groEL1 ΔC) merodiploid strains harbouring different expression *ssrA*<sup>HIS</sup> vectors.
3.7.1 Purification of His-tagged proteins from (BCG$^{9\text{His}A}$) pK.$ssrA^{9\text{His}}$ lysate

The BCG$^{9\text{His}A}$ merodiploid was grown in a volume of 500ml of Roisin’s minimal media containing 30µg ml$^{-1}$ of kanamycin until an OD of 1. The culture was spun and the cell pellet was 3 times washed in ice cold PBS. The cells were re-suspended in a lysis buffer containing protease inhibitors (1mM PMSF and 1mM EGTA) and mechanically disrupted. The lysate was twice spun before passing through the 1ml nickel column. The protein concentration in the eluted sample was measured using the Nanodrop spectrophotometer. The highest protein concentration obtained was 0.4mg/ml. Two of the His pull-down samples were run in 12% polyacrylamide gels along with neat BCG str. Pasteur merodiploid lysate, wild-type BCG and $M$. $tuberculosis$ H37rv wild-type neat lysate. Figure 19 shows a coomassie stain of the gel and reveals that the nickel column has purified a smear of proteins of a wide molecular mass range but dominated by a dense band at approximately 55kDa. A replicate gel was transferred to PVDF membrane. The membrane was blocked with 10% milk (dissolved in 0.1% Tween 20 TBS buffer) and treated with anti-his HRP conjugated antibodies (1 in 1000) and developed using Clarity™ Western ECL Blotting substrate.
Figure 19. Western blot and Protein gel for nickel column purified protein from BCG^{9HisA}

Picture (A) Western blot for detection of histidine tagged protein (as a result of possible ssrA tagging) seen in picture B. Lane 1&2 are the histidine column purified protein. Lane 3, 4 and 5 are the neat lysate from BCG str. Pasteur merodiploid (BCG^{9HisA}), wild type BCG and wild type M. tuberculosis H37rv respectively. Lane 6 is the positive control for histidine tag using the recombinant Acr1 protein. The bands seen in picture (B) lane 1-6 correspond to the lanes on picture (A).

The Western Blot showed anti-His immunoreactivity only for the histidine column purified proteins and the neat lysate of the BCG merodiploid (BCG^{9HisA}). The non-nickel purified neat lysate of BCG wild type and M. tuberculosis H37rv had no immunoreactivity, which indicated the absence of any naturally histidine rich protein background. The nickel purified protein was subjected to trypsin digest and LC-MS and the data analysed by mascot to identify the proteins.
Mascot result

BCG str. Pasteur merodiploid (BCG\textsuperscript{9HisA}) lysate was passed through nickel column to pull down any possible histidine tagged protein. The obtained protein was analysed by mass spectrophotometry. The predominant identified protein was GroEL1. The letters in red are the amino acids identified by the mass spectrophotometry and the black letters are those predicted when compared to Mycobacteria proteins database.

Mascot results indicated the presence of a dominant protein, GroEL1, which is an abundant non-essential heat shock protein (Figure 19). The most obvious feature of GroEL1 is that it has a histidine rich C-terminal in its genomic coding sequence. This feature may help explain why it is purified on the nickel column and why it is reactive with anti-His antibodies in the Western blot. The reason why no positive band can be seen in the BCG str. Pasteur wild-type and M. tuberculosis H37rv may be because these wild-type cultures were not stressed sufficiently to produce a detectable level of GroEL1 which is a stress inducible chaperone. This would suggest that expression of the altered ssrA induces a stressed state in the
recombinant mycobacterium. Further attempts were made using different BCG merodiploids harbouring different (Table 4). These are described in the following sections.

3.7.2 Detection of ssrA\(^{9\text{His}}\) His-tagged protein in \textit{M. smegmatis} groEL1ΔC harbouring pKssrA\(^{9\text{His}}\), pMV261 ssrA\(^{9\text{His}}\) and pMV361 ssrA\(^{9\text{His}}\) expression vectors

The failure to pull down any ssrA\(^{9\text{His}}\) His-tagged proteins in BCG str. Pasteur other than the naturally occurring His-rich GroEL1 may have been due to the abundance of GroEL1 either saturating the nickel column or masking the detection of less abundant proteins by LC-MS. To counter this potential problem, \textit{Mycobacteria smegmatis} groEL1ΔC (Noens \textit{et al.}, 2011), lacking the histidine rich C terminal region, was obtained from the European Molecular Biology Laboratory (EMBL) Hamburg in Germany. The genotype of the strain was checked by PCR and sequencing of the GroEL1 gene, which confirmed removal of the terminal histidine residues (data not shown). Although this was a different species of mycobacteria we anticipated that the \textit{Mycobacterium tuberculosis} H37rv ssrA would be functional in this strain. Merodiploids of \textit{Mycobacterium smegmatis} groEL1ΔC were produced using the same construct as before (pKssrA\(^{9\text{His}}\)) and also several new constructs in which the ssrA\(^{9\text{His}}\) variant gene was cloned under the control of other mycobacterial promoters in the replicative shuttle vectors pMV261 (Bolla \textit{et al.}, 2012) and integrative pMV306 (Greendyke \textit{et al.}, 2002) (Table 4). The resultant merodiploids were grown as described above in the BCG section (3.7.1). Protein was isolated from the cultures and nickel columns used to pull down any his-tagged proteins. Very little protein was purified from the column as viewed by coomassie staining (an example is shown in Figure 21) and Western blotting with an anti-His antibody did not reveal any His-tagged proteins (an example is shown in Figure 22).
Figure 21. SDS-PAGE of whole cell lysates and His-tag purified proteins from *M. smegmatis* GroELΔC and *M. smegmatis* groELΔC merodiploids harbouring constructs that express ssrA<sup>9His</sup>.

SDS-PAGE shows coomassie stained whole lysate (lane 2, 4 and 6) from *M. smegmatis* groELΔC, *M. smegmatis* groELΔC<sup>9His</sup> (harbouring pK.<sup>ssrA<sup>9His</sup></sup>) and *M. smegmatis* groELΔC<sup>9His</sup> (harbouring pMV261.<sup>ssrA<sup>9His</sup></sup>) respectively. Lanes 1, 3 & 5 show the nickel column pull down for the respective strains.

Figure 22. Western blot for detection of the histidine tagged protein from *M. smegssrA<sup>9His</sup>B* and *M. smegssrA<sup>9His</sup>A* His-pull down preps.

Western blot for the detection of histidine tagged protein in the nickel column pull down fractions from *M. smegmatis* groELΔC, *M. smegmatis* groELΔC<sup>9HisA</sup> (harbouring pK.<sup>ssrA<sup>9His</sup></sup>) and *M. smegmatis* groELΔC<sup>9HisB</sup> (harbouring pMV261.<sup>ssrA<sup>9His</sup></sup>) in lanes 1, 3 & 5 respectively. Lanes 2, 4, and 6 are the whole lysate from the respective strains. Lane 7 is a his-tagged positive control protein.
3.8 Co-expression of the 

The constructs for the expression of \( ssrA^{9\text{His}} \) that were used in the previous section did not include its binding protein, SmpB, encoded by the \( smpB \) gene. SmpB protein is not essential for the aerobic growth of \( M.\,\text{tuberculosis} \) (Personne and Parish, 2014), which indicates that it might be dispensable for the tmRNA tagging. SmpB has been shown to be an important factor for determination of the initial codon for tag-translation in \( E.\,\text{coli} \) and damaged SmpB failed to promote peptidyl transfer (Konno et al., 2007). Also, deleting the \( smpB \) gene led to the absence of tmRNA in \( Streptomyces\,\text{coelicolor}\,\Delta smpB \) suggesting it may be required for tmRNA accumulation (Yang and Glover, 2009). To accommodate for the possibility that the co-expression of \( smpB \) with the tmRNA\(^{9\text{His}}\) variant may increase the tagging efficiency, \( smpB \) was cloned along with the \( ssrA \) in co-expression constructs.

As shown in Figure 23, Rv3099c (a hypothetical protein) is located in-between \( ssrA \) and \( smpB \). These three genes are all oriented in the same direction in a putative single transcriptional unit suggesting that they may be functionally related in the tmRNA protein tagging system. Therefore, to avoid any possible transcriptional defects, the whole segment containing the \( smpB \), Rv3099c and \( ssrA \) was PCR amplified using primers P37&P38 and cloned into pKinta to produced pK-\( smpB\)-Rv3099c-\( ssrA \) (Appendix 8.8) employing the same protocol as in section (3.3). The primers used to amplify this segment contained \( SacI \) and \( EcoRI \) at the 5’ of the forward primer and \( SacI \) and \( HindIII \) at the 5’ of the reverse primer to allow for the cloning in of pKinta, and the subsequent sub-cloning into pMV261 (replicative expression vector containing hsp promoter) and pMV361 (integrative expression vector containing hsp promoter). Then the native \( ssrA \) gene was replaced with the \( ssrA^{9\text{His}} \) variant.
using the same strategy as described in section (3.5). The resulting construct was designated pk-\textit{smpB-Rv3099c-ssrA}^{9\text{His}} (Figure 24). From this plasmid the \textit{smpB-Rv3099c-ssrA}^{9\text{His}} fragment was cleaved using \textit{EcoRI} and \textit{HindIII} restriction enzymes and directionally cloned in front of the heat shock promoter (hsp60) in pMV361 (Appendix 8.9) plasmid as well as into the pMV261 plasmid to produce pmv261-\textit{smpB-Rv3099c-ssrA}^{9\text{His}} (Figure 25).

\textbf{Figure 23.} Schematic diagram (A) showing the position of \textit{smpB}, MSMEG\_2092 and MSMEG\_2093 (tmRNA) and their orientation on the \textit{M. smegmatis} genome obtained from http://mycobrowser.epfl.ch/smegmasearch.php?gene+name=smpB&submit=Search. Schematic diagram (B) showing the position of \textit{smpB}, Rv3099c and \textit{ssr} (\textit{ssrA}) and their orientation on the \textit{M. tuberculosis} genome obtained from http://tuberculist.epfl.ch/quicksearch.php?gene+name=smpB&submit=Search.
Figure 24. pk-smpB-Rv3099c-ssrA\textsuperscript{9His} construct

pk-smpB-Rv3099c-ssrA\textsuperscript{9His} construct map showing the smpB-Rv3099c-ssrA\textsuperscript{9His} gene complex cloned at the SacI site. The map shows the location of the Hist-tag within the ssrA gene sequence.

Figure 25. pMV261-smpB-Rv3099c-ssrA\textsuperscript{9His} construct

pMV261-smpB-Rv3099c-ssrA\textsuperscript{9His} construct map showing the smpB-Rv3099c-ssrA\textsuperscript{9His} gene complex directionally cloned at HindIII and EcoRI sites. This construct was introduced into \textit{M. smegmatis groEL1ΔC parental strain} and \textit{M. smegmatis groEL1ΔC Δlon} to produce smpB-Rv3099c-ssrA\textsuperscript{9His} merodiploid strains.
These constructs were used to transform *M. smegmatis* groEL1ΔC using the procedures described in section (3.3) to produce merodiploids harbouring either pK-*smpB*-Rv3099c-*ssrA*<sup>9His</sup> or pmv261-*smpB*-Rv3099c-*ssrA*-<sup>9His</sup> (Table 4). The successful merodiploids were grown and harvested as described in section (3.7.2).

### 3.9 Validation of *ssrA*<sup>9His</sup> expression in the *M. smegmatis* groEL1ΔC merodiploids harbouring pk-*smpB*-Rv3099c-*ssrA*<sup>9His</sup> or pMV261-*smpB*-Rv3099c-*ssrA*<sup>9His</sup> expression vectors

Before embarking on the protein purification experiments from the “new” merodiploids harbouring pk-*smpB*-Rv3099c-*ssrA*<sup>9His</sup> and pMV261-*smpB*-Rv3099c-*ssrA*<sup>9His</sup>, it was decided to check for the expression of *ssrA*<sup>9His</sup> by RT-PCR. The merodiploids and the parent strain (*M. smegmatis* groEL1ΔC) were inoculated into 10ml 7H9 containing 10% ACD and 0.1% Tween 80 in triplicate and incubated shaking at 37°C overnight. Two hours before extracting the RNA the replicate cultures were treated thus:

1. A final concentration of 16μg/ml erythromycin was added to one of the three cultures for each strain. This was done because it was reported that exposure to ribosome targeting antibiotics increases expression of tmRNA in Mycobacteria (Andini and Nash, 2011)

2. A final concentration of 0.5mM of H<sub>2</sub>O<sub>2</sub> was added to stress the culture

3. Untreated

RNA was extracted using the ThermoFisher Scientific Trizol®Plus RNA purification system. The RNA samples were DNAase treated before the RT-PCR was performed. The cDNA was used as the template to detect the presence of the his-tag sequence. Positive
detection of an 80 bp product would confirm the presence and expression of $ssrA^{9\text{His}}$ in the respective merodiploid using primers P35&P36 (Table 3). No amplification should result from the native $ssrA$ transcript.

As seen in the agarose gel (Figure 26), the no reverse transcriptase control for DNA contamination in the RNA (lanes 10, 11 &12) did not show a band thereby confirming that no DNA was present in the RNA samples prior to RT PCR and therefore, all the PCR products were from the mRNA transcripts only. Two positive controls were used (lanes 13 and 14 pk.$smpB$-$Rv3099c$-$ssrA^{9\text{His}}$ and pMV261.$smpB$-$Rv3099c$-$ssrA^{9\text{His}}$ DNA) to check the PCR primer specificity and the amplification. They were also used to double check that the constructs do have the $ssrA^{9\text{His}}$ variant constructs. Both constructs showed the expected 80bp band although an 600bp product is also observed in the pK.$smpB$-$Rv3099c$-$ssrA^{9\text{His}}$ (lane 13) construct. This could be due to the primers having a non-specific binding to a sequence within the pKinta plasmid.
Figure 26. PCR of cDNA from RT PCR of different RNA preps from *M. smegmatis* groEl1ΔC and *M. smegmatis* groEl1ΔC ssrA merodiploids harbouring either pk.smpB-Rv3099c-ssrA<sup>θHis</sup> or pMV261-smpB-Rv3099c.ssra<sup>θHis</sup>

Lanes 10, 11 and 12 show the PCR result for the no reverse transcriptase control for DNA contamination in the RNA sample. Lanes 1, 4 and 7 show no amplification products from RT-PCR of RNA from *M. smegmatis* groEl1ΔC wild type either untreated, treated with 16µg/ml<sup>-1</sup> erythromycin, or treated with 0.5mM H<sub>2</sub>O<sub>2</sub> respectively. RT-PCR of the *M. smegmatis* groEl1ΔC merodiploid harbouring pk.smpB-Rv3099c-ssrA<sup>θHis</sup> (lane 2; no treatment, lane 5; 16µg/ml<sup>-1</sup> Erythromycin, lane 8; 0.5mM H<sub>2</sub>O<sub>2</sub>) show the expected band for the presence of the His-tag sequence confirming the expression of ssrA<sup>θHis</sup> variant in all the preparations. RT-PCR of the *M. smegmatis* groEl1ΔC merodiploid harbouring pMV261-smpB-Rv3099c.ssra<sup>θHis</sup> (lane 3; no treatment, lane 6; 16µg/ml<sup>-1</sup> Erythromycin, lane 9; 0.5mM H<sub>2</sub>O<sub>2</sub>) shows the presence of the His-tag confirming the expression of ssrA<sup>θHis</sup> in two of the preps but, the third failed to amplify. The two positive controls of plasmid DNA amplified 80bp indicating the presence of His-tag in the constructs but pk.smpB-Rv3099c-ssrA<sup>θHis</sup> (lane 13) showed other bands of 600 which was absent in pMV261-smpB-Rv3099c.ssra<sup>θHis</sup> (lane 15). The negative control PCR mix with no template added (lane 14) produced no product indicating that the reagents were free from DNA contamination.
RT-PCR of RNA (Figure 26) from the *M. smegmatis groElΔC* merodiploid harbouring pK.smpB-Rv3099c-ssrA^9His^ (lane 2; no treatment, lane 5; 16µg/ml erythromycin, lane 8; 0.5mM H_2O_2) and the *M. smegmatis groElΔC* merodiploid harbouring pMV261.smpB-Rv3099c.ssrA^9His^ (lane 3; no treatment, lane 9; 0.5mM H_2O_2) showed the expected band (80 bp) for the presence of the His-tag sequence confirming the expression of ssrA^9His^ variant in all the recombinant strains. However, one of the samples from *M. smegmatis groElΔC* harbouring pMV261.smpB-Rv3099c.ssrA^9His^ (lane 6, erythromycin treated) failed to amplify an ssrA product which may have been due to the presence of inhibitors in this sample.

3.10 Detection of ssrA^9His^ variant His-tagged proteins using *M. smegmatis groElΔC* harbouring pK.smpB-Rv3099c-ssrA^9His^, pMV261.smpB-Rv3099c-ssrA^9His^ expression vectors

After it was confirmed that the ssrA^9His^ variant was being expressed in the *M. smegmatis groElΔC* merodiploids harbouring pK.smpB-Rv3099c-ssrA^9His^ and pMV261.smpB-Rv3099c-ssrA^9His^ expression vectors, the strains were grown as described before in section (3.7.2) but, either with single or combined stress condition/s (heat shock at 42°C and sublethal concentration of erythromycin (16ug/ml) and H_2O_2 (0.5mM)) for three hours prior to protein extraction. The protein extraction was carried out as described in section (3.7.2). Unfortunately, as previously experienced, western blotting showed no band for any His-tagged protein (Data not shown). Thus, co-expression of the SmpB protein and Rv3099c with ssrA^9His^ did not increase His-tagging of protein substrates.

