Determining the effect of treatment with an exogenous host defence peptide on *Mycobacterium marinum*-zebrafish infection

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Declaration of Originality

I declare that all work presented in this thesis is my own, and that any ideas, information, data, results and figures from another source or from collaborations have been appropriately referenced or acknowledged.

Rebecca Louise Price
6th June 2017

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Abstract

We urgently require novel antimicrobial therapies for TB infections as a third of the world’s population is infected with latent TB and cases of multidrug-resistant TB are increasing. Naturally occurring host defence peptides (HDPs), components of the innate immune system, provide protection against infections in vivo through their direct antimicrobial and immunomodulatory functions. The effect of one HDP, bovine bactenecin-5 (Bac5), on mycobacterial infections was investigated in this study using the M. marinum-zebrafish TB infection model. Bacterial two-component systems (TCSs) regulate gene expression in response to external environmental changes, controlling physiological processes including virulence. Generation of TCSs deletion mutants in Mycobacterium marinum was attempted, unsuccessfully, by mycobacteria-adapted recombineering and sucrose counter-selection, with a view to studying the effects of targeting these systems during mycobacterial infections using zebrafish as a model organism.

The M. marinum-zebrafish embryo model permits the monitoring of pathogenesis in real time through live-imaging. Bacterial Distance Distribution and Fluorescent Pixel Count protocols were developed or optimised in Icy to quantify bacterial dissemination and bacterial burden from images of zebrafish embryo infections using fluorescently labelled bacterial strains. These techniques, coupled with zebrafish leukocyte recruitment quantification using transgenic zebrafish lines and our image processing protocol, permit simultaneous analysis of multiple infection progression parameters during longitudinal studies.

Injection with exogenous Bac5 alone and co-injection with M. marinum led to a significant initial increase in macrophage recruitment to the zebrafish injection site. We also observed trends following injections of zebrafish embryos with Bac5 for increased initial neutrophil recruitment, increased expression of the chemokine cxcl-c1c and pro-inflammatory cytokines IL-1β and MMP-9, and reduced M. marinum burdens. These results provide a basis for further investigations into the efficacy of Bac5 treatment of mycobacterial infections, in particular the optimal timings and dosage, with a view to using this exogenous HDP to treat human TB infections.
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## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>Ac</td>
<td>Acetamide</td>
</tr>
<tr>
<td>AES</td>
<td>Allelic Exchange Substrate</td>
</tr>
<tr>
<td>Bac5</td>
<td>Bactenecin-5</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony Forming Unit</td>
</tr>
<tr>
<td>CO</td>
<td>Carbon monoxide</td>
</tr>
<tr>
<td>CRAMP</td>
<td>Cathelin-related antimicrobial peptide</td>
</tr>
<tr>
<td>DCs</td>
<td>Dendritic Cells</td>
</tr>
<tr>
<td>dpf</td>
<td>Days post fertilisation</td>
</tr>
<tr>
<td>dpi</td>
<td>Days post injection</td>
</tr>
<tr>
<td>DR</td>
<td>Downstream homology Region</td>
</tr>
<tr>
<td>DsRed</td>
<td><em>Discosoma</em> Red fluorescent protein</td>
</tr>
<tr>
<td>eGFP</td>
<td>Enhanced Green Fluorescent Protein</td>
</tr>
<tr>
<td>EPI</td>
<td>Epinecidin-1</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-related kinase</td>
</tr>
<tr>
<td>FPC</td>
<td>Fluorescent Pixel Count</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>HBV</td>
<td>Hindbrain ventricle</td>
</tr>
<tr>
<td>HDP</td>
<td>Host Defence Peptide</td>
</tr>
<tr>
<td>HygR</td>
<td>Hygromycin resistance gene</td>
</tr>
<tr>
<td>IDR</td>
<td>Innate Defence Regulator</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>LL-37</td>
<td>Human cathelicidin</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Broth</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LTBI</td>
<td>Latent tuberculosis infection</td>
</tr>
<tr>
<td>LysC</td>
<td>Lysozyme C</td>
</tr>
<tr>
<td>MCETs</td>
<td>Mast Cell Derived Extracellular Traps</td>
</tr>
<tr>
<td>MCP</td>
<td>Macrophage Chemotactic Protein</td>
</tr>
<tr>
<td>MDR</td>
<td>Multidrug-resistant</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum Inhibitory Concentration</td>
</tr>
<tr>
<td>Mmar</td>
<td><em>Mycobacterium marinum</em></td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
</tr>
<tr>
<td>Mpeg-1</td>
<td>Macrophage-expressed gene 1</td>
</tr>
<tr>
<td>Mpx</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>Msm</td>
<td><em>Mycobacterium smegmatis</em></td>
</tr>
<tr>
<td>MTBC</td>
<td><em>Mycobacterium tuberculosis</em> complex</td>
</tr>
<tr>
<td>NETs</td>
<td>Neutrophil Extracellular Traps</td>
</tr>
<tr>
<td>NfkB</td>
<td>Nuclear Factor Kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NK cell</td>
<td>Natural Killer cell</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>NRP</td>
<td>Non-replicating persistence</td>
</tr>
<tr>
<td>OADC</td>
<td>Oleic acid albumin dextrose catalase supplement</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative Real-Time PCR</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol-12-myristate-13-acetate</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of Interest</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>RR</td>
<td>Response Regulator</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe Combined Immunodeficiency</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>SK</td>
<td>Sensor Kinase</td>
</tr>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>TCS</td>
<td>Two-component signal transduction system</td>
</tr>
<tr>
<td>Tg</td>
<td>Transgenomic</td>
</tr>
<tr>
<td>Th1/Th2</td>
<td>Type 1/2 T-helper cells</td>
</tr>
<tr>
<td>THP-1</td>
<td>Human monocytic leukaemia cell line</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>UR</td>
<td>Upstream homology Region</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
</tbody>
</table>
Table of Contents