This negative result raised another question: could it be possible that the tagged protein was degraded? One of the proteases that is directly involved in the degradation of the tmRNA tagged-proteins is the Lon protease which is encoded by the *lon* gene. This protease is an
ATP-dependent protease that has the ability to recognise determinants in the N-terminal of tmRNA-tagged proteins (Choy et al., 2007). Choy et al showed that deleting Lon caused the accumulation of the His6-tagged proteins in mutants harbouring a tmRNA^His^ variant. Although, this work was done in E. coli, M. smegmatis has the Lon protease, therefore, it was decided to delete lon in M. smegmatis groEL1ΔC and repeat the experiments with the ssrA^His^ expression.

3.11 Deletion of the lon (MSMEG_3582) gene from M. smegmatis groEL1ΔC

M. tuberculosis H37rv and BCG str. Pasteur do not have the lon gene but it is present in M. smegmatis groEL1ΔC. Therefore, it was possible that the Lon protease was involved in degradation of the His-tagged protein. Therefore, the lon gene was deleted from M. smegmatis groEL1ΔC using a one-step knockout strategy using pG5 suicide plasmid as describe in section (3.2). The lon gene flanks were PCR amplified with the relevant restriction enzymes at both ends to clone them in to flank the hygromycin gene on pG5 (Figure 27).
Figure 27. The pG5lon::hyg construct for gene-replacement of \textit{M. smegmatis groEL1AC \textit{Δlon}}.

PG5lon::hyg map showing the hygromycin gene flanked by DNA from the \textit{lon} gene genome region. Hygromycin is the positive selection marker and \textit{SacB} is the counter selection.

The pG5Lon::hyg construct was introduced into \textit{M. smegmatis groEL1AC} by electroporation. The resultant clones were screened for the absence of the \textit{lon} gene by PCR and confirmed by restriction digestion and sequencing (Figure 29).
Figure 28. Lon gene homologous recombination schematic diagram

Diagram (A) illustrates the location of the *lon* gene in *M. smegmatis groEL ΔC* genome. Diagram (B) illustrates the addition of a *Bgl*II site in the knockout strains which would give a restriction pattern of the PCR product between primers P34/P33 enabling the differentiation between the wild-type and gene-replacement genotypes.

Two clones gave a PCR product of 6350bp indicative of *lon* gene replacement by hygromycin (as illustrated in Figure 28). The PCR products were then restriction digested with *Bgl*II which produced three fragments of 2837bp, 1880bp and 713bp confirming the gene replacement of *lon* (Figure 29).
Figure 29. PCR amplification and BglII digest to determine gene-replacement of lon.

Agarose gel (1%) picture showing the BglII restriction digest of the PCR fragment amplified from the outside flanks of the lon gene using primers (P33&P34). The clone with the successful gene replacement (Lane 4 and 5) produced a fragment of 5430bp whereas the wild type (lane 3) produced a fragment of 6350bp. The BglII digest of the fragments in lane 1 & 2 produced the expected bands of 2837bp, 188bp and 713bp to verify the gene replacement.
3.12 Detection of ssrA\(^{9\text{His}}\) variant tagged protein using \textit{M. smegmatis groEl1ΔCΔCΔlon} harbouring pK.smpB-Rv3099c-ssrA\(^{9\text{His}}\), pMV261.smpB-Rv3099c-ssrA\(^{9\text{His}}\) expression vectors

\textit{M. smegmatis groEl1ΔCΔlon} was transformed with either pK.smpB-Rv3099c-ssrA\(^{9\text{His}}\) or pmv261.smpB-Rv3099c-ssrA\(^{9\text{His}}\) to produced merodiploid strains harbouring wild-type \textit{ssrA} and \textit{ssrA}\(^{9\text{His}}\) genes and designated as \textit{M. smegmatis groEl1ΔCΔlon}\(^{9\text{HisK}}\) and \textit{M. smegmatis groEl1ΔCΔlon}\(^{9\text{HisL}}\) respectively. The successful merodiploids were grown and harvested as described earlier in section (3.7.2). The protein was extracted as described in section (3.7.2). Once again, western blotting for the His-tagged proteins indicated unsuccessful pull-down of any tagged protein (Data not shown). This suggested that the Lon protease was not the cause for failure of detecting any \textit{ssrA}\(^{9\text{His}}\) tmRNA variant tagged protein.
3.13 The construction of the ssrA\(^6\)His gene construct

In the ssrA\(^9\)His variant gene used above, the proteolytic tag ADSHQRDYaLaA Stop sequence was replaced with ADShHHHHHHHHH Stop in which glutamine and arginine were lost. Wower and colleagues (Wower \textit{et al.}, 2014) produced a \textit{M. tuberculosis} tmRNA\(^6\)His variant that had tagging activity in \textit{E. coli}. The tmRNA\(^6\)His variant tag sequence they presented was ADShQRHHHHHHH which was different to the Tag used in the present study so far as it retained the glutamine and arginine and replaced the DyALAA codons of the proteolytic tag with six histidine codons. To accommodate for a possible negative effect of losing the codons for glutamine and arginine which may be important for the \textit{tmRNA} variant tagging efficiency or its stability, I restored these codons in a modified construct. Site directed mutagenesis (section 2.16) was used to change the sequence of the ssrA\(^9\)His variant tag from ADShHHHHHHHHH Stop to ADShQRRHHHHHH Stop (Figure 30). This variant gene was designated as ssrA\(^6\)His.

**Version 1 ssrA\(^9\)His sequence (ADShHHHHH HHH)**

GGATCGAGGGGCT (XmnI restriction site) GAAACGGTTTCACTTCGCACATGAATCAAGGAAGCGTGGCGTGGCAAGCAAGACCCGATGAAACGCTGCGGCTGAACGGTTTCGACTTCGCACATTACATCACAACCAACACCAACCAACTTACTTACTAATCGTCTGCTGTGCAAGCGAGCGGAACCCGCG GTGCCGCCGGACCTGGCATCGCTAGAGGGATCCACCGATGAGTCCGGTCGCGGGACTCCTCGGGACACCACAGCG ACTGGGATCTGTCATCTGGCTAGTTCGCTGACCAGGGAGATCCCAAGACAGCATAGCGAAGACAAGCCCTGCGACG

**Version 2 ssrA\(^6\)His sequence (ADShQRRHHHHHH)**

GGATCGAGGGGCTGAACGGTTTCGACTTCGCACATGAATCAAGGAAGCGTGGCGTGGCAAGCAAGACCCGATGAAACGCTGCGGCTGAACGGTTTCGACTTCGCACATTACATCACAACCAACACCAACCAACTTACTTACTAATCGTCTGCTGTGCAAGCGAGCGGAACCCGCG GTGCCGCCGGACCTGGCATCGCTAGAGGGATCCACCGATGAGTCCGGTCGCGGGACTCCTCGGGACACCACAGCG ACTGGGATCTGTCATCTGGCTAGTTCGCTGACCAGGGAGATCCCAAGACAGCATAGCGAAGACAAGCCCTGCGACG

**Figure 30. ssrA\(^6\)His variant sequence compared to ssrA\(^9\)His variant**

The ssrA\(^9\)His variant was modified to restore the glutamine and arginine codons of the tag sequence. Version 1 is the sequence of the ssrA\(^9\)His variant with 9 Histidine (Two CAT and seven CAC codons), Version 2 is sequence modification after site directed mutagenesis containing 6 Histidine (five CAC and one CAT codon). The underlined sequences in red are the ssrA\(^9\)His tag sequence (version 1) and the ssrA\(^6\)His tag sequence (version 2).
The mutagenesis was directly done on pk-smpB-Rv3099c-ssrA<sup>9His</sup> and pmv261-smpB-Rv3099c-ssrA<sup>9His</sup>. The resulted plasmids were propagated into E. coli DH5α, extracted and sent for sequencing to confirm the restoration of the arginine and glutamine codons (Appendix 8.9). The sequencing confirmed the intended mutations and the constructs were designated as pK-smpB-Rv3099c-ssrA<sup>6His</sup> and pMV-smpB-Rv3099c-ssrA<sup>6His</sup> which then were introduced <i>M. smegmatis</i> <i>groEL1ACΔlon</i> (Table 4). These constructs were also introduced into <i>M. smegmatis</i> <i>groEL1AC</i> but due to the time shortage it was not possible to continue further work with these four.

Four successful transformants were selected for protein extraction; two harbouring pK-smpB-3099c-ssrA<sup>6His</sup> designated <i>M. smegmatis</i> <i>groEL1ACΔlon</i><sup>6His</sup> I & IV and two harbouring pMV-smpB-3099c-ssrA<sup>6His</sup> designated <i>M. smegmatis</i> <i>groEL1ACΔlon</i><sup>6His</sup> W 2 & 3. All the four merodiploid were grown in different stress conditions as described in section (3.10) and harvested as previously described in section (3.7.2). Again western blotting indicated the absence of any His-tagged protein in the bacterial whole lysate and there was no protein pulled down by the nickel column (Data not shown).

Despite this disappointment, it was observed that the merodiploids showed a growth phenotype. The merodiploid <i>M. smegmatis</i> <i>groEL1ACΔlon</i><sup>6His</sup> failed to grow on 7H11 without glycerol, unlike the parental strains and the previous merodiploid. Therefore, this was further investigated by doing a growth curve comparing <i>M. smegmatis</i> <i>groEL1AC</i>, <i>M. smegmatis</i> <i>groEL1ACΔlon</i> and <i>M. smegmatis</i> <i>groEL1ACΔlon</i><sup>6His</sup>.
3.14 Growth Phenotype of *Mycobacterium smegmatis Δlon*<sup>6HisV</sup>

The growth phenotype of *M. smegmatis groEL1ΔCΔlon*<sup>6HisV</sup> was observed in the carbon source limited media, so it was decided to perform a growth curve by growing the different strains in 7H9 containing 0.4% glycerol and 0.1% Tween 80 without the addition of any antibiotics. *M. smegmatis groEL1ΔCΔlon*<sup>6HisV</sup> IV merodiploid was selected to compare its growth rate with the parental strain *M. smegmatis groEL1ΔCΔlon* and the wild type *M. smegmatis groEL1C*. As shown in the growth curve (Figure 31), *M. smegmatis groEL1ΔCΔlon*<sup>6HisV</sup> grew much slower and has a very long lag phase compared to *M. smegmatis groEL1 ΔC* and *M. smegmatis groEL1C Δlon*.

![Growth curve](image)

**Figure 31.** *M. smegmatis groEL1 ΔC, M. smegmatis groEL1CΔlon* and *M. smegmatis groEL1CΔlon*<sup>6HisV</sup> (ssrA<sup>6His</sup> variant merodiploid) growth curves by OD 600.

*M. smegmatis groEL1 ΔC, M. smegmatis groEL1CΔlon and M. smegmatis groEL1CΔlon*<sup>6HisV</sup> IV were grown in 7H9 containing 0.4% glycerol and 0.1% Tween 80 only and incubated at 37°C shaking. Each point is an average OD<sub>600</sub> of 3 biological replicates. The error bars are the standard error of the mean.
There is a slight difference in the generation time between *Mycobacterium smegmatis Δlon* and its parental strain *M. smegmatis groEL1ΔAC* (85 and 101 minutes respectively). In contrast, the *M. smegmatis groEL1ΔACΔlon* IV merodiploid had a doubling time of 125 minutes. The difference in the generation time between the *M. smegmatis groEL1ΔACΔlon* and its parent *M. smegmatis groEL1AC* suggests that deleting the *lon* gene may have slightly impaired it in the conditions used in this experiments. Furthermore, the difference in the generation time between *M. smegmatis groEL1ACΔlon* IV merodiploid and its parent *Mycobacterium smegmatis groEL1AC Δlon* may also suggest that harbouring the *ssrA*<sup>6His</sup> variant may be the cause for the very long lag phase and slower generation time.

![Graph](image)

**Figure 32.** *M. smegmatis groEL1AC, M. smegmatis groEL1AC Δlon* and *M. smegmatis groEL1ACΔlon*<sup>6His</sup> IV (ssrA<sup>6His</sup> variant merodiploid) growth curves by cfu.

*M. smegmatis gro1ΔC, M. smegmatis groEL1ACΔlon* and *M. smegmatis groEL1ACΔlon*<sup>6His</sup> IV were grown in 7H9 containing 0.4% glycerol and 0.1% Tween 80 only and incubated at 37°C shaking. Miles and Misra counts were done on LBA. Each point is an average log10 of 3 biological replicates.
The resumption of the almost similar growth to the wild and the parent strains after the long lag phase suggested some kind of changes occurred in the merodiploid that enabled it to grow almost as normal. It was hypothesized that these changes could have been due to a mutation of the *M. tuberculosis* H37rv tmRNA ssrA<sup>6His</sup> variant to inhibit its expression or through the induction of compensatory mechanisms to overcome the burden caused by its presence.

To test for the *possibility* that the merodiploid may excise or destroy the entire plasmid, the growth curve was repeated with *M. smegmatis groEL1ΔC Δlon* without kanamycin and four different clones *M. smegmatis groEL1ΔC Δlon*<sup>6HisV</sup> (I&IV) and *M. smegmatis groEL1ΔC Δlon*<sup>6HisW</sup> (2 & 3) with 50µg of kanamycin to prevent the loss of the plasmid and maintain the expression of *M. tuberculosis ssrA*<sup>6His</sup> variant.
Figure 33. *M. smegmatis* groEL1ΔCΔlon, *M. smegmatis* groEL1ΔCΔlon6HisV, *M. smegmatis* groEL1ΔCΔlon6HisW2 & 3 (ssrA6His variant merodiploid) growth curves by OD 600.

*M. smegmatis* groEL1ΔCΔlon, *M. smegmatis* groEL1ΔCΔlon6HisV, *M. smegmatis* groEL1ΔCΔlon6HisW2 & 3 growth curves by OD600. These strains were grown in 7H9 containing 0.4% glycerol and 0.1% Tween 80 only and incubated at 37°C shaking. *M. smegmatis* groEL1ΔCΔlon was grown without adding kanamycin as media control. The reset of the merodiploids were grown in the presence of 50µg of kanamycin.
As shown in Figure 33, *M. smegmatis groEL1ΔCΔLon* grew as previously seen replicating the earlier result of the first growth curve shown in Figure 31. In contrast, 3 of the merodiploids grown in the presence of kanamycin did show a very long lag phase again replicating the earlier result of *M. smegmatis groEL1ΔCΔLon*<sup>6HisIV</sup> (Figure 31). However, one of the four failed to grow. These observations suggested that different clones respond differently to the stress imposed by the presence of *M. tuberculosis tmRNA ssrA<sup>6His</sup> variant.

The variability in the length of the lag phase between the different clones of merodiploids could also be due to the variability in the level of expression of *M. tuberculosis H37rv tmRNA ssrA<sup>6His</sup>. The presence of kanamycin might have some effect too as it might cause ribosome stalling even though the merodiploids have the *aph* gene as a selection marker. Kanamycin may add further stress that might contribute to the slow growth or may increase the expression of *ssrA<sup>6His</sup> leading to even to slower growth. The CFU count was also performed to check the viability of *M. smegmatis groEL1ΔCΔLon*<sup>6HisIV</sup>, which maintained a growth rate of 5x10<sup>3</sup> to 3x10<sup>4</sup> cfu/ml up to day 4 but then showed no growth on day 5. It is possible that the merodiploid became non-viable or non-culturable at this point but this will require further work in the future to confirm.

The viable count was done on LBA plates with and without kanamycin. All the merodiploids grew in the presence of the kanamycin and there was no difference in the CFU count on LBA plates with and without kanamycin. This indicates that the merodiploids did not lose the plasmid.

In an attempt to verify the suggestion that the merodiploid harbouring the *M. tuberculosis tmRNA ssrA<sup>6His</sup> variant were being modified, 10 individual colonies from the CFU count plate of *M. smegmatis groEL1ΔCΔlon*<sup>6HisIV</sup> late log phase (grown in the absence of
Kanamycin) were picked. Each colony then was streaked on LB agar without antibiotics and then on another LB agar containing 50µgml⁻¹ kanamycin and incubated for two days. All of the picked colonies grew on both plates indicating they still possessed the aph gene and hence the plasmid. Then a colony PCR was done to amplify the cloned construct which was then sent for sequencing. The sequencing results did not indicate any sequence changes that might be seen as a cause for the overcoming of the effect of the presence of the *M. tuberculosis* H37rv *ssrA*⁶*His* variant.

Taken together, these observations and finding may suggest:

1. The presence of the *M. tuberculosis* H37Rv *ssrA*⁶*His* had an impact on the growth rate of the harbouring merodiploid that is possibly due to the essentiality of the tmRNA tagging function
2. The failure to detect any His-tagged protein may have been due to the cessation of the growth of the bacterial cells expressing *M. tuberculosis ssrA*⁶*His* and as a result there was no detectable accumulation of the tagged protein
3.15 Discussion

Understanding the function of tmRNA in *M. tuberculosis* H37rv is very important as it might open a window to finding new drug targets. One important approach to assign function to a gene/DNA region is to delete it and then observe any phenotypic changes in the bacterium. Unfortunately, we were unable to delete the *ssrA* gene that encodes the tmRNA of *M. tuberculosis* H37rv unless we provided a second functional copy elsewhere on the genome. It was not possible to delete the *ssrA* when a second recombinant was provided with the protease tag coding sequence replaced by a sequence encoding a run of histidines (*ssrA*₉His). This suggested that tmRNA is essential in *M. tuberculosis* H37rv and moreover that the protease tagging function of tmRNA is an essential function. However, there are two points that might initiate a doubt in this conclusion. Firstly, when the knockout construct (pG5hyg::*ssrA*) was made the size of the *M. tuberculosis* H37rv tmRNA had been annotated to have a sequence size of 607bp but, recently this was corrected to 338bp. This means that this construct would have deleted 269bp from downstream of the tmRNA as well as the intended gene and in doing so might have negatively affected the downstream genes. This might have a negative impact on the viability of the transformed *M. tuberculosis* H37rv and hence no transformants with the gene replacement would be detected. Therefore, a revisit of the strategies used in the attempt to delete *ssrA* in this study must be done in order to clear any doubts about the conclusion that tmRNA is an essential molecule. In addition, a recent paper published since the experiments in the present study were started, demonstrated that the deletion of *smpB* in *M. tuberculosis* H37rv (Personne and Parish, 2014) did not affect *in vitro* growth. This might suggest that tmRNA is not essential for rescuing the stalled ribosome. This would necessitate the presence of an alternative ribosome recycling mechanism in mycobacteria, but none have so far been identified. *E. coli* possesses
alternative ribosome rescue proteins, namely; ArfA, (Alternative ribosome-rescue factor A) which is encoded by the yhdL gene and shown to have properties consistent with tmRNA-independent system (Chadani et al., 2010) and ArfB, (YaeJ) paralogous to the release factors (RFs) (Chadani et al., 2012) and the putative stop-codon independent release factor known as PrfH whose function could be redundant with ArfB (Baranov et al., 2006).

Expressing E. coli ArfA in Shigella flexneri made it possible to delete its ssrA which is essential (Ramadoss et al., 2013). ssrA is not essential for the survival of Caulobacter crescentus ssrA and it does not have an arfA homolog but has been reported to have a protein known as CC1214 that had homology to E. coli ArfB and could hydrolyse peptidyl-tRNA in nonstop translation complexes in vitro (Feaga et al., 2014). Thus, future work in mycobacteria should include studies to identify alternative ribosome recycling systems

Personne and Parish also demonstrated the deletion of ssrA from a merodiploid strain of M. tuberculosis H37rv carrying a second recombinant ssrA which had a double glycine instead of a terminal double alanine in the protease tag sequence. This recombination showed no effect on growth and no effect on mutant sensitivity to a ribosome inhibitory drug Pyrazinamide (PZA) suggesting that tmRNA protease tagging is not an essential function in M. tuberculosis H37rv but that ribosome rescue is. These findings are in contrast to those in the present study where it was possible to delete ssrA from wild type-ssrA merodiploid, however, it was not possible to delete it from ssrA9His variant merodiploid which replaced the protease tag with a run of histidines. One study in support of an essential function for the protease tagging role of tmRNA is that the Clp protease itself is essential for bacterial viability (Sassetti et al 2003; Raju et al 2011). Perhaps this contradiction may suggest that ssrADD variant tagged proteins are more easily degraded by other protease systems than the
His tagged proteins. These contrasting results could also be explained if the \textit{ssrA}^{9\text{His}} produced a completely non-functional tmRNA that was unable to interact appropriately with the translation machinery.

The other aspect of this chapter was an attempt to identify the protein substrates of the tmRNA tagging system. The first approach was to replace the wild-type \textit{ssrA} with \textit{ssrA}^{9\text{His}} and then purify the His-tagged protein repertoire of the cell and identify by LC-MS. As explained above, this strain was not viable, so it was attempted to purify His-tagged proteins from merodiploids carrying both the wild-type and His-variant \textit{ssrA} genes. This study failed to identify any His-tagging of truncated proteins despite an exhaustive set of experiments in which \textit{ssrA} His-tagging genes (His6 and His9) were expressed from different promoters, from episomal vectors, from integrated constructs, in a GroEL1 mutant background, and in a Lon protease deficient background. Towards the end of these experiments we observed that expression of His-tmRNA was detrimental to bacterial growth in media with low glycerol content. One possible explanation for this is that tmRNA protease tagging and ClpP degradation is so important that even a proportion of truncated protein being His-tagged is damaging to the cell. Indeed, this is consistent with observations in \textit{Caulobacter crescentus ssrA} mutants (Keiler and Shapiro, 2003) in which slow growth in limited medium was related to the disruption of cell cycle as a result of the absence of the wild type \textit{ssrA} tagging activity and not due to the accumulation of stalled ribosomes because an \textit{ssrA}^{DD} variant did not complement the growth defect. It was also noticed that the \textit{Caulobacter crescentus ssrA}^{DD} merodiploid showed a similar effect but not as strong as the effect seen in \textit{Caulobacter crescentus ΔssrA} suggesting tagging not ribosome rescue is the reason for the phenotype. In some other bacteria in which the \textit{ssrA} is non essential, deleting it produced a
mildly defective phenotype and its function can be complemented by the $ssrA^{DD}$ variant (Keiler, 2008; Moore and Sauer, 2007). Further supportive of this is that *E. coli* ssrA mutants showed a growth defect under nutrient starvation (Oh and Apirion, 1991) (Komine *et al.*, 1994). This may indicate the possible involvement of *M. tuberculosis* tmRNA in cell growth regulation directly by (1) interacting with the respective growth factor by tagging it for degradation thereby directly interfering with its expression or (2) indirectly via regulation of its regulatory mechanisms or (3) by leading to accumulation of un-degraded truncated proteins that interact with many cellular proteins and disrupt the physiology of the cell. Alternatively, the results are also consistent with $ssrA^{His}$ producing a non-functional tmRNA that interacts appropriately with the translation machinery and inhibits translation.

Further work is clearly needed to elucidate the role of tmRNA. Analysis of the accumulation of tagged proteins in $ssrA^{DD}$ and $ssrA^{His}$ expressing mycobacterial strains will help to understand the contrasting results reported in this and the study of Personne et al 2014. In addition, research should incorporate analysis of the interaction of tmRNA with the translation machinery possibly with an affinity tagged tmRNA (not His!) to pull down interacting proteins and RNAs.
Chapter 4

Ultrastructural localisation of α-crystallins in *M. tuberculosis* complex bacteria
4.0 Introduction

*M. tuberculosis* H37rv is a successful pathogen due to its ability to evade and resist the killing mechanisms employed by the immune system of its host. Indeed, it is able to persist in the body for decades in an apparently dormant state which is often termed latent tuberculosis infection (LTBI). This persistent *M. tuberculosis* H37rv infection occurs in the majority of the 2 billion people who are infected with *M. tuberculosis* H37rv worldwide. Thus understanding how *M. tuberculosis* H37rv is able to resist stress and maintain viability in latent TB is important to the development of novel control therapies.

There are many conditions that are potentially hostile to the bacterium during infection: for example, nutrient starvation; hypoxia; temperature; reactive nitrogen intermediates; reactive oxygen intermediates and low pH. Molecular chaperones represent one group of proteins that are prominent in the bacterial stress response to any condition that results in the denaturation of proteins. In chapters 4 and 5 of this thesis I study two small heat shock proteins (sHSP) belonging to the α–crystallin family of chaperones - Acr1 and Acr2.

The α–crystallins like Acr1 and Acr2 protect other proteins from thermal aggregation although they may not prevent the loss of enzyme activity in the target protein. They act in an ATP-independent manner and therefore, they are frequently developmentally regulated normally when and where metabolic activity is minimal, such as in the eye lens (Arrigo and Simon, 2010). α–crystallins are reported to be found in sporulating microorganisms where they are involved in the assembly of the spore’s outer coat (Henriques et al., 1997). Although *M. tuberculosis* H37rv does not sporulate, both *acr1* and *acr2* genes have been reported to be up-regulated in non-replicating *M. tuberculosis* H37rv (Stewart et al., 2005) but, their
role in *M. tuberculosis* survival and pathogenicity is complex and remains poorly understood.

The Acr1 protein (also known as the 16kDa antigen) is one of 5 proteins that were identified by mass fingerprinting and immunodetection in the lysate of *M. tuberculosis* grown under low oxygen conditions (Rosenkrands et al., 2002). It is reported to be required for optimal mycobacterial growth within macrophages (Yuan et al., 1996) and is up-regulated during hypoxia (Desjardin et al., 2001). Acr1 is under the control of the two component response regulator known as DosR (Rv3133c/Rv3132c) which upregulates over 50 genes in response to microaerophilic conditions or low concentrations of NO (Voskuil et al., 2004).

Acr2 is a parologue of Acr1 which shares 30% overall homology in amino acid sequence to Acr1 and 41% homology when comparing the residues of the α-crystallin core. Acr2 was first described as a stress protein that associated with the ribosomal fraction of heat stressed *M. tuberculosis* (Ohara et al., 1997; Pang and Howard, 2007; Stewart et al., 2002a; Tabira et al., 2000). The *acr2* gene is transcriptionally upregulated more than any other gene during heat shock (Stewart et al., 2002b) and is under the control of regulatory systems that coordinate its gene expression in response to a variety of different stresses including starvation, palmitic acid, diamide or hydrogen peroxide (H$_2$O$_2$), nitric oxide, naïve and activated macrophages and detergent (Kennaway et al., 2005; Wilkinson et al., 2005). The mechanisms of Acr2 regulation have been studied in detail. It is regulated by the repressor HspR and the activator ClgR in response to heat/denatured protein (Estorninho et al., 2010; Stewart et al., 2002b) and by the sigma factor SigE and the two component response regulator MprA/B in response to cell surface stress such as surfactant (He et al., 2006; Manganelli et al., 2004; Pang and Howard, 2007; XiaoZhen et al., 2011). It is also regulated
by the two-component system PhoP which is important for mycobacterial virulence (Singh et al., 2014a). Further indicative of its importance in infection, *acr2* expression is upregulated during the first 24hrs post-infection of the macrophage (Wilkinson et al., 2005).

There is little detail on the roles or substrates of the \(\alpha\)-crystallins other than their general function as molecular chaperones. Some insight into the role of Acr1 has been gained by examination of its subcellular location. Electron microscopy has shown that Acr1 may be associated with fibrous peptidoglycan-like structures, intracellular and peripheral clusters as well as the cell envelope. Its association with the cell envelope is strongest in stationary phase bacteria under hypoxia and it has been suggested that the chaperone may play an important role in thickening and stabilising the cell structure helping the cell to survive the hypoxic environment (Cunningham and Spreadbury, 1998). Although *M. tuberculosis* is a non-capsule producing bacteria, it has been observed to be surrounded by capsule-like structure which is seen as electron-transparent zone (ETZ) in pathogenic mycobacteria (*M. tuberculosis* and *M. avium*) but absent in non-pathogenic mycobacteria (*M. smegmatis* and *M. aurum*). Acr1 has been identified to be among the major presumed “capsular” proteins of *M. tuberculosis* (Daffé and Etienne, 1999).

Before this thesis, there was no evidence that the Acr2 protein was similarly involved in the thickening or maintenance of the cell wall, rather that it may be important for maintenance of the protein synthesis machinery under stress conditions. Evidence for this was derived from its apparent purification with ribosomes from mycobacteria exposed to high temperature (Ohara et al., 1997; Pang and Howard, 2007). However, its induction by regulators that respond to cell surface stress (MprA/B and SigE) may indicate some
involvement in cell wall biology as well as being a ribosome chaperone. Further evidence to support its cellular function would be provided by knowledge of its subcellular localisation. In contrast to Acr1, up to the date of writing this thesis, there is no such data regarding Acr2 localisation.

One of the main hypotheses of this thesis is that there is functional redundancy between the two α-crystallins. This chapter attempts to add information to test this hypothesis by comparing the subcellular localisation of the α-crystallins.

The specific objectives of this chapter are to,

1. Detect Acr1 and Acr2 in different cellular fractions (culture supernatant, cell pellet, cytosolic and plasma membrane factions) by Western blot.
2. Examine the intracellular localisation of Acr2 by Immuno-electron microscopy.
4.1. Localisation of $\alpha$-crystallins by Western blotting in *M. bovis* BCG sub-cellular fractions

It was not possible to use *M. tuberculosis* H37rv for the cell fractionation and protein extraction due to the technical difficulties related to the volume of culture required inside the CL3 laboratory. Therefore, BCG str. Pasteur, the close *M. tuberculosis* relative, was used instead making it possible to do the required work in the CL2 laboratory where the required facilities and permissions exist. A starter culture of BCG str. Pasteur was grown in 7H9 containing 10% ADC and 0.1% Tween 80 to mid log out of which a volume of 500ml of Roisin’s minimal medium was inoculated. This medium was important because it does not contain albumin like 7H9, which would interfere with the cellular protein analysis. The BCG culture was grown for 4 weeks until late log phase OD 1.0. The cells were harvested, lysed and the lysate was fractionated using ultracentrifugation at 35000RPM, 2hours for each step. The four fractions that were obtained were (1) culture supernatant, (2) solid cell pellets after cell disruption (3) cytosolic and (4) plasma membrane fractions. The fractions were run on 12% SDS PAGE (picture not shown) and the proteins transferred to PVDF membrane. The $\alpha$-crystallin proteins were detected by Western blot using the respective polyclonal antibodies.
4.1.2 Western blot localisation of α-crystallin 1 in subcellular fractions of BCG

Figure 34: Detection of Acr1 by Western in different BCG culture fractions

Western blot showing the presence of Acr1 protein (16 kDa) in the BCG culture supernatant, solid cell pellet, cytosolic and plasma membrane fractions (lanes 1-4 respectively). Lane 5 is the positive control of BCG whole lysate.

Western blotting showed the presence of Acr1 in all the subcellular fractions tested (Figure 34). Although this technique is only semi-quantitative at best, Acr1 appeared to be most abundant in the cell pellet and plasma membrane fractions. From the intensity of the immunoreactivity detected, the Acr1 concentration seemed to be lower in the cytosolic and culture supernatant fractions. The detection of Acr1 in the plasma membrane fraction may indicate its association with the plasma membrane components or its associated proteins. Thus Acr1 appears to be widely distributed in the cell and its presence in the cell pellet and plasma membrane fraction is consistent with the previous electron microscopy studies which indicated its presence in the cell wall (Cunningham and Spreadbury, 1998). It’s presence in the supernatant could result from lysis of bacteria in the culture but it is also reasonable to
assume that cell wall associated proteins may be shed into the culture from whole replicating bacteria

**Figure 35. Detection of Acr2 by Western blotting in different BCG culture fractions**

Western blot showing the presence of Acr2 protein (18kDa) in the solid cell pellet and plasma membrane fraction (lane 2 and 4 respectively) and its absence in the culture supernatant and cytosolic fractions (lane 1 and 3 respectively). Lane 5 is the positive control of BCG whole lysate.

Unlike Acr1, Acr2 was only detected in the solid cell pellet and plasma membrane fractions (Figure 35). This suggests that Acr2 functions exclusively in the plasma membrane and cell wall compartments in the bacterium and is not secreted from the cell nor required in the cytoplasm. The result may also support the idea that Acr2 is required for the process of protein synthesis via its interaction with the ribosome 30S subunit. The finding in the present study agrees with what Ohara and colleagues reported, that the 18-kDa ribosome associated protein HrpA (synonymous with Acr2) was found in the membrane and ribosome fractions (Ohara *et al.*, 1997). In the present study, further fractionation of the plasma membrane
fraction was not performed and therefore, although the findings are consistent with Ohara, they cannot confirm the association of Acr2 with the ribosome.

4.1.3. Localisation of Acr1 and Acr2 in *M. tuberculosis* H37rv by immuno-electron microscopy

Visualising the distribution of Acr1 and Acr2 using immuno-electron microscopy was carried out using an aged culture of *M. tuberculosis* H37Rv. Acr1 is highly expressed in stationary phase and Acr2 is also known to be expressed in non-replicating bacteria (Stewart *et al.*, 2005; Yuan *et al.*, 1996). WT *M. tuberculosis* H37rv was grown in 7H9 containing 10% ADC and 0.01% Tween 80 and incubated for 90 days shaking at 37°C. The culture was spun down and the cell pellet resuspended in 4% formaldehyde and 0.2% glutaraldehyde. After fixation overnight, the cells were washed and embedded in LR white acrylic resin as described in the methods (section 2.17). LR white is hydrophilic and thus allows for the interaction between antibodies in solution with antigen in the embedded samples. Ultrathin sections were cut and stained with rabbit anti Acr1 and Acr2 serum and goat anti-rabbit secondary antibody conjugated to 10 nm gold particles as described in the methods (Section 2.17).

The specificity control, using non-immune rabbit serum in place of the primary antiserum to check for non-specific binding of the antibodies (Figure 36), showed some non-specific binding but at a very low gold particle density of <1 particles per 0.12µm area. This gives
good confidence that most of the particles seen in the Acr1 and Acr2 specific immuno-gold staining is due to specific binding of the primary or secondary antibodies.

Figure 36. Immunogold electron micrograph of *M. tuberculosis* H37rv stained with non-immune rabbit serum (specificity control)

The micrographs for the control testing for the non-specific binding of primary and secondary antibodies. The samples slides were treated with only the secondary anti-bodies-Gold particle conjugate.

The ultrastructural preservation of the cells was inadequate with bacterial outlines visible but without visible membranes in most places. The reason for this was that osmium tetroxide could not be used to stain membranes as electron dense structures because osmium has the effect of masking antigenicity. It was attempted to retain as much membrane structure as possible by using uranyl acetate staining *en bloc* but this produced only low contrast staining. The cell wall was not well defined and in most micrographs is visible as an electron translucent zone surrounding the bacteria although occasional areas with better preservation of the layers were present. The staining for both Acr1 (Figure 37) and Acr2 (Figure 38) produced a significantly higher density of gold particles (27 and 15 gold particles per 0.12µm respectively) than staining performed with a non-immune rabbit serum in place of the Acr1
and Acr2 primary antisera (Figure 36). This indicated that the Acr1 and Acr2 immunoreactivity was specific for the intended antigens.

Acr1 immunoreactivity was mostly associated with the cytoplasmic region of the bacteria although there was also a lower level of staining visible on the periphery of the cells (Figure 37). The latter may indicate staining of the cell wall and this is consistent with the findings of Spreadbury et al (Cunningham and Spreadbury, 1998).

**Figure 37. Acr1 immunogold electron micrograph**

Immunogold Electron Micrograph showing the localisation and distribution of Acr1 immunoreactivity in *M. tuberculosis* H37Rv. The *M. tuberculosis* was grown in 7H9 containing 10% ADC and 0.1% Tween80 and incubated for 90 days shaking at 37°C. Although the preservation process was not good enough to show cell wall details, the bacillus cell wall appears as an electron translucent zone surrounding the darker cytoplasmic part of the cell. Acr1 immunoreactivity is associated with both the cytoplasmic area and the cell periphery. The density of the gold particle is 27 particles per 0.12µm.