Declaration of Originality ................................................................................................................. 2
Copyright Declaration ......................................................................................................................... 2
Abstract ............................................................................................................................................. 3
Acknowledgements ............................................................................................................................ 4
Funding ................................................................................................................................................ 4
Abbreviations ..................................................................................................................................... 5
Table of Contents ............................................................................................................................... 7
List of Figures ...................................................................................................................................... 13
List of Tables ....................................................................................................................................... 17

1 Introduction .................................................................................................................................... 19

1.1 Mycobacterium tuberculosis and Tuberculosis (TB) ................................................................. 19

1.1.1 M. tuberculosis, TB, latent TB and multi-drug resistant (MDR) TB ................................. 19

1.1.2 M. tuberculosis lung pathogenesis and granuloma formation ........................................... 20

1.1.3 Requirement for novel therapies and vaccines ................................................................. 24

1.2 TB infection models .................................................................................................................... 26

1.2.1 Established TB infection models ....................................................................................... 26

1.2.2 The M. marinum-zebrafish model ..................................................................................... 28

1.2.2.1 M. marinum as a model for M. tuberculosis pathogenesis ........................................... 28

1.2.2.2 Zebrafish as a model organism for studying infections and drug treatments 29

1.2.2.3 Insights into mycobacterial pathogenesis and granuloma formation from the M. marinum-zebrafish model ........................................................................................................... 31

1.3 The assessment of M. marinum infection progression in THP-1 cells and the zebrafish .................................................................................................................................................. 33

1.3.1 Assessment of infection progression in THP-1 cells .......................................................... 33

1.3.2 Quantification of infection progression in the M. marinum-zebrafish model ............... 34

1.3.2.1 Quantification of infected zebrafish bacterial burden ............................................... 35

1.3.2.2 Quantification of leukocyte recruitment to the infection site .................................... 36

1.3.2.3 Quantification of bacterial dissemination in infected zebrafish ................................ 36

1.4 Host defence peptides (HDPs) .................................................................................................... 37

1.4.1 Introduction to HDPs ........................................................................................................... 37

1.4.2 Mammalian and teleost HDPs – cathelicidins, piscidins and synthetic innate defence regulator (IDR) peptides ........................................................................................................ 38