Acr2 immunoreactivity contrasted with Acr1 because it was clearly associated with the periphery of the cell, particularly the clear electron translucent zone that surrounded the
bacteria which is presumed to be the cell wall (Figure 38). There was very little Acr2 gold staining associated with the cytoplasm of the bacteria.

Figure 38. Acr2 immunogold electron micrograph

Immunogold Electron Micrograph showing the localisation and distribution of Acr2 in M. tuberculosis H37Rv. M. tuberculosis was grown in 7H9 containing 10% ADC and 0.01% Tween 80 and incubated for 90 days shaking at 37°C. Although the preservation process was not good enough to show cell wall details, the bacillus cell wall appears as an electron translucent zone surrounding the darker cytoplasmic part of the cell. Acr2 immunoreactivity was associated with the cell wall and not the cytoplasm. The density of the gold particle is 15 particles per 0.12 µm.
4.2 Discussion

The α-crystallins Acr1 and Acr2 are small heat shock proteins (sHSPs) that function primarily as molecular chaperones preventing improper protein association (Abgar et al., 2001). They are able to bind to non-native proteins under various stress situations leading to inhibition of their irreversible aggregation and prevention of cell damage (Lindner et al., 2000). These proteins belong to a class of heat shock proteins known as holdases and they bind to their protein clients in an ATP-independent manner. When the stress condition is lifted the bound proteins are released for refolding with the help of the foldases (Basha et al., 2012; Haslbeck et al., 2005). They are promiscuous chaperones which means they can bind to a wide range of unfolded proteins (Tyedmers et al., 2010). Expression of both Acr1 and Acr2 proteins increases in response to conditions such as stationary phase and various stresses although there are key differences in the stresses which induce their expression indicating that they may have different roles in the cell (Rosenkrands et al., 2002; Stewart et al., 2005) (Yuan et al., 1996).

Cunningham and Spreadbury (1998) in their study concluded that the Mycobacteria may adapt to hypoxic condition by developing a thick cell wall. Using Immunogold electron microscopy, they have shown the distribution pattern and localisation of Acr1 in M. bovis BCG bacilli that were incubated anaerobically for different lengths of time. They compared the localisation of Acr1 in cells grown for 4, 12 and 26 weeks. They observed that after 26 weeks, Acr1 is localised at various parts of the cell including in the cell wall, and in fibrous structures and peptidoglycan like fibres. After incubating for 4 weeks, the Acr1 was seen clustered and associated with the cytoplasm and the periphery of the cell. After 12 weeks of incubation, Acr1 was shown to be more heavily localised in the cell periphery. In the present
study, *M. tuberculosis* H37rv was incubated for 12 weeks with shaking to allow gradual depletion of oxygen and nutrients and entry of the bacterium into stationary phase. The Acr1 immunogold electron microscopy showed staining in the cytoplasm of some cells and some heavier peripheral staining in other cells. Although the cells in my study were grown for 90 days, the similarity of the result to cells from various aged cultures in Spreadbury and Cunningham’s study, suggests that different populations of bacilli existed within my 90 days’ culture. In other words, some of the *M. tuberculosis* cells were still replicating and had not switched to a non-replicating state where staining in the periphery is more pronounced. Unfortunately, it was not possible to view the fibrous details of the cell which may not have been preserved during the processing of the cells either at the fixation or dehydration step or may not be visible due to the low contrast of the sections.

The immune-electron microscopy of *M. tuberculosis* H37rv was supported by Western blotting of subcellular fractions of stationary phase BCG, which showed that Acr1 was present in the cytoplasmic, cell wall and plasma membrane fractions as well as in the culture supernatant. Therefore, Acr1 seems to be present throughout the bacterial cells in growth conditions when there is presumably less metabolic activity in the cells. Given the fact that Acr1 acts in an ATP independent manner, it could be the best option to transport and protect essential proteins for survival in this low metabolic state.

That Acr1 was found in the culture supernatant may be an important observation with regard to its potential as a vaccine antigen as this appears to be an important quality of most mycobacterial vaccine candidates that give protection, e.g., Esat-6, Cfp10, Ag85A/B (Langermans et al., 2005; Maue et al., 2007; Xu et al., 2008). Acr1 is known to be
immunogenic and it has been reported to provide high long term protection against *M. tuberculosis* infection in mice when used in multistage subunit vaccine containing Acr1, ESAT6, Ag85B and MPT64 (Niu et al., 2015). Also Acr1 protein alone that was purified from the *M. tuberculosis* cell lysate, protected the mice against aerosol challenge and improved protective efficacy of BCG, when used as a booster vaccine. However, recombinant Acr1 protein expressed in *E. coli* was used alone as a prophylactic vaccine, failed to protect mice against aerosol challenge. This suggests that Acr1 may chaperone one or more immunogenic and protective antigens (Taylor et al., 2012). Because Acr1 is a chaperone which acts in an ATP independent manner in non-replicating “dormant” cells, its potential as a chaperone/carrier of important dormancy antigens may be important in efforts to produce a vaccine against latent tuberculosis.

In contrast to Acr1, Acr2 seems to be distributed almost exclusively on the periphery of the cells in the electron translucent zone, that we presume to be the cell wall, or on the cell membrane beneath it. One previous study suggested that Acr2 is a ribosome binding protein (Ohara et al., 1997) aiding translation initiation because it was associated with 30S subunit of the ribosome (Ohara et al., 1997; Stewart et al., 2002a; Tabira et al., 2000). The data in the present study does not support this because if Acr2 was associated with ribosomes, one would expect it to be localised in the cytoplasm. Here, the Western blotting and immune-electron microscopy indicate that Acr2 associates with the plasma membrane (and cell wall) and it is possible that membrane fractions containing Acr2 co-migrated with ribosomes in the study by Tabira, which led to the conclusion that Acr2 was associated with the ribosome.
Acr2 is known to be upregulated in various stress conditions especially in heat shock and stresses such as surfactant which are associated with damage to the cell surface. Indeed, two of the regulators that control its expression, SigE and MprA/B, are known to govern the cell surface stress response. Thus the localisation of Acr2 to the cell wall and plasma membrane makes sense, implicating Acr2 as a major chaperone important for maintenance of the mycobacterial cell wall during stress. Just as for Acr1, the ATP-independence of Acr2, unlike many other classes of chaperone, makes it perfect for this function on the external periphery of the cell.

Thus, the localisation of Acr1 and Acr2 to different areas of the bacterial cell suggests that these two holdase chaperones have different cellular functions. However, there is some overlap in their subcellular localisation, as both can be found in the cell wall/periphery of stationary phase bacteria (although Acr2 appears more abundant). Thus there is potential for some level of functional redundancy. This will be further investigated by examining the phenotype of α-crystallin mutant strains of *M. tuberculosis* H37rv in Chapter 5.
Chapter 5

Gene replacement of the *M. tuberculosis* \(\alpha\)-crystalline genes and phenotypic characterisation of the mutant strains
5.0 Introduction

The immuno-electron microscopy and Western blotting experiments explained in chapter 4 suggested that the two α-crystallin chaperones under investigation were not localised in the same manner in the cell. Acr1 was found in all the subcellular fractions and the electron micrographs showed its wide distribution all over the cytoplasm of the cell and the cell wall. In contrast, the Acr2 was only detected in the cell pellet and plasma membrane fractions (further fractionation was not done to determine which fraction of the plasma membrane) and the electron micrographs showed an almost exclusive distribution in the cell wall/plasma membrane with little or no staining in the cytoplasm or nucleolus. Although this difference in cellular location must represent a difference in function, the overlap in distribution in the cell wall could still allow for some functional redundancy in this peripheral area of the cell. The classic approach to associate phenotypes to genes is to generate knockout mutant strains and compare the phenotypes of mutant and wild-type. Deletion of acr1 has previously been performed in M. tuberculosis H37rv in two different studies and the mutants were shown to be partially attenuated for growth in macrophages and have an altered growth phenotype in mice in which infection reached a higher bacterial load (Hu et al., 2006; Yuan et al., 1996). Deletion of acr2 was also performed in M. tuberculosis H37rv and this mutant was shown to be moderately attenuated in human macrophages, and in mice caused an infection which reached a similar bacterial load but with altered pathology and reduced loss of animal weight (Stewart et al., 2005; Wilkinson et al., 2005).

If there is functional redundancy for Acr1 and Acr2, deletion of one chaperone may be partially compensated by the remaining paralogue. In this chapter, an acr1 M. tuberculosis H37rv mutant strain and its wild-type parent were obtained from a collaborating laboratory;
St. George’s Hospital laboratory (London), and the \textit{acr2} gene was deleted from both to give isogenic single mutants in \textit{acr1} and \textit{acr2} and a double mutant deleted of both \textit{acr1} and \textit{acr2}. This chapter further describes the genetic complementation of these mutants and presents and interprets data obtained from different stress assays and macrophage infection used to test for a phenotypic difference between the different mutants. In particular, the focus was on whether the double mutant had a greatly reduced resistance/survival to any stress which may indicate redundancy between Acr1 and Acr2.
5.1. Gene replacement

5.1.1. α-crystalline 1 gene knockout

The strains *M. tuberculosis* H37rv wild type and *M. tuberculosis* H37rv Δacr1 (ΔhspX) mutant (Hu *et al.*, 2006) were the parental strains for the single deletion (Δacr2) and double deletion (Δacr1 Δacr2) respectively. The un marked deletion of the *acr1* gene in *M. tuberculosis* H37rv Δacr1 (obtained from St. George’s Hospital laboratory, London) was checked using primers that amplified the *acr1* region (primers P19&P20, Table 3) to produce a 591bp (435bp, 148bp upstream and 8bp downstream) DNA product in WT *M. tuberculosis* H37rv but a 156bp product in the *acr1* deletion mutant (Figure 39). Figure 40 shows that PCR of the *M. tuberculosis* H37rv Δacr1 single mutant (lane 2) and a *M. tuberculosis* H37rv Δacr1Δacr2 double mutant (made later in the chapter) (lane 4) produced <250bp (the smallest product size in the 1kb DNA ladder) products, which is consistent with deletion of the *acr1* gene sequence (156bp). In contrast, when *M. tuberculosis* H37rv wild-type (lane 1) and *M. tuberculosis* H37rv Δacr2 single mutant (lane 3) strain DNA was used as template, the amplified PCR size was consistent the expected 591bp product size indicative of the presence of the wild-type *acr1* (Figure 40)
Figure 39. Schematic diagram illustrating the position of acr1 in the *M. tuberculosis* H37rv genome and PCR primers for verifying the deletion of acr1

Diagram (A) illustrates the position of the acr1 gene in *M. tuberculosis* H37rv genome. Diagram (B) illustrates the unmarked deletion of acr1 gene. Primers 19 and 20 (Table 1) were used to verify deletion of the acr1 gene.

Figure 40. Deletion of acr1 gene - confirmation by PCR

Gel (1% agarose, TAE) picture showing PCR products using DNA templates from *M. tuberculosis* H37Rv wild type, *M. tuberculosis* H37Rv Δacr1 single mutant, *M. tuberculosis* H37Rv Δacr2 single mutant and *M. tuberculosis* H37Rv Δacr1Δacr2 double mutant strains to confirm the deletion of acr1 gene. The primers P19 and P20 produce a 596bp PCR product in the *M. tuberculosis* H37Rv wild-type strain (lane 1) and the *M. tuberculosis* H37Rv Δacr2 single mutant (lane 3). The *M. tuberculosis* H37Rv Δacr1 single mutant (lane 2) and *M. tuberculosis* H37Rv Δacr1Δacr2 double mutant (lane 4) produced a smaller 156bp product consistent with the loss of the acr1 gene.
The deletion of *acr2* was done using the suicide plasmid construct known as pSMT183 which was constructed and described by Stewart *et al* (2005). This plasmid is used in a one-step knockout strategy with the *hyg* gene as a positive selection marker and the *sacB* gene as counter selection marker. The deletion of the *acr2* gene after electroporation with pSMT183 was confirmed by PCR screening of the transformant colonies growing in the presence of 50 µg hygromycin ml⁻¹ and 2% sucrose of 7H11 agar supplemented with 5% Oleic Acid Albumin Dextrose Complex (OADC). The PCR primers (P23 and P24  Table 3) were designed to anneal outside the flanking regions of the *acr2* gene and amplify across the *acr2* region. These primers should produce a fragment of 4361bp if the gene was replaced with the *hyg* gene or 3711bp if it was not (Figure 41).

![Diagram](image-url)

**Figure 41. Genome position of the *acr2* gene and the PCR based strategy for verification of *acr2* gene replacement.**

Diagram (A) illustrates the position of *acr2* gene in the *M. tuberculosis* H37Rv genome and the position of the primers used to amplify from the outside of the cloned flanks of *acr2* in the recombination vector, pSMT183, inward across the *acr2* gene. Diagram (B) illustrates the genomic arrangement and PCR products after *acr2* is replaced by the *hyg* gene. *BglII* digestion of the putative *acr2* gene replacement products give 3 restriction fragments of 2375bp, 1330bp and 656bp, confirmatory of gene replacement.
Four transformants colonies were screened for \textit{acr2} deletion and an example PCR to demonstrate gene replacement in one colony is shown in Figure 42. This figure shows the 4361bp PCR products indicative of gene replacement to make the \textit{M. tuberculosis H37Rv} Δacr2 single mutant strain (lane 3) and \textit{M. tuberculosis H37Rv} Δacr1Δacr2 double mutant strain (lane 4). For comparison, PCR using WT \textit{M. tuberculosis H37rv} (lane 1) and \textit{M. tuberculosis H37Rv} Δacr1 single mutant (lane 2) strains as template produced single products 3711bp, indicative of the presence of the \textit{acr2} gene.

![Figure 42. PCR screening for the \textit{acr2} gene deletion](image)

\textbf{Figure 42. PCR screening for the \textit{acr2} gene deletion}

Gel (1% agarose, TAE) picture shows the PCR products amplified by using primers P23&P24 that were designed to anneal outside the flaking regions of the \textit{acr2} genes and amplify inwards across it. wild type \textit{M. tuberculosis H37rv} (lane 1) \textit{M. tuberculosis H37rv} Δacr1 single mutant (lane 2) strains produced 3711bp PCR products which indicated the presence of the \textit{acr2} gene. Lanes 3 and 4 show 4361bp products which indicate the replacement of the \textit{acr2} gene with the \textit{hyg} gene to produce the \textit{M. tuberculosis H37rv} Δacr2 single mutant (lane 3) and \textit{M. tuberculosis H37rv} Δacr1Δacr2 double mutant (lane 4) strains. These are the same PCR products digested with \textit{BglII} restriction enzyme shown in Figure 43.

To further confirm the gene replacement, the resulting PCR products were digested using \textit{BglII} restriction enzyme which would produce different restriction fragments in wild-type and disrupted genomes (Figure 41&Figure 43)

The agarose electrophoresis in (Figure 43), shows that the PCR products from the WT \textit{M. tuberculosis H37rv} (lane 1) and \textit{M. tuberculosis H37rv} Δacr1 single mutant (lane 2) strains
produced two *BglII* restriction fragments, however, three fragments are seen in lanes 3 and 4 indicative of replacement of the *acr2* gene to produce the *M. tuberculosis* H37rv Δacr2 single mutant and *M. tuberculosis* H37rv Δacr1Δacr2 double mutant strains respectively.

![Figure 43. Confirmation of *acr2* gene deletion by BglI1 digest of PCR products encompassing the *acr2* region](image)

Gel (1% agarose, TAE) picture shows the result of *BglII* digest of the PCR product of the region from outside of the *acr2* gene flanks inward across the target gene. The PCR products from wild type *M. tuberculosis* H37rv (lane 1) and *M. tuberculosis* H37rv Δacr1 single mutant (lane 2) strains produced two bands of 3705bp and 656bp. The PCR products from the *M. tuberculosis* H37rv Δacr2 single mutant (lane3) and *M. tuberculosis* H37rv Δacr1 Δacr2 double mutant (lane 4) strains produced three restriction fragments of 2375bp, 1330bp and 656bp indicating the replacement of the *acr2* gene with the *hyg* gene.

The PCR products from *M. tuberculosis* H37rv Δacr2 single mutant and *M. tuberculosis* H37rv Δacr1Δacr2 double mutant strains were also sent for sequencing to further confirm the replacement of the *acr2* gene with the *hyg* gene as well as to check for any undesired mutation that might have occurred in the surrounding sequence during the crossover process. The sequence has confirmed the replacement of the *acr2* gene with the *hyg* gene and ruled out the presence of undesired mutation (Appendix 8.7).
5.1.2. Confirmation of the absence of α-crystallin expression in \textit{M. tuberculosis} H37rv Δacr1, \textit{M. tuberculosis} H37rv Δacr2 and \textit{M. tuberculosis} H37rv Δacr1 by Western blotting

The absence of α-crystallin protein in the mutant \textit{M. tuberculosis} H37rv strains was tested by Western blotting using polyclonal antibodies to \textit{acr1} and \textit{acr2}. Therefore, one clone of each knockout mutant was grown in 20ml of 7H9 with 0.1% Tween 80 and 10% ADC until late log phase. The cultures were spun down and re-suspended in 1ml of sterile PBS followed by boiling for 10min before taken out of the Cat.3 laboratory. Cell lysates were prepared as described in the methods (2.9), proteins separated on 12\% acrylamide gels by SDS-PAGE (2.11), transferred to PVDF membranes and probed with the respective primary antibodies (1 in 1000) followed by secondary HRP conjugated antibodies (1 in 10,000). The chemiluminescent substrate used was ECL. α-crystallin 1 was not detected in \textit{M. tuberculosis} H37rv Δacr1 (Figure 44 A, lane 2) as well as in \textit{M. tuberculosis} H37rv Δacr1 Δacr2 (Figure 44 A, lane 4) but was detected in \textit{M. tuberculosis} H37rv Δacr2 (Figure 44A, lane 3) and WT \textit{M. tuberculosis} H37rv (Figure 44A, lane 1). α-crystallin 2 was not detected in \textit{M. tuberculosis} H37rv Δacr2 (Figure 44B, lane 2) nor was it detected in \textit{M. tuberculosis} H37rv Δacr1 Δacr2 (Figure 44B, lane 4) but detected in \textit{M. tuberculosis} H37rv Δacr1 (Figure 44B, lane 3) and WT \textit{M. tuberculosis} H37rv (Figure 44B, lane 1). This result further confirmed the successful gene replacement of the α-crystallins in their respective knockout mutants.
Figure 44. Western detection of Acr1 and Acr2 in different knockout strains and WT *M. tuberculosis* H37Rv.

(A) Western blot for Acr1 protein in WT *M. tuberculosis* H37rv (lane 1); *M. tuberculosis* H37rv Δacr1 (lane 2); *M. tuberculosis* H37rv Δacr2 (lane 3); and *M. tuberculosis* H37rv Δacr1 Δacr2 (lane 4)

(B) Western blot for Acr2 protein in WT *M. tuberculosis* H37rv (lane 1); *M. tuberculosis* H37rv Δacr1 (lane 3); *M. tuberculosis* H37rv Δacr2 (lane 2); and *M. tuberculosis* H37rv Δacr1 Δacr2 (lane 4)

5.1.3. Constructing acr1, acr2 and acr1&acr2 expression constructs for complementation of mutants strains

The integrating mycobacterial plasmid, pKINTA, used in Chapter 3, was also the backbone plasmid for expression of *acr1*, *acr2* and both *acr1* and *acr2* to complement *M. tuberculosis* H37rv Δacr1, *M. tuberculosis* H37rv Δacr2 and *M. tuberculosis* H37rv Δacr1 Δacr2 strains. To construct the pK-acr1 construct, the *acr1* gene with its putative promoter (596bp) was PCR amplified using primers P19&P20 (Table 3) containing *SacI* restriction enzyme sequences at both ends and non-directionally cloned into pKINTA at the *SacI* restriction site (Figure 45). To construct the pK-acr2 construct, the *acr2* gene with its putative promoter (713bp: 219bp upstream, 480bp *acr2* gene and 14bp downstream) was PCR amplified with primers P21&P22 (Table 3) containing *NotI* restriction enzyme sequences at both ends and non-directionally cloned into pKINTA at the *NotI* site (construct not shown). To construct the pK-acr1acr2 construct, the *acr2* gene with its putative promoter was cloned at the *NotI* site in the pK-acr1 construct (Figure 46). Therefore, pK-acr1acr2 has both α-crystallin genes each with its own putative promoter. The finished constructs were screened and confirmed by PCR and digestion with the relevant restriction enzymes (Figure 47).
Figure 45. pK-acr1 construct

Construct map showing the acr1 gene with its putative promoter cloned at SacI site. This construct was introduced into M. tuberculosis H37rv Δacr1 to compliment the acr1 deletion.

Figure 46. pK-acr1acr2 construct

Construct map showing the acr1 and acr2 genes with their putative promoters cloned at the SacI and NotI sites respectively. This construct was introduced into M. tuberculosis H37rv Δacr1 Δacr2 to compliment the acr1 and acr2 mutations.
Figure 47. Confirmation of pk-acr1, pk-acr2 and pk-acr1&2 plasmid construction by restriction digest

Gel (1% agarose, TAE) picture shows the screening result for the cloning of acr1 and acr2 with their native promoters into pKinta. The different constructs were digested with SacI alone (lane 3), Not I alone (lane 1) or SacI&NotI (lane 2) of pk-acr1 (lane 3) pK-acr2 (lane 1) and pk-acr1&acr2 (lane 2). The fragments excised from the different constructs confirm the presence of right sized cloned fragments of acr1 and its native promoter (596bp) in pk-acr1, acr2 and its native promoter (713bp) in pk-acr2 and acr1&acr2 (double band) in pk-acr1&acr2. The top fragments (4870bp) are the pKinta backbone.
5.1.4. Confirming the reconstitution of α-crystallin expression in *M. tuberculosis* H37rv Δacr1, *M. tuberculosis* H37rv Δacr2 and *M. tuberculosis* H37rv Δacr1&2 respective complemented strains

PCR screening was used to confirm the integration of the integrative plasmids carrying the respective α-crystallin genes into the *attB* site to restore the function of the deleted native genes. Following successful electroporation with pK-acr1, pK-acr2 and pK-acr1acr2, the transformants were selected (Kanamycin resistant) and grown in broth to late log phase before collection of cells, lysis and SDS-PAGE and Western blotting as in (2.11). Expression of α-crystallin 1, as it is shown in Figure 48 was restored in *M. tuberculosis* H37rv Δacr1 comp (lane 3) & *M. tuberculosis* H37rv Δacr1 Δacr2 comp (lane 7), whereas it was absent in the knockout strains *M. tuberculosis* H37rv Δacr1 and *M. tuberculosis* H37rv Δacr1 Δacr2 (lane 2 & 6) respectively.

![Western blot](image)

**Figure 48:** Western blot detection of Acr1 showing complementation of Acr1 expression in null mutants.

Acr1 expression is restored in *M. tuberculosis* H37rv Δacr1 comp, which carries pKacr1, (lane 3) and in *M. tuberculosis* H37rv Δacr1 Δacr2 comp, which carries pKacr1acr2 (lane 7). Acr1 is absent in the empty knockout strains (*M. tuberculosis* H37rv Δacr1, lane 2 & *M. tuberculosis* H37rv Δacr1 Δacr2, lane 6). Wild type H37rv lane 1 and *M. tuberculosis* H37rv Δacr2 (lane 4) *M. tuberculosis* H37rv Δacr2 comp (pKacr2) (lane 5).
Western blotting (Figure 49) also confirmed the successful expression of α-crystallin 2 in the respective strains *M. tuberculosis* H37rv Δacr2 comp (lane 4) and *M. tuberculosis* H37rv Δacr1Δacr2 comp (lane 6) which carry pKacr2 and pKacr1acr2 complimenting constructs respectively.

**Figure 49. Western blot detection of Acr2 showing complementation of Acr2 expression in null mutants.**

Acr2 protein was detected in *acr2* mutant strains which had been complemented with the pKacr2 and pKacr1acr2 constructs (*M. tuberculosis* H37rv Δacr2 comp lane 4 & *M. tuberculosis* H37rv Δacr1Δacr2 comp lane 6). Acr2 immunoreactivity was absent in the respective empty knockout strains (*M. tuberculosis* H37rv Δacr2 lane 5 & *M. tuberculosis* H37rv Δacr1Δacr2 lane 7). Wild type *M. tuberculosis* (lane 1); *M. tuberculosis* H37rv Δacr1 (lane 2); *M. tuberculosis* Δacr1 pKacr1 (lane 3).

The expression of alpha crystalline 2 in the double complimentary strain *M. tuberculosis* H37rv Δacr1Δacr2 comp (Figure 49) is not as strong as alpha-crystalline 1 in the *M. tuberculosis* H37rv Δacr1Δacr2 comp (Figure 49). This level of expression may not be enough to restore the function of the native *acr2* in stress conditions.
5.2 Growth of the α-crystallin mutants in shaken broth culture

To investigate the effect of the loss of one or both genes on the growth of the different mutants in comparison to the wild type, two biological replicates of each strain were inoculated into 7H9 containing 10% ADC and 0.1% Tween80. An OD 600 reading and samples for viability counting were taken at certain time points during a three weeks’ period. As seen in the OD growth curve (Figure 50), *M. tuberculosis* H37rv Δacr2 has similar growth as the WT *M. tuberculosis* H37Rv. However, *M. tuberculosis* H37rv Δacr1 and *M. tuberculosis* H37rv Δacr1Δacr2 have a slower growth curve which plateaus at a much lower OD value (Figure 50).

![Figure 50. Growth curve (OD 600) of WT M. tuberculosis H37rv and α-crystallin knockout mutants grown in 7H9 containing 10% ADC and 0.1% Tween80. Two biological replicate for each](image-url)
Although there is a distinctive difference in the shape of *M. tuberculosis H37rv Δacr1* and *M. tuberculosis H37rv Δacr1Δacr2* OD growth curve from the WT *M. tuberculosis H37rv* and *M. tuberculosis H37rv Δacr2*, the strains appear to have similar growth when assessed by viability count Figure 51. It has previously been reported that the *M. tuberculosis H37RvΔacr1* has the same growth rate as the wild type *M. tuberculosis H37rv* (Yuan et al., 1998). Therefore, the observed slow OD growth curve shape could be misleading and may be the result of high aggregation as a result of the loss of α-crystallin 1.

![Growth curve (viability assessed by cfu) of WT M. tuberculosis H37rv and α-crystallin knockout mutants grown in 7H9 containing 10% OADC and 0.1% Tween80. Two biological replicate for each.](image)

**Figure 51.** Growth curve (viability assessed by cfu) of WT *M. tuberculosis H37rv* and α-crystallin knockout mutants grown in 7H9 containing 10% OADC and 0.1% Tween80. Two biological replicate for each.

If the *acr1* mutant strains are more aggregative (clumpy) than the other strains including the WT *M. tuberculosis H37Rv*, further phenotypic experiments involving quantitative
assessment of bacterial numbers may be affected. Therefore, it is necessary to take into account such possibility when interpreting the experiments in this chapter.

5.3 Heat Shock resistance of the acr1, acr2 and acr1acr2 mutant M. tuberculosis H37Rv

This experiment tested for the effect of the loss of one or both acr genes on the ability of the mutant to resist killing by heat in comparison to the wild-type. The different strains were grown to mid log phase and the OD adjusted to 0.2 for all of them. Each culture was split into two duplicates of 1ml for each time point. This means for each sampling point two tubes for each strain were removed from the water bath, sampled and then discarded. This was done to enable sampling without affecting the culture temperature during the process and also to minimize the risk of culture contamination via repeated sampling. It was not possible to process more than duplicate samples for each strain at each time point. It was noted that any delay in plating by putting the samples on ice to enable more replicates to be processed resulted in great variation in the replicates dependent on how long the samples had rested on ice. However, despite the absence of statistical analysis, it was clear that the strains lacking, M. tuberculosis H37rv Δacr1 and M. tuberculosis H37rv Δacr1Δacr2 were less heat resistant than WT M. tuberculosis H37rv or the acr2 mutant. Indeed 60 minutes at 53°C resulted in 3-4 log reduction in the M. tuberculosis H37rv or the acr2 mutant strains but a complete 7 log kill of the acr1 deficient strains (Figure 52)
Figure 52. Heat resistance *M. tuberculosis* H37rv α-crystalline mutants

The different strains were incubated for two hours in 53°C. A sample was taken at different time points for viability count by plating on 7H11 media and counting cfu after 21 days. Two technical replicate for each. Error bars show the standard error of the mean. The threshold of detection is 167 cfu/ml.

The *acr1* gene is not inducible by heat and *M. tuberculosis* H37rv Δacr1 has previously been shown to have the same survival after 2 hrs in 55°C as the wild type (Hu et al., 2006). However, the temperature Hu *et al* used in their experiment is 2°C higher than the temperature (53°C) used in this study and thus the present study may have detected a more subtle difference. The α-crystallin 2 has been purported to protect *M. tuberculosis* H37rv from heat (Ohara *et al.*, 1997) and is highly induced when *M. tuberculosis* H37rv is subjected to a heat shock of 45°C for 30 minutes (Stewart *et al.*, 2002b). Thus, it was surprising that the *M. tuberculosis* H37rv Δacr2 strain had wild-type resistance to heat.
5.4 H$_2$O$_2$ stress response of the *acr1*, *acr2* and *acr1acr2* mutant *M. tuberculosis* H37Rv

The different strains were grown in 7H9 broth containing 10% ADC and 0.1% Tween80 to mid-log phase. The cultures were adjusted to OD 0.4 and washed once with sterile water to remove any residual growth media and were re-suspended in sterile water with the same starting OD. 100µl of the washed re-suspended cells were added to a set of 1ml tubes of Roisin’s medium containing 0mM, 10mM or 20mM freshly prepared H$_2$O$_2$ for each strain in triplicate. After incubation for 2hrs at 37°C without shaking, a sample was taken from each tube and serially diluted for viable count.

As shown in Figure 53, there is variability between the strains in the cfu number retrieved from incubation in medium alone (0mM), ranging from 1x10$^5$- 3x10$^5$ cfu/ml, which might be due to the fact that there is some difference in aggregation between the strains. Apart from the *M. tuberculosis* H37rv Δacr2 and its complement, 10mM H$_2$O$_2$ induced significant killing in all strains (p 0.0001). This is most clearly seen in Figure 54 which shows percentage survival in the different H$_2$O$_2$ concentrations. Survival of wild type *M. tuberculosis* H37Rv, *M. tuberculosis* H37rv Δacr1 and *M. tuberculosis* H37rv Δacr1Δacr2, *M. tuberculosis* H37rv Δacr1 comp, *M. tuberculosis* H37rv Δacr1Δacr2 was reduced by 80% but *M. tuberculosis* H37rv Δacr2 and its complemented strain were only reduced by 70-80%.
Figure 53. H₂O₂ resistance of *M. tuberculosis* H37RV  α-crystallin mutants

Wild type *M. tuberculosis* H37Rv, *M. tuberculosis* H37rv Δacr1, *M. tuberculosis* H37rv Δacr2 and *M. tuberculosis* H37rv Δacr1Δacr2 and their complemented strains were grown to mid log phase, washed and diluted to an OD of 0.4. The different strains were incubated for two hours in 0mM 10mM and 20mM H₂O₂. A sample was taken at different time points for viability counting on 7H11 media. Three biological replicates for each. Error bars represent standard error of the mean of three independent replicates. Statistical significance was assessed by Tukey’s multiple comparisons test.

This result disagrees however with Stewart et.al (2006) who previously reported that the loss of the acr2 gene rendered the knockout strain to be more susceptible to oxidative stress when subjected to 1mM and 10mM for 8hours. This difference may reflect the different parental strain used in these experiments or the different H₂O₂ concentrations and exposure times.
The elevated resistance of the *M. tuberculosis* H37rv Δacr2 and its compliment in the present experiment may indicate that a secondary mutation has been selected for during deletion of acr2 and that this overcompensates for any stress related functional deficiency in the oxidative stress response. This explanation is further supported by the observation that deletion of the acr2 gene in the acr1 mutant background does not increase resistance to H2O2. Further evidence to support the theory that the single acr2 mutant has acquired a secondary mutation would require genome sequencing of the parent strain and acr2 mutant, but this is beyond the scope of the present study.

At the higher concentration of 20mM H2O2, there was a slightly higher percentage level of killing and no significant difference between strains except for the *M. tuberculosis* H37rv Δacr2 complement which survived at significantly higher levels than the wild-type (Figure 54)

Deletion of the acr1 gene had no effect on resistance to H2O2. *M. tuberculosis* H37rv Δacr1 had the same resistance/survival rates as wild type *M. tuberculosis* H37rv (Figure 54). This is in agreement with a previous study which reported that deletion of acr1, which is not induced by H2O2 (Hu et al., 2006), did not significantly increase the sensitivity to H2O2 in comparison to wild type *M. tuberculosis* H37rv (Yuan et al., 1998). The most important question for this study was whether deletion of both α-crystallins had a cumulative or synergistic effect on resistance. The double mutant strain showed the same sensitivity to H2O2 as the wild-type *M. tuberculosis* H37rv (Figure 54) (Hu et al., 2006)
Figure 54. Percentage survival of *M. tuberculosis* H37RV \(\alpha\)–crystallin mutants in H\(_2\)O\(_2\)

Wild type *M. tuberculosis* H37Rv, *M. tuberculosis* H37rv \(\Delta\text{acr1}\), *M. tuberculosis* H37rv \(\Delta\text{acr2}\) and *M. tuberculosis* H37rv \(\Delta\text{acr1}\Delta\text{acr2}\) and their complemented strains were grown to mid log phase, washed and diluted to an OD of 0.4. The different strains were incubated for two hours in 0mM, 10mM and 20mM H\(_2\)O\(_2\). A sample was taken at different time points for viability counting on 7H11 media. Survival is shown as percentage of survival for each strain in Roisin’s medium alone. Three biological replicates for each. Error bars represent standard error of the mean of three independent replicates. Statistical significance was assessed by Tukey’s multiple comparisons test.
5.5 Resistance to low pH in acr1, acr2 and acr1acr2 mutant M. tuberculosis H37Rv

The different strains were grown in 7H9 broth containing 10% ADC and 0.1% Tween80 to mid-log phase. The cultures were adjusted to OD_{600} 0.4 and washed once with sterile water to remove any residual growth media and resuspended in sterile water with the same starting OD. 100µl of the washed resuspended cells were added to a set of 1ml aliquots of Roisin’s medium pH adjusted to 4.4 or 6.6 for each strain in triplicate. After static incubation for 7 days at 37°C, a sample was taken from each tube and serially diluted for viable cell counting. The growth of different strains indicated by the increase in the cfu count after 7 days (Figure 55) of incubation from the input cfu count suggest the medium at pH6.6 is supportive for their growth.

Figure 56 shows the percentage survival of the α-crystallin mutants. Statistically there was no difference between the survival of the strains in pH4.4 after 7 days of incubation. However, there appeared to be a trend towards the M. tuberculosis H37rv Δacr2 and M. tuberculosis H37rv Δacr1Δacr2 having a higher percentage of survival in comparison to the wild type M. tuberculosis H37Rv, M. tuberculosis H37rv Δacr1, and their respective complements. There was no trend towards any change in acid resistance in the single acr1 mutant, which is in agreement with previous studies (Hu et al., 2006). Thus, the deletion of acr2 again seems to induce compensatory changes which increase resistance. In this case, they are reversed by complementation which restored the wild type sensitivity to low pH stress.
Figure 55. Acid pH resistance of *M. tuberculosis* H37rv α-crystalline mutants

Growth/survival of wild type *M. tuberculosis* H37Rv, *M. tuberculosis* H37rv Δacr1, *M. tuberculosis* H37rv Δacr2 and *M. tuberculosis* H37rv Δacr1Δacr2 and their complemented strains in different pH. The different strains incubated in Roisin’s medium at pH 6.6 and 4.4 for 7 days and cell viability assessed by plating on 7H11 and counting cfus. Three biological replicates for each. Error bars represent standard error of the mean of three independent replicates. Statistical significance was assessed by Tukey’s multiple comparisons test.