1.4.3 General properties of HDPs ............................................................................................... 39
### 1.4.4 Mechanisms of action of HDPs ........................................................................ 41
  1.4.4.1 Direct antimicrobial activities of HDPs ......................................................... 41
  1.4.4.2 Immunomodulatory activities of HDPs ......................................................... 42
  1.4.4.3 Studies of the protective effects of immunomodulation by HDPs against infections in vivo ......................................................................................... 46

### 1.4.5 HDPs as novel therapeutics for TB infections ....................................................... 49
  1.4.5.1 Advantages and limitations of HDPs as novel therapeutics ........................... 49
  1.4.5.2 Cathelicidins as novel therapeutics for TB infections ................................... 52

### 1.4.6 HDPs selected for study .................................................................................... 55

### 1.5 Bacterial two-component signal transduction systems (TCSs) .............................. 56
  1.5.1 Introduction to bacterial TCSs and their general properties ............................. 56
  1.5.1.1 Regulation of TCSs ....................................................................................... 57
  1.5.1.2 Specificity and cross-talk in TCSs ................................................................. 58
  1.5.1.3 *M. tuberculosis* TCSs ................................................................................ 59
  1.5.1.4 The DosR/S/T TCSs of *M. tuberculosis* – hypoxia adaptation and latency ..... 60

### 1.5.2 TCSs as targets in novel therapies ................................................................... 61

### 1.5.3 TCSs selected for study .................................................................................... 64

### 1.6 Aims of this study ............................................................................................... 70

#### 2 Materials and methods ....................................................................................... 71

### 2.1 General materials and methods ......................................................................... 71
  2.1.1 Bacterial strains and plasmids ......................................................................... 71
  2.1.2 Primers ............................................................................................................ 73
  2.1.3 qRT-PCR probes ............................................................................................ 78
  2.1.4 Kits ................................................................................................................. 78
  2.1.5 Chemicals and reagents ................................................................................. 79
  2.1.6 Enzymes ........................................................................................................ 80
  2.1.7 Zebrafish lines ............................................................................................... 81

### 2.2 Microbiology methods ....................................................................................... 82
  2.2.1 Bacterial culture conditions and antibiotics .................................................... 82
  2.2.2 Optical density (OD) measurement .................................................................. 82
  2.2.3 Growth curves ............................................................................................... 83
  2.2.4 Passaging to investigate rate of pJV53 loss .................................................... 83
  2.2.5 96-well plate host defence peptides (HDP) treatments assay ......................... 83

### 2.3 Molecular biology methods ................................................................................. 84
  2.3.1 Competent bacteria ....................................................................................... 84
3 Optimisation of techniques for the assessment of *M. marinum* infection progression in THP-1 cells and the zebrafish infection model................................................. 99
3.1 Introduction...........................................................................................................................................99
3.2 Aims.........................................................................................................................................................99
3.3 Results.....................................................................................................................................................100
  3.3.1 Optimisation of THP-1 cell infection with *M. marinum* and quantification of infection progression ..................................................................................................................100
    3.3.1.1 Overview......................................................................................................................................100
    3.3.1.2 Optimisation of THP-1 cell seeding density for 96-well plate assays.................................100
    3.3.1.3 Optimisation of multiplicity of infection (MOI) for *M. marinum* infection of THP-1 cells ..................................................................................................................................100
    3.3.1.4 Optimisation of qRT-PCR sampling of THP-1 cells for 96-well plate assays 102
  3.3.2 Summary of zebrafish infection model optimisations performed during MRes rotation 104
  3.3.3 Visualisation of initial interactions between infecting bacilli and the zebrafish immune system following HBV injection.........................................................................................................................104
  3.3.4 Quantification of zebrafish infection progression from fluorescence images using the bioimage informatics platform Icy ........................................................................................................................................106
    3.3.4.1 Overview......................................................................................................................................106
    3.3.4.2 Quantification of infected zebrafish bacterial burden..............................................................106
    3.3.4.3 Determination of *M. marinum* dosage for zebrafish infection experiments.115
    3.3.4.4 Development of an image processing method to permit simultaneous analysis of bacterial burden and leukocyte recruitment in the HBV region .........................118
    3.3.4.5 Quantification of zebrafish leukocyte recruitment to the HBV injection site.....
      ..........................................................................................................................................................120
    3.3.4.6 Quantification of bacterial dissemination in infected zebrafish by development of a bacterial distance distribution protocol.................................................................................126
  3.3.5 Quantification of zebrafish immunity gene expression in response to *M. marinum* infection by qRT-PCR ........................................................................................................................................131
  3.4 Discussion................................................................................................................................................132