Figure 55 shows that the acidic pH of 4.4 has an effect on the survival of *M. tuberculosis* H37rv reducing the viable count of all strains in comparison to their input counts and their viability at pH6.6.
Figure 56. Percent survival of *M. tuberculosis* H37rv α-crystallin mutants in acid stress

Growth/survival of wild type *M. tuberculosis* H37Rv, *M. tuberculosis* H37rv Δacr1, *M. tuberculosis* H37rv Δacr2 and *M. tuberculosis* H37rv Δacr1Δacr2 and their complemented strains in different pH. The different strains incubated in Roisin’s medium at pH 6.6 and 4.4 for 7 days and cell viability assessed by plating on 7H11 and counting cfus. Survival is shown as percentage of the input count for each strain. Three biological replicates for each. Error bars represent standard error of the mean of three independent replicates. Statistical significance was assessed by Tukey’s multiple comparisons test.
5.6 Intracellular phenotype of acr1, acr2 and acr1acr2 mutant M. tuberculosis H37rv in THP-1 macrophages.

Macrophages play a significant role in the defence against *M. tuberculosis* H37rv infection and at the same time *M. tuberculosis* H37rv has adapted to overcome the killing mechanisms it faces within the macrophages. The human acute monocytic leukemia cells (THP-1) which are used in this study are one of many model cell lines used to study *in vitro* the host cell- *M. tuberculosis* H37rv interaction and they are considered to be a good model of a peripheral blood monocyte (Theus *et al.*, 2004). The THP-1 cells were grown in RPMI 1640 medium supplemented with 10% FCS, 10mM L-glutamine and 10mM pyruvate in 5% CO2 at 37°C for 4 days. A concentration of 10^6 ml⁻¹ THP-1 cells was adjusted to the required volume of RPMI containing 100ng ml⁻¹ PMA and added to 96 well plates to give about 2x10⁵ cells per well. The cells then incubated for 3 days to differentiate and adhere to surface of the well to produce a monolayer. The cells were then washed three times with warm RPMI and infected with the different *M. tuberculosis* H37rv strains diluted in warm RPMI (with10% FCS, 10mM L-glutamine and 10mM pyruvate) medium at an MOI of 0.1 and incubated in 5% CO2 at 37°C for the required period of time. Different plates were set up for different time points of 2hrs, 24hrs, 48hrs and 7 days. At each time point the respective plate was taken out of the incubator, all the wells were washed with warm PBS then 3 wells were assessed for CFU counts. Additionally, three infected wells and 6 uninfected wells were fixed with 4% formaldehyde and stained with crystal violet to determine the number of macrophage cells present at each time point and to assess the level of bacterial induced cytotoxicity.
Figure 57: Intracellular survival of *M. tuberculosis* H37rv α-crystallin mutants in THP-1 macrophages

Viable bacterial counts from THP-1 macrophages infected at MOI of 0.1 with *M. tuberculosis* H37Rv, *M. tuberculosis* H37rv Δacr1, *M. tuberculosis* H37rv Δacr2 and *M. tuberculosis* H37rv Δacr1Δacr2 and their complemented strains. Three technical replicate for each. Error bars represent standard error of the mean of three independent replicates. Statistical significance was assessed by Tukey’s multiple comparisons test.
As indicated in Figure 57 there was no statistically significant difference between the strains in CFU count after 2hrs of incubation, which represents the time allowed for phagocytosis. However, by eye it appears that the *M. tuberculosis* H37rv Δacr1 and *M. tuberculosis* H37rv Δacr1Δacr2 strains have been taken up in higher amounts compared to other strains. This could represent a difference in the inocula or a difference in the efficiency of phagocytosis of these strains. The viable/CFU count of all the strains increased significantly after 24 hrs and remained almost the same at 48 hrs post-infection. The *M. tuberculosis* H37rv Δacr1 and *M. tuberculosis* H37rv Δacr1Δacr2 were present in significantly higher loads than the wild-type at both 24 and 48 hrs post-infection but at 84 hrs were significantly lower than the wild-type. There was no significant difference between the complemented mutant strains and the wild-type at any time point. There was no significant difference between the *M. tuberculosis* H37rv Δacr2 and its complemented strain at any time point. Thus, mutants defective in the *acr*1 gene, either as a single mutant or as a double with *acr*2 deletion, had an enhanced intracellular survival over the first 48 hours which then crashed by 84 hours to levels below the WT *M. tuberculosis* H37Rv. These results are partially consistent with a previous study which showed that *M. tuberculosis* H37rv Δacr1 has been reported to have the same infectivity but substantial reduction in CFU count compared to the wild type after 10 day of THP-1 infection (Yuan et al., 1998). Also, in monocyte derived macrophages (MDM) *M. tuberculosis* H37rv Δacr2 the growth was the same as wild-type up to day 4 post-infection (Wilkinson et al., 2005).

To examine deeper into the dynamics of the intracellular growth of the α–crystallin mutants, we assessed the number of macrophages remaining in the monolayers throughout the experiment (Figure 58). There was no significant difference between the number of
macrophages in the different strain infections at 2, 24 or 48 hrs post-infection. However, there was considerable cytotoxicity by 84 hrs. There was less cytotoxicity in macrophages infected with the acr2 mutant strain compared to those infected with wild-type M. tuberculosis. Wild-type cytotoxicity was not recovered in M. tuberculosis H37rv Δacr2 infected cells by reintroduction of acr2 in the complemented strain. The complemented M. tuberculosis H37rv Δacr1Δacr2 strain also showed reduced cytotoxicity. This may indicate that α-crystallin 2 is required for M. tuberculosis to express full cytotoxicity. This phenotype is not simply reflective of an altered bacterial load because the M. tuberculosis H37rv Δacr2 load was equivalent to WT M. tuberculosis H37rv throughout the infection. The inability of the complementing construct to restore this cytotoxic effect may be due to the only partial restoration of Acr2 expression in the complemented strains (Figure 49, lanes 4 and 6). Indeed, partial complementation using a similar integrating Acr2 expression construct has been observed before by Stewart et.al (2006).
Figure 58. Cytotoxicity of infection THP-1 macrophages infected with *M. tuberculosis* H37Rv α-crystallin mutants.

Number of THP-1 macrophages assessed by crystal violet staining in in vitro infections at MOI of 0.1 with *M. tuberculosis* H37Rv, *M. tuberculosis* H37Rv Δacr1, *M. tuberculosis* H37Rv Δacr2 and *M. tuberculosis* H37Rv Δacr1Δacr2 and their complemented strains. Three technical replicate for each. The error bars are the standard error of the mean of three independent replicates.
5.7 Growth/survival of \textit{acr1}, \textit{acr2} and \textit{acr1acr2} mutant \textit{M. tuberculosis} H37rv in hypoxia/microaerophilic conditions

The \textit{in vivo} environment presents \textit{M. tuberculosis} H37rv with a number of different stressful conditions, one of which is hypoxia which can occur inside the tubercle lesion or granuloma (Duque-Correa \textit{et al.}, 2014; Qualls and Murray, 2016). Hypoxic conditions are inimical to mycobacterial replication but \textit{M. tuberculosis} H37rv has adapted to persist in this environment and become, so called, non-replicating persisters (NRP) (Fattorini \textit{et al.}, 2013). This adaptation involves the regulation of many genes including those involved in stress responses and metabolism and includes many prominent antigens. One of the many genes which is strongly induced by hypoxia is the \textit{acr1} gene encoding the heat shock (HspX) protein, which is regulated as part of the DosR regulon (Sherman \textit{et al.}, 2001; Tabira \textit{et al.}, 1998; Voskuil \textit{et al.}, 2004; Yuan \textit{et al.}, 1998). Interestingly, \textit{acr2} which is not part of the DosR hypoxic response, is also upregulated during non-replicating persistence (Stewart \textit{et al.}, 2005). Thus, it was chosen to study the functional role of \(\alpha\)-crystallins in hypoxia by examining the survival of the \textit{acr} deficient mutants under \textit{in vitro} hypoxic conditions.

Due to technical difficulty, it was not possible to employ the \textit{in vitro} hypoxia model used by Wayne (Wayne and Hayes, 1996) in this study. Therefore, a modified method was used where sterile mineral oil was used to overlay static broth cultures to prevent oxygen dissolving in the culture during incubation and thus microaerophilic/hypoxic conditions would progressively develop. The different strains of \textit{M. tuberculosis} H37rv were grown to mid-log phase in 7H9 medium containing 10\% ADC and 0.1\% Tween80 at 37\(^\circ\)C shaking. After adjusting all the cultures to the same OD, 1ml aliquots were distributed into 2ml O-ringed tubes in triplicates for each sampling time. To each tube 0.5ml of sterile mineral oil was layered on top of the 1ml of the culture and incubated at 37\(^\circ\)C. Tubes were only sampled
once to avoid aeration of cultures. Methylene blue indicator at concentration of 1.5 µg/ml was used as an indicator of oxygen depletion in one replicate of each strain culture and in a medium only control. The blue colour should disappear in the absence of oxygen. Figure 59 shows that the blue colour in the culture tubes was significantly less intense than in the medium alone tube, indicating that there was a level of oxygen depletion in the bacterial cultures but that it did not reach 100% oxygen depletion. It may be that the static conditions of the culture did not encourage sufficient bacterial growth to deplete the oxygen levels fully. The Wayne model uses stirred cultures to overcome this issue but due to the technical difficulty it was not possible to perform this in this experiment. Thus the bacilli which were sedimented at the bottom of the tube were present in a microaerophilic environment at which the first stage NRP-1 may have occurred but, it was probably not sufficient to exert the stress that might trigger the bacilli to enter dormancy and NRP-2 stage. For the bacilli to enter the microaerophilic non replicating persisters (NRP) stage 1 the oxygen content of the solution should drop below 1% (Wayne, 2001) and if the level of the dissolved oxygen drops below 0.06% the NRP-2 stage occurs (Wayne and Hayes, 1996). As it was not technically possible to quantify the amount of the dissolved oxygen in this experiment, it is not possible to directly compare the results in this experiment with the NRP model reported by Wayne.
Figure 59: Methylene blue indication of oxygen depletion

Tube (1) contains just sterile 7H9 media, tubes 2,3,4&5 contain 1ml of WT *M. tuberculosis* H37Rv, *M. tuberculosis* H37rv Δacr1, *M. tuberculosis* H37rv Δacr2 & *M. tuberculosis* H37rv Δacr1 Δacr2 cultures respectively at day 30. The difference in colour between tube 1 and the rest of the tubes indicate that most of the oxygen has been consumed by the living bacteria but, 100% oxygen depletion was not achieved.

All the bacterial strains grew significantly during the first 10 days of culture in the oil-overlayed broths. At this 10 day time point, all three *acr* mutant strains had a significantly lower number of bacteria, as measured by cfu/ml, compared to the wild type *M. tuberculosis* H37rv (*p*<0.001) (Figure 60). By 20 days only the *acr1* mutant strain had a lower number of bacteria than the wild-type. Between 20 and 30 days, there was a significant reduction in the number of bacteria in all cultures (1.5-2 log) and there was no difference in survival between the strains at this time point. This experiment was repeated (Appendix 8.4) and the result was confirmed that there was no statistically significant difference in survival between the different strains after 30 days of incubation. Therefore, it can be concluded that neither *acr1* nor *acr2* are essential for survival in non-replicating microaerophilic culture.
Figure 60: Growth/survival of *M. tuberculosis* H37rv α-crystallin mutants in microaerophilic culture

The graph shows the result of microaerophilic/hypoxia stress on the different *M. tuberculosis* H37rv strains. The different *M. tuberculosis* H37rv strains were grown in 7H9 containing 10% ADC and 0.1% Tween80 until mid-log phase. The cultures were adjusted to the same OD. Replicate 1 ml volumes of the respective cultures were distributed into 2ml O-ringed screw capped tubes and layered with 0.5ml of sterile mineral oil. The tubes were incubated still at 37°C for 30 days. Results show the mean of triplicate cultures at each time point. Error bars represent standard error of the mean of three independent replicates. Statistical significance was assessed by Tukey’s multiple comparisons test.
5.8 Discussion

In this chapter, the acr2 gene was successfully deleted from WT *M. tuberculosis* to produce a *M. tuberculosis* H37rv Δacr2 single knockout mutant and from *M. tuberculosis* H37rv Δacr1 to produce the *M. tuberculosis* H37rv Δacr1Δacr2 double knockout mutant. The knockout of acr2 gene from the respective mutant was successfully confirmed by PCR, restriction enzyme digest of the PCR product and sequencing. The deletion of acr1 was also verified by PCR. Further to this, Western blotting was used to confirm the absence of Acr1 and Acr2 proteins from the respective mutant bacteria. The complementation of the respective genes in the knockout strains was also successfully confirmed by Western blotting although wild-type levels of expression were not achieved.

*M. tuberculosis* H37rv Δacr2 had a similar growth curve to wild type in 7H9 broth when assessed by optical density. In contrast the *M. tuberculosis* H37rv Δacr1 and the double mutant *M. tuberculosis* H37rv Δacr1Δacr2 had an apparently slower than the wild-type growth, reaching a lower optical density. However, this difference was not observed in the viable colony count growth curve in which all the strains showed similar growth kinetics. Our findings by optical density are in agreement with a previous study in which deletion of acr1 was reported to have slower growth than the wild type parent strain (Yuan et al., 1998). The similarity between growth of all the strains assessed by viability count may suggest that the loss of acr1 causes a change in the aggregation of the cells which results in the absence of correlation between cfu and optical density. Although this was not confirmed experimentally and also the growth curve for the complement strains was not done, it should be taken into consideration when interpreting the results of other in vitro stress conditions.
experiments. Most relevant to the aim of this chapter was the observation that there was no additive or synergistic effect of deleting both α-crystallins. Thus we can conclude that the chaperones do not have an essential but redundant function for normal growth.

The *M. tuberculosis* H37rv Δacr1 and *M. tuberculosis* H37rv Δacr1Δacr2 strains were more sensitive to heat than WT *M. tuberculosis* H37rv or the single Δacr2 mutant. This is indicated by a 7 log reduction in cfu of single Δacr1 and the double Δacr1Δacr2 mutants compared to a 3-4 log reduction in cfu observed in wild type and single Δacr2 mutant at 53°C. This is perhaps surprising given the published data that acr1 is not inducible by heat and a mutant was shown to have the same sensitivity to heat stress, albeit a higher temperature (Hu *et al.*, 2006) whereas, acr2 is massively induced by heat (Stewart *et al.*, 2005). One explanation for the enhanced sensitivity of the acr1 mutant is that it functions to control protein aggregation in the cytoplasm, as indicated by its localisation in the previous chapter, whereas Acr2 is only present in the cell wall. If in the short term, protein aggregation in the cytoplasm is more detrimental to cell viability than aggregation outside of the cell in the cell wall, then an absence of constitutive Acr1 may lead to a greater heat sensitivity than an absence of Acr2. Once again, the double mutant had no additive loss of fitness in this condition indicating that the chaperones do not perform essential but redundant roles during heat stress.
The *M. tuberculosis* H37rv response to oxidative stress is complex and it uses wide range of molecules to either detoxify ROI and RNI before they cause stress or by repairing the damage they cause (Sandgren *et al.*, 2009). There are various proteins including AhpC, superoxide dismutase (SOD), KatG, Mel2, Rv2136c, Rv224c, PonA2, Acr1 and Acr2 that directly or indirectly are necessary for *M. tuberculosis* complex (MTBC) species to survive the oxidative and nitrosative stresses. Rv2136c, Rv224c, PonA2 are thought to contribute to the cell wall integrity which may suggest that the mycobacterial lipid-rich cell envelope may act as an effective barrier against the entry of RNI and ROI (Forrellad *et al.*, 2013). In this study, *M. tuberculosis* H37rv Δacr2 and its complement showed significantly increased survival (p 0.0001) in H$_2$O$_2$ compared to the other tested strains. This contrasts with previous reports that the loss of *acr2* increased the susceptibility of *M. tuberculosis* H37rv to oxidative stress (Stewart *et al.*, 2006). Again the experimental conditions, concentration of H$_2$O$_2$, the strains used and the length of exposure time may explain these different findings. Alternatively, this may suggest that *M. tuberculosis* H37rv Δacr2 in this study may have acquired some kind of compensatory mutation during the process of deleting *acr2*, which is also observed in the response of its complement strain. Such a theory is partially supported by the fact that the double Δacr1Δacr2 mutant is not more resistant to oxidative than the single Δacr1. The confirmation of a secondary mutation will require genome sequencing of the parent strain and *acr2* mutant (not performed in this study).

*M. tuberculosis* H37rv can resist phagosomal pH and it was reported to have survived pH 4.5 in a simple buffer *in vitro* (Vandal *et al.*, 2008). However, the interpretation of Mycobacteria resistance to acid in *in vitro* experiments is complicated. This is because the
survival of the bacteria in acid is dependent on culture conditions including bacterial density and composition of the media being used (Bodmer et al., 2000; Sung and Collins, 2003; Vandal et al., 2009). The optimal growth pH in liquid medium for *M. tuberculosis* H37Rv reported to between about 6.2-7.3 (Chapman and Bernard, 1962) and its growth is inhibited below pH5.5 (Heifets and Iseman, 1985). In this study, all the strains tested showed sensitivity to the acid pH4.4 which was indicated by reduction of the cfu in comparison to the input number. Although there is no statistical significant difference in the response to the acid pH, there is a trend of more resistance by *M. tuberculosis* H37rv Δacr2 and *M. tuberculosis* H37rv Δacr1Δacr2 mutants observed by naked eye. This trend was not observed in the respective complementary strains. This is surprising but consistent with the occurrence of compensatory physiological adaptations to the loss of Acr2. Once again, it is noteworthy that there are no synergistic or additive effects of deletion of both α–crystallins.

Hypoxia is one of the stresses that *M. tuberculosis* H37rv is facing within the tubercle lesion or granuloma (Duque-Correa et al., 2014; Qualls and Murray, 2016). *M. tuberculosis* H37rv is able to evade such detrimental hypoxic condition by halting their replication and become non-replicating (NRP) persisters (Fattorini et al., 2013). HspX protein encoded by acr1 which part of the DosR regulon and highly induced hypoxia (Sherman et al., 2001; Tabira et al., 1998; Voskuil et al., 2004; Yuan et al., 1998). Although acr2 is not part of the DosR hypoxic response, is also upregulated during non-replicating persistence (Stewart et al., 2005). It was thus of great interest to examine the phenotype of the α–crystalline mutants in hypoxic of microaerophilic non-replicating culture.
In this study the *acr* deficient mutants were grown in hypoxic conditions modified from the Wayne *in vitro* hypoxia model (Wayne and Hayes, 1996) due to technical constraints. The modification applied did not allow the 100% depletion of oxygen as indicated by presence of the methylene blue oxygen depletion indicator colour which should decolourise in the event of 100% absence of oxygen. However, there was a degree of hypoxia achieved and the replication of bacteria was inhibited in the cultures. It seems likely that the bacilli sedimented at the bottom of the tube were present in microaerophilic conditions sufficient to cause the bacilli to enter the non-replicating persistence, NRP-1, stage. However, such a microaerophilic environment may not be sufficient to cause the bacilli to enter NRP-2 stage. For the bacilli to enter the microaerophilic non replicating persisters (NRP) stage 1 the oxygen content of the solution should drop below 1% (Wayne, 2001) and if the level of the dissolved oxygen drops below 0.06% the NRP-2 stage occurs (Wayne and Hayes, 1996). As it was not technically possible to quantify the amount of the dissolved oxygen in this experiment, it is not possible to directly compare the results in this experiment with the NRP model reported by Wayne.

All the bacterial strains grew significantly during the first 10 days of culture. Between 20 and 30 days, there was a significant reduction in the number of bacteria in all cultures (1.5-2 log) and there was no difference in survival between the strains at this time point. This experiment was repeated (Appendix 8.4) and result was confirmed that there was no statistically significant difference in survival between the different strains after 30 days of incubation. Therefore, it can be concluded that neither Acr1 nor Acr2 are essential for survival in non-replicating microaerophilic culture and that they do not confer redundant function in providing for survival in this condition.
In summary, except for Acr1 in heat shock, deficiency of neither α-crystallin was important for survival in any of the stress conditions tested and no redundancy in function was detected in which the presence of at least one α-crystallin could compensate for loss of the other and the loss of both engendered a weakened phenotype. In retrospect, the obvious omission from these experiments was the inclusion of a cell wall specific stress such as a detergent or surfactant. These are known to induce expression of Acr2 specifically via SigE and MprA/B and given the knowledge from chapter 4 that Acr2 is localised almost exclusively in the cell wall, it could be postulated that there would be a detectable phenotype in cells deficient in Acr2. Investigating if Acr1, with its lesser localisation in the cell wall, could compensate for any sensitivity to cell surface stress would be an important experiment.

There have been contradictory reports about the importance of α-crystallins to *M. tuberculosis* during infection of host cells. In the present experiment, loss of *acr1* in both the single mutant and in combination with deletion of *acr2* led to an elevated bacterial load at 24hr and 48hrs. However, consistent with previous studies (Yuan et al., 1998) the mutants lacking Acr1 survived less well than wild-type bacteria at the later stage of infection (84 hours). This contrasted with the Δacr2 mutant which survived equally as well as wild-type throughout the experiment. Arguably the most interesting observation was that the Acr2 deficient strain, despite an equal bacterial load, caused less host cell cytotoxicity than the WT *M. tuberculosis* H37Rv. These results are consistent with a previous study which showed that in MDM *M. tuberculosis* H37rv Δacr2 growth was the same as wild-type up to day 4 post-infection (Wilkinson et al., 2005). Also, the reduced cytotoxicity is consistent with the altered pathology in murine infections with *M. tuberculosis* H37rv Δacr2 which
resulted in a reduction in disease symptoms despite similar bacterial load (Stewart et al., 2005).

Taken together, the experiments performed in Chapters 4 and 5 strongly indicate that the two α-crystallins of *M. tuberculosis* have different functions: they are localised in different parts of the cell and their individual deletion confers different phenotypes during macrophage infection. Importantly, in none of the phenotypic characterisation experiments was there observed a phenotype that was present in the double mutant but not in either single mutant. Thus there appears to be no or limited redundancy between the α-crystallins. Future experiments should include comparison of *in vivo* phenotypes of the single and double mutant strains during animal infection and also in other in vitro stress conditions such as surfactant cell surface stress.
Chapter 6

General discussion & conclusion
6.0 General Discussion

Tuberculosis remains a disease with high morbidity and mortality caused by *M. tuberculosis* (WHO, 2015). Humans get infected by inhaling microscopic aerosol droplets containing small numbers of bacilli (Kaufmann, 2001). In the terminal alveoli, the invading *M. tuberculosis* are engulfed by macrophages which migrate into the parenchyma of the lung and initiate formation of granulomatous lesions. The in vivo environment has the capacity to subject *M. tuberculosis* to many stresses, examples of which include nutrient limitation, hypoxia, oxidative stress, nitric oxide and low pH (Duque et al., 2014; Qualls and Murray, 2016; Rohde et al., 2007; Schnappinger et al., 2003; Voskuil et al., 2003). To survive this, the bacterium modulates the host immune response to limit exposure to the stresses (Ehrt and Schnappinger, 2009; Ehrt et al., 2001; Mahajan et al., 2012; McDonough et al., 1993) and also undergoes genome-wide changes in gene expression, which results in a changed physiological and metabolic state, such as dormancy (Fattorini et al., 2013; Gengenbacher and Kaufmann, 2012; Kelly and O'Neill, 2015; Mariotti et al., 2013; McKinney et al., 2000) These changes also result in the production of specific resistance mechanisms to the various stresses. A major requirement for the bacterial cell during stress is to up-regulate/activate systems that provide homeostasis for the protein component of the cell. This may include enzyme systems to degrade denatured, misfolded or truncated proteins generated under stress and also may include a group of so called heat shock proteins (Hsps). These Hsps are the molecular chaperones which function to bind denatured proteins to stop them aggregating and either encourage them to refold or traffic them to the aforementioned proteases.
Hsps are among the most up-regulated *M. tuberculosis* proteins following infection of the macrophage and some have been demonstrated to be essential for optimal growth in the *in vivo* environment (Monahan *et al.*, 2001; Sherman *et al.*, 2001; Stewart *et al.*, 2005; Stewart *et al.*, 2002a; Voskuil *et al.*, 2004). This thesis considers the function of two molecular chaperones from a class of proteins known as the α-crystallins. Acr1 (HspX) is one of the many proteins which is regulated as part of the DosR regulon and is strongly induced by hypoxia (Sherman *et al.*, 2001; Tabira *et al.*, 1998; Voskuil *et al.*, 2004; Yuan *et al.*, 1998). Acr2 is also up-regulated during non-replicating persistence, including that induced under hypoxia, but it is not part of the DosR regulon. Instead it is regulated by a number of other systems that leads to its induction in response to many stresses (Stewart *et al.*, 2005) and it has been suggested to be associated with ribosomes during stress. It is up-regulated more than any other gene during macrophage infection (Schnappinger *et al.*, 2003). Thus, both α-crystallins have been implicated as important during infection but there is a lack of detail regarding their specific functions and the degree of redundancy between them. Thus this thesis set out to test the below hypothesis:

**Hypothesis A**

There is some functional redundancy between the two α-crystallins and that one can compensate to some extent for the loss of the other.

To begin to test Hypothesis A, this investigation compared the subcellular localisation of the α-crystallins in *M. tuberculosis H37rv* from stationary phase cultures. The presence of Acr1 and Acr2 was determined in different cellular fractions (culture supernatant, cell pellet, cytosolic and plasma membrane factions) by Western blot and the intracellular localisation of Acr1 and Acr2 was examined by immuno-electron microscopy.
The Acr1 immunogold electron microscopy showed localisation in all parts of the cell, with localisation biased to the cytoplasm of some cells and to the periphery in others. This result largely agreed with the findings of Cunningham and Spreadbury (1998) and were consistent with Western blotting of the subcellular fractions of stationary phase BCG, in which Acr1 was detected in all the fractions including the culture supernatant. In contrast, Acr2 was localized almost exclusively around the periphery of the cells and possibly associated with the presumed cell wall (that is seen as electron translucent zone) or on the cell membrane beneath it. Western blotting confirmed this localization with the immuno-reactivity only detected in the cell wall and plasma membrane fractions. Although, it has been reported that Acr2 is a ribosome associated protein and associated with the ribosomal 30S subunit (Ohara et al., 1997; Stewart et al., 2002a; Tabira et al., 2000), the findings in this study are not consistent with this and it is possible that the conclusions in the earlier studies (Ohara et al., 1997; Tabira et al., 2000) resulted from the co-migration of Acr2-containing membrane/cell wall aggregates with ribosomes.

Thus there were clearly differences in the subcellular distribution of the two *M. tuberculosis/M. bovis* (BCG str. Pasteur) α-crystallins. However, their overlapping subcellular localization on the cell wall/periphery of stationary phase bacteria may suggest some level of functional redundancy. Unlike eukaryotic cells where damage is concentrated in the nuclei during heat shock, prokaryote heat shock is associated with the cytoplasmic membrane (Lindquist, 1986). Thus the identification that both Acr1 and Acr2 are associated with the cell wall puts them in the front line against heat shock and other surface stress. The α-crystallins belong to a class of heat shock proteins known as holdases which bind to their client proteins in an ATP-independent manner (Basha et al., 2012). Therefore, their presence
in the cell wall/periphery of the cell is a logical assumption. This ATP-independence also makes sense for these chaperones which are highly expressed in dormant bacilli with a very low metabolic activity.

If there is functional redundancy for Acr1 and Acr2, it was anticipated that deletion of one chaperone would be partially compensated for by the remaining parologue. To test this, we compared isogenic single mutants in acr1 and acr2 and a double mutant deleted of both acr1 and acr2 in several in vitro stresses and during intracellular growth in macrophages. Except for Acr1 in heat shock, deficiency of neither of the two α-crystallins was important for survival in any of the stress conditions tested and no redundancy in function was detected. In retrospect, the obvious omission from these experiments was the inclusion of a cell wall specific stress such as a surfactant. These are known to induce expression of Acr2 specifically via SigE and MprA/B and given the knowledge from the immune-electron microscopy and Western blots that Acr2 is localised almost exclusively in the cell wall, it would be reasonable to suggest that cells deficient in Acr2 may be sensitive to surface stress. Investigating if Acr1, with its lesser localisation in the cell wall, could compensate for any sensitivity to cell surface stress would be an important experiment to determine functional redundancy between the α-crystallins.

In macrophages, loss of acr1 in both the single mutant and in combination with deletion of acr2 led to an elevated bacterial load at 24hr and 48hrs but a reduction in bacterial survival at 84 hrs. This is consistent with previous studies (Yuan et al., 1998) and contrasted with the Δacr2 mutant which survived equally as well as the wild-type throughout the experiment but induced less host cell cytotoxicity than the wild type M. tuberculosis H37Rv.
Taken together, the experiments in this thesis strongly indicate that the two α-crystallins of *M. tuberculosis* H37rv have different functions: they are localised in different parts of the cell and their individual deletion confers different phenotypes during macrophage infection. Importantly, in none of the phenotypic characterisation experiments was there observed a phenotype that was present in the double mutant but not in either single mutant. Thus we have no evidence to support Hypothesis A that “There is some functional redundancy between the two α-crystallins and that one can compensate to some extent for the loss of the other”. However, future experiments, including comparison of the *in vivo* phenotypes of the single and double mutant strains during animal infection and also in other *in vitro* stress conditions, such as surfactant cell surface stress, could yet reveal at least some overlap in function.

The other aspect of this thesis is the transfer-messenger RNA (tmRNA) system of *M. tuberculosis* which has been shown in several bacteria to play an essential role in protein homeostasis in stress conditions (Keiler, 2008; Keiler and Shapiro, 2003; Komine et al., 1994; Moore and Sauer, 2007; Oh and Apirion, 1991). It functions to rescue stalled ribosomes concurrently with tagging the truncated nascent protein to target it for degradation by the stress responsive Clp protease. Of particular interest tmRNA mediated trans-translation may play a role in the expression of major Hsp/chaperones in the actinomycete *Streptomyces coelicolor* (Barends et al., 2010) and in *M. tuberculosis* is reported to play a role in its persistence (Shi et al., 2011). However, at the beginning of the present project there was little known about tmRNA in Mycobacteria because there was no description in the literature of the *M. tuberculosis* tmRNA target proteins and no study of the phenotype of
tmRNA null mutants. This information may be useful in the identification of new therapeutic targets against TB. Therefore, this study was designed to test the following hypothesis:

**Hypothesis B**
Transfer messenger RNA (tmRNA) is involved in the translation of stress proteins during the stress response

To test Hypothesis B that “Transfer messenger RNA (tmRNA) is involved in the translation of stress proteins during the stress response”, it was attempted to delete the ssrA gene which encodes tmRNA from *M. tuberculosis* H37Rv. In the present study, it was not possible to delete *ssrA* unless a second functional copy of *ssrA* was provided elsewhere on the genome. This suggested that tmRNA is an essential molecule for *M. tuberculosis* H37Rv. It was not possible to delete the *ssrA* when a second recombinant was provided with the protease tag coding sequence replaced with sequence encoding a run of histidines (*ssrA*9His). This suggested that tmRNA is essential in *M. tuberculosis* H37rv and moreover that the protease tagging function of tmRNA is an essential function. Since starting this project, a paper was published (Personne and Parish, 2014) that similarly describes experiments that indicate that tmRNA is essential although in this case the authors provided evidence that the protease tagging function was not an essential role. This discrepancy between conclusions is discussed further in Chapter 3, but the most important finding is the essentiality of tmRNA as this highlights this molecule as a potential target for anti-mycobacterial drugs. Indeed, it has been revealed that tmRNA is a target for pyrazinamide (Shi et al., 2011; Yang et al., 2015)
To try to reveal the protein substrate targets for tmRNA and to determine if it is directly involved in translation of stress induced molecular chaperones as observed in the related actinomycete, Streptomyces (Barends et al., 2010) we generated merodiploid strains of mycobacteria that expressed the $ssrA^{His}$ genes in addition to the wild-type $ssrA$. The intention was to generate recombinant strains in which a proportion of the tmRNA protein substrates would be tagged with histidines instead of the Clp protease recognition tag. Subsequently, the His tagged proteins could be purified and identified as performed in Streptomyces (Barends et al., 2010). Despite an exhaustive array of experiments with different $ssrA^{His}$ expression constructs and recombinant mycobacterial strains, the investigations failed to identify any His-labelled proteins. Experiments with some of the $ssrA^{His}$ recombinant strains suggested that expression of the $ssrA^{His}$ inhibited bacterial growth. Thus, the experiments described do not provide any evidence to support Hypothesis B that the mycobacterial tmRNA is directly involved in the translation of stress proteins as demonstrated in Streptomyces.

Further work is clearly needed to elucidate the role of tmRNA. Analysis of the accumulation of tagged proteins in $ssrA^{DD}$ and $ssrA^{His}$ expressing mycobacterial strains will help to understand the contrasting results reported in this and in the study of Personne et al 2014. In addition, research should incorporate analysis of the interaction of tmRNA with the translation machinery, possibly with an affinity tagged tmRNA to pull down interacting proteins and RNAs. Experiments examining the interactions between tmRNA, toxin-antitoxin systems and ClpP may also help to understand the role of tmRNA in the growth regulation of mycobacteria.
6.1 Conclusion

In conclusion although both acr1 and acr2 are important for Mycobacterium tuberculosis infection, in this study they were shown to be localised in different parts of the cell and individual deletion did confer a different phenotype in macrophage infection. As the double mutant did not show a different phenotype from the individual deletion in the different characterisation experiments therefore, there is no evidence to support the hypothesis that “There is some functional redundancy between the two α-crystallins and that one can compensate to some extent for the loss of the other”

As regards to the deletion of the ssrA, it was not possible to delete it unless a second functional copy was present elsewhere in the genome which agreed with what has since been described in literature. However, the findings of this study disagree with the literature as regards to the essentiality of the degradation tagging. Whilst Personne et al 2014 are of the opinion that tagging is not essential, the findings of this study has led us to believe that it is. However, there is insufficient evidence to support hypothesis B “Transfer messenger RNA (tmRNA) is involved in the translation of stress proteins during the stress response”. Therefore, further work is recommended for fully elucidate the role of tmRNA in transl-translation of stress proteins.
7. References


Baranov, P.V., B. Westergaard, T. Hamelryck, R.F. Gesteland, J. Nyborg, and J.F. Atkins. 2006. Diverse bacterial genomes encode an operon of two genes, one of which is an
unusual class-I release factor that potentially recognizes atypical mRNA signals other than normal stop codons. *Biology direct*. 1:28.


Study the Persistence of *Mycobacterium tuberculosis* Using Vitamin D(3) and Retinoic Acid Activated THP-1 Macrophages. *Front Microbiol.* 2:67.


Guidice, E., K. Mace, and R. Gillet. 2014. Trans-translation exposed: understanding the structures and functions of tmRNA-SmpB. Frontiers in Microbiology. 5.


Kumar, A., J.C. Toledo, R.P. Patel, J.R. Lancaster, and A.J.C. Steyn. 2007. Mycobacterium tuberculosis DosS is a redox sensor and DosT is a hypoxia sensor.


Shrivastava, R., A.K. Ghosh, and A.K. Das. 2009. Intram- and intermolecular domain interactions among novel two-component system proteins coded by Rv0600c,
Rv0601c and Rv0602c of \textit{Mycobacterium tuberculosis}. \textit{Microbiology}. 155:772-779.


8. Appendices
Appendix 8.1. pH repeat experiment graphs

Growth/survival of wild type *M. tuberculosis* H37Rv, *M. tuberculosis* H37Rv Δacr1, *M. tuberculosis* H37Rv Δacr2 and *M. tuberculosis* H37Rv Δacr1Δacr2 and their complemented strains except *M. tuberculosis* H37Rv Δacr1 comp in different pH. The different strains incubated in Roisin’s medium at pH 6.6 and 4.4 for 7 days and cell viability assessed by plating on 7H11 and counting cfus. Three biological replicates for each. Error bars represent standard error of the mean of three independent replicates. Statistical significance was assessed by Tukey’s multiple comparisons test.
Growth/survival of wild type *M. tuberculosis* H37Rv, *M. tuberculosis* H37rv Δacr1, *M. tuberculosis* H37rv Δacr2 and *M. tuberculosis* H37rv Δacr1Δacr2 and their complemented strains except *M. tuberculosis* H37rv Δacr1 comp in different pH. The different strains incubated in Roisin’s medium at pH 6.6 and 4.4 for 7 days and cell viability assessed by plating on 7H11 and counting cfus. Survival is shown as percentage of the input count for each strain. Three biological replicates for each. Error bars represent standard error of the mean of three independent replicates. Statistical significance was assessed by Tukey’s multiple comparisons test.
Appendix 8.2. H$_2$O$_2$ repeat experiment graphs

Wild type *M. tuberculosis* H37Rv, *M. tuberculosis* H37rv Δacr1, *M. tuberculosis* H37rv Δacr2 and *M. tuberculosis* H37rv Δacr1Δacr2 and their complemented strains except *M. tuberculosis* H37rv Δacr1 comp were grown to mid log phase, washed and diluted to an OD of 0.4. The different strains were incubated for two hours in 0mM 10mM and 20mM H$_2$O$_2$. A sample was taken at different time points for viability counting on 7H11 media. Three biological replicates for each. Error bars represent standard error of the mean of three independent replicates. Statistical significance was assessed by Tukey’s multiple comparisons test.
Wild type *M. tuberculosis* H37Rv, *M. tuberculosis* H37Rv Δacr1, *M. tuberculosis* H37Rv Δacr2 and *M. tuberculosis* H37Rv Δacr1Δacr2 and their complemented strains except *M. tuberculosis* H37Rv Δacr1 comp were grown to mid log phase, washed and diluted to an OD of 0.4. The different strains were incubated for two hours in 0mM, 10mM and 20mM H₂O₂. A sample was taken at different time points for viability counting on 7H11 media. Survival is shown as percentage of survival for each strain in Roisin’s medium alone. Three biological replicate for each. Error bars represent standard error of the mean of three independent replicates. Statistical significance was assessed by Tukey’s multiple comparisons test.
Appendix 8.3. THP-1 repeat experiment graphs

Viable bacterial counts from THP-1 macrophages infected at MOI of 0.1 with *M. tuberculosis* H37Rv, *M. tuberculosis* H37Rv Δacr2, *M. tuberculosis* H37Rv Δacr1Δacr2 and *M. tuberculosis* H37Rv Δacr1Δacr2 and their complemented strains except *M. tuberculosis* H37Rv Δacr1comp. Three technical replicates. Error bars represent standard error of the mean of three independent replicates. Statistical significance was assessed by Tukey’s multiple comparisons test.
Number of THP-1 macrophages assessed by crystal violet staining in in vitro infections at MOI of 0.1 with *M. tuberculosis* H37Rv, *M. tuberculosis* H37rv Δacr1, *M. tuberculosis* H37rv Δacr2 and *M. tuberculosis* H37rv Δacr1Δacr2 and their complemented strains except *M. tuberculosis* H37rv Δacr1 comp. The error bars are the standard error of the mean.
Time hours

- WT *M. tuberculosis* H37Rv
- *M. tuberculosis* H37Rv Δacr2
- *M. tuberculosis* H37Rv Δacr1Δacr2
- *M. tuberculosis* H37Rv Δacr2 comp
- *M. tuberculosis* H37Rv Δacr1Δacr2 comp
Appendix 8.4. Hypoxia repeat experiment

The graph shows the result of microaerophilic/hypoxia stress on the different M. tuberculosis H37rv strains. The different M. tuberculosis H37rv strains were grown in 7H9 containing 10% ADC and 0.1% Tween80 until mid-log phase. The cultures were adjusted to the same OD. Replicate 1 ml volumes of the respective cultures were distributed into 2ml O-ringed screw capped tubes and layered with 0.5ml of sterile mineral oil. The tubes were incubated still at 37°C for 30 days. Results show the mean of triplicate cultures at each time point. Error bars represent standard error of the mean of three independent replicates. Statistical significance was assessed by Tukey’s multiple comparisons test.
Appendix 8.5. Heat shock repeat experiment

The different strains were incubated for two hours in 53°C. A sample was taken at different time points for viability count by plating on 7H11 media and counting cfu after 21 days. Two technical replicate for each. Error bars show the standard error of the mean. The threshold of detection is 167 cfu/ml.
Appendix 8.6. Checking *M. smegmatis* groEL1ΔC

To check that the histidine at the C-terminal of the GroEL was actually removed from the strain received, the PCR primers were designed 65bp upstream (forward primer) and 196bp down stream (reverse primer) amplify inward across GroEL1 gene to give a product size of 1842bp. As it can be seen in the gel picture (Figure 62) the right size fragment was amplified.

Figure 61: GroEL1 down and up-stream schematic diagram

Schematic diagram representing *M. smegmatis* groEL1ΔC genome showing the annealing sites of the two primers used to amplify inward across the GroEL1 gene for sequencing. The successful amplification should produce a PCR fragment of 1842bp.

Figure 62: *M. smegmatis* groEL1ΔC PCR fragment

Gel (1% agarose, TAE) picture shows the PCR products amplified by using primers xxx that were designed to anneal outside the flaking regions of *M. smegmatis* groEL1ΔC genes inward across it. The expected 1824bp PCR products is shown.
The amplified PCR product was sent for sequencing and the obtained sequence confirmed the deletion of the histidine rich region of the *M. smegmatis* groEL1ΔC GroEL1 C-terminal

**WT Mycobacteria smegmatis GroEL1 sequence**

```
GCCTCGACGGTTCCGTCGTGGTGAACAAGGTCTCCGAACTTCCCAAGGGGCAG
GGCTTCACGCCGCCACACGTGGATTGCCTCTCCGGCGCGTCTGC
CGATCCGCGCAAGGTTGCCCTCGGCTTTCATCAACGCCGCTCGGGCTGCTGGAGTTCGGTGATCTGGTCTCCGCCGGC
CGATCCGGCCAAGGGCAGGGCTTCAACGCCGCCACGCTGGAGTTCGGTGATCTGGTCTCCGCCGGC
```

PADEDEHGHGHHGHHAHStop

**M. smegmatis groEL1ΔC obtained sequence**

```
CGGCTCCGCTCTACTGGATCGCCACCAACGCGCGGCTCGACGGTTCCGTCGTG
GTAAACAAGGTCTCCGAACCTTCACAAAGGCGAGGGCTTCAACGCACGCCACGCT
GGAGTTGCTGATCTGGCTCCTCCGGCGCCGCTCGGATCCGGCCAAGGGTGAACC
GCTCGGCTGTTTCTCAACGCCGCTCAGCCTGGTCGGCCCGATGATCTCTAGACGACGGAG
ACCGCCGTCGACAAAGCGGCGCGACGAGGACGAGTCTAGAACCAGGTGTATC
CTAAAT
```

PADEDESRTGVSStop

*Figure 63 M. smegmatis groEL1ΔC sequence*

A segment of the C-terminal sequence of GroEL1 ΔC seen in red after being sequenced compared to the WT *M. smegmatis* GroEL1 sequence.

The sequencing confirmed that the C-terminal histidine region was indeed deleted from the GroEL of the received strain. Although *M. smegmatis* groEL1ΔC parent strain did not have histidine rich C-terminal, the western results suggested there is still some residual Histidine rich proteins that are in adequate concentration to give positive bands.
Appendix 8.7. *acr2* deletion sequence confirmation

Alignment output for *M. tuberculosis Δacr2* vs. WT *M. tuberculosis*

```plaintext
# bin/lalign36 -E 10.0 -f -12 -g -2 ./wwwtmp/lalign26729.1.seq ./wwwtmp/lalign26729.2.seq -J -K 3
LALIGN finds non-overlapping local alignments
version 36.3.5e Nov, 2012 (preload8)
Please cite:
Parameters not available for: +5/-4: -12/-2
Query: ./wwwtmp/lalign26729.1.seq
Library: ./wwwtmp/lalign26729.2.seq
1260 residues in 1 sequences
Statistics: (shuffled [500]) MLE statistics: Lambda= 0.0418; K=0.01246 statistics sampled from 1 (1) to 500 sequences
Threshold: E() < 10 score: 178
Algorithm: Smith-Waterman (SSE2, Michael Farrar 2006) (7.2 Nov 2010)
Parameters: +5/-4 matrix (5:-4), open/ext: -12/-2
Scan time:  0.340
>>WT M. tuberculosis 1260 bp                              (1260 nt)
Waterman-Eggert score: 1905;  121.3 bits; E(1) <  4.2e-31
89.5% identity (89.5% similar) in 486 nt overlap (578-100:1-472)

KO     TCCGCAGCGACCACGCACGCCGCTCAGCGACCCGTCCGCGTCACGGGCACGCACCGCTTCGACCAGCCGATGGCCCACCA
:::::::::::::::::::::::::::::::::::::::::::::::
WT     TCCGCAGCGACCACGCACGCCGCTCAGCGACCCGTCCGCGTCACGGGCACGCACCGCTTCGACCAGCCGATGGCCCACCA

KO     TGCCGTGGCCGACCACGACGATCTCGCGAG
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
WT     TGCCGTGGCCGACCACGACGATCTCGCGAGCCGCGGCAGGGCGCGCGGAACTCCCAGGC

KO     TAGGCATGTCGTGAGGTTATGAGGGGGCAATTACCGAAAATCAGCCAATGTGACGCCACA
:::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
WT     TAGGCATGTCGTGAGGTTATGAGGGGGCAATTACCGAAAATCAGCCAATGTGACGCCACA

KO     TATGACGCTGTGATCACACCTGGCGACCGGCTGTGTGACAGTGTTCGCTTCCGGTGAACG
::::::::::::::::::::::::::::::::::::::::::::::::
WT     TATGACGCTGTGATCACACCTGGCGACCGGCTGTGTGACAGTGTTCGCTTCCGGTGAACG

KO     CCGAGGTGGAACTTAAGCGTGGTCGACTCAG
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
WT     CCGAGGTGGAACTTAAGCGTGGTCGACTCAGGTTCTTGGTATAGCGGGCCGGCAACGCG

KO     CGGACACAGCTAACCCTGCTTCCGGTGAGGAGATAACCATGAACAATCTCGCATTGTG
```

237
The bases in red are the last few bases upstream of the \textit{acr2} after which the hygromycin gene replaced the \textit{acr2} gene indicating the successful gene replacement.

\textbf{Mycobacterium tuberculosis H37Rv, complete genome}

Sequence ID: ref\textit{|NC_000962.3|} Length: 4411532 Number of Matches: 1

\begin{table}[h]
\begin{tabular}{|c|c|c|c|c|}
\hline
Score & Expect & Identities & Gaps & Strand \\
\hline
134 bits(72) & 5e-29 & 72/72(100\%) & 0/72(0\%) & Plus/M \\
\hline
\end{tabular}
\end{table}

Features:
\begin{itemize}
\item heat shock protein
\end{itemize}

Query 1  \texttt{CGGACACAGCTAACTCAACACGGAACGATGAGGAGATAACCATAAATCTCGCATTTGG}  60

Sbjct 302692  \texttt{CGGACACAGCTAACTCAACACGGAACGATGAGGAGATAACCATAAATCTCGCATTTGG}  302633

Query 61  \texttt{GTCGGGTCCCGT}  72

Sbjct 302632  \texttt{GTCGGGTCCCGT}  302621

These are the bases seen in red in the alignment above confirming the presence of the \textit{acr2} in the WT \textit{M. tuberculosis} and its absence from \textit{M. tuberculosis} \textit{\Deltaacr2}.
Alignment output for *M. tuberculosis Δacr2* vs. WT *M. tuberculosis*

```
# bin/lalign36 -E 10.0 -f -12 -g -2 ./wwwtmp/lalign4352.1.seq ./wwwtmp/lalign4352.2.seq -J -K 3
LALIGN finds non-overlapping local alignments
version 36.3.5e Nov, 2012(preload8)
Please cite:

Parameters not available for: +5/-4: -12/-2
Query: ./wwwtmp/lalign4352.1.seq
Library: ./wwwtmp/lalign4352.2.seq
1260 residues in 1 sequences

Statistics: (shuffled [500]) MLE statistics: Lambda= 0.0482; K=0.008354
statistics sampled from 1 (1) to 500 sequences
Threshold: E() < 10 score: 146
Scan time: 0.370

>>WT M. tuberculosis 1260 bp (1260 nt)
Waterman-Eggert score: 2357; 170.7 bits; E(1) < 5.4e-46
88.2% identity (88.2% similar) in 618 nt overlap (3-613:652-1260)

<table>
<thead>
<tr>
<th>Query</th>
<th>Library</th>
</tr>
</thead>
<tbody>
<tr>
<td>KO</td>
<td>WT</td>
</tr>
</tbody>
</table>

KO: GCCCGCGAACCTGCTCGCC

WT: GCGTGAGATCCGCTACGGATCA

KO: GCTGGATCTCTC

WT: AGCGAGGCCATCGCGGCTTC

KO: CCGG

WT: GCGCCTACAAGGCCCCAGGCCGAAACTCAGG

KO: CGCGATACGGCATGATCCG

WT: CGCGATACGGCATGATCCGGCCGGGCCGATGGGTCCGCCGGATCATGCCATTGGGCCGAG

KO: TAGACCCAACGACTCCGTCCGTCGATAGCTAGTATCGGGAGCGTACTGTGATCTGTAACA

WT: TAGACCCAACGACTCCGTCCGTCGATAGCTAGTATCGGGAGCGTACTGTGATCT

KO: CCGCCAGCCCAGGGAGCACCCATTGTCCACGACTGCCGAACTCGCCGAACTGCACGACCT

WT: CCGCCAGCCCAGGGAGCACCCATTGTCCACGACTGCCGAACTCGCCGAACTGCACGACCT

KO: TAGACCCAACGACTCCGTCCGTCGATAGCTAGTATCGGGAGCGTACTGTGATCTGTAACA

WT: TAGACCCAACGACTCCGTCCGTCGATAGCTAGTATCGGGAGCGTACTGTGATCT

KO: CCGCCAGCCCAGGGAGCACCCATTGTCCACGACTGCCGAACTCGCCGAACTGCACGACCT

WT: CCGCCAGCCCAGGGAGCACCCATTGTCCACGACTGCCGAACTCGCCGAACTGCACGACCT
```
The bases in red are the few bases down stream of acr2 after the sequence of hygromycin gene that replaced the acr2 gene indicating the successful gene replacement.

**Mycobacterium tuberculosis H37Rv, complete genome**

Sequence ID: ref|NC_000962.3| Length: 4411532 Number of Matches: 4

<table>
<thead>
<tr>
<th>Score</th>
<th>Expect</th>
<th>Identities</th>
<th>Gaps</th>
<th>Strand</th>
</tr>
</thead>
<tbody>
<tr>
<td>56.0 bits(28)</td>
<td>3e-06</td>
<td>28/28(100%)</td>
<td>0/28(0%)</td>
<td>Plus/W</td>
</tr>
</tbody>
</table>

Features:
- heat shock protein

Query 1:  
<table>
<thead>
<tr>
<th>CGCAGCGCATCGCCATCAGGAAGTAGTT</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Sbjct 302198:  
| CGCAGCGCATCGCCATCAGGAAGTAGTT    | 302171 |

These are the bases seen in red in the alignment above confirming the presence of the acr2 in the WT M. tuberculosis and its absence from M. tuberculosis Δacr2
Appendix 8.8. pK.\textit{smpB-Rv3099c-ssrA} construct map

\begin{center}
\includegraphics[width=0.8\textwidth]{construct_map.png}
\end{center}

pK-\textit{smpB-Rv3099c-ssrA} construct map showing the \textit{smpB-Rv3099c-ssrA}^{9\text{His}} gene complex cloned at the \textit{SacI} site. This construct was used to produce \textit{smpB-Rv3099c-ssrA}^{9\text{His}} which then was introduced into \textit{M. smegmatis groEL1ΔC} parental strain and \textit{M. smegmatis groEL1ΔC Δon} to produce \textit{smpB-Rv3099c-ssrA}^{9\text{His}} merodiploid strains.
Appendix 8.9. pMV361-\textit{smpB-Rv3099c-ssrA}^{9\text{His}}\text{ construct map}

\text{pmv361-\textit{smpB-Rv3099c-ssrA}^{9\text{His}}} construct map showing the \textit{smpB-Rv3099c-ssrA}^{9\text{His}} gene complex directionally cloned at \text{HindIII} and \text{EcoRI} sites. This construct was introduced into \textit{M. smegmatis} \text{groEL1\text{AC}} parental strain and \textit{M. smegmatis} \text{groEL1\text{AC} \text{A}lon} to produce \textit{smpB-Rv3099c-ssrA}^{9\text{His}} merodiploid strains.
Appendix 8.10. \textit{ssrA}^{6\text{His}} sequence comparison with \textit{ssrA}^{9\text{His}}

The sequence to confirm the successful mutagenesis to restore the arginine and glutamine and produce the \textit{ssrA}^{6\text{His}} variant.