4 Investigating the effect of selected host defence peptides (HDPs) on *M. marinum* infection progression in THP-1 cells and the zebrafish.............................................137
  4.1 Introduction............................................................................................................................................137
  4.2 Aims.........................................................................................................................................................137
  4.3 Results.....................................................................................................................................................138
    4.3.1 Selected HDPs demonstrate direct bacteriocidal activity *in vitro* ...........................................138
    4.3.2 Effect of HDPs on *M. marinum* infection progression in THP-1 cells ........................................139
      4.3.2.1 Effect of HDPs on host immunity gene expression of infected and uninfected THP-1 cells..................................................................................................................................................139
4.3.3 Effect of Bac5 on *M. marinum* infection progression in zebrafish following Bac5 and *M. marinum* co-injection to the HBV.............................................................140

4.3.3.1 Effect of Bac5 on macrophage recruitment and host immunity gene expression following co-injections to the zebrafish HBV.............................................................141

4.3.3.2 Effect of Bac5 on neutrophil recruitment and bacterial burden following co-injections to the zebrafish HBV................................................................................152

4.3.4 Effect of Bac5 on *M. marinum* infection progression in zebrafish following sequential injections of *M. marinum* and Bac5 to the HBV ...............................................................166

4.3.4.1 Effect of Bac5 on neutrophil recruitment and bacterial burden following sequential injections to the zebrafish HBV................................................................................166

4.3.4.2 Effect of Bac5 on host immunity gene expression following sequential injections to the zebrafish HBV................................................................................172

4.4 Discussion ............................................................................................................175

4.4.1 Bac5 lacks direct antimycobacterial activities ....................................................175

4.4.2 Bovine Bac5 is capable of immunomodulation of human THP-1 cells independent of *M. marinum* infections ..............................................................................................175

4.4.3 Bovine Bac5 increases initial macrophage recruitment during the early stages of *M. marinum* infection of zebrafish embryos ...........................................................................176

4.4.4 Bovine Bac5 does not affect *M. marinum* infection progression of zebrafish embryos at the early granuloma formation stage .....................................................................180

4.4.5 Implications for Bac5 as a novel TB therapy ....................................................182

5 Generation of TCSs deletion mutants in *M. marinum* ........................................188

5.1 Introduction .......................................................................................................188

5.2 Aims .................................................................................................................188

5.3 Results ............................................................................................................189

5.3.1 Investigation of TCSs relatedness between *M. tuberculosis* and *M. marinum* ......189

5.3.1.1 Overview ...................................................................................................189

5.3.1.2 Investigation of TCSs relatedness between *M. tuberculosis* and *M. marinum*......189

5.3.2 Attempted generation of *M. marinum* ΔTCSs operon mutants by mycobacteria-adapted recombineering .............................................................................................194

5.3.2.1 Overview ...................................................................................................194

5.3.2.2 Mycobacteria-adapted recombineering .......................................................195

5.3.2.3 Characterisation of putative TCSs deletion mutant strains: Overview ...........199

5.3.2.4 Characterisation of putative TCSs deletion mutant strains obtained through initial rounds of recombineering ..................................................................................200

5.3.2.5 Investigation of the efficacy of recombineering in *M. marinum* .......................204
5.3.3 Attempted generation of $M.\text{marinum} \Delta$TCSs operon mutants by sucrose counter-selection ................................................................. 210
  5.3.3.1 Overview ........................................................................................................ 210
  5.3.3.2 Sucrose counter-selection .............................................................................. 211
  5.3.3.3 Characterisation of putative TCSs single crossover strains obtained through sucrose counter-selection ......................................................... 215
  5.3.3.4 Characterisation of putative TCSs double crossover strains obtained through sucrose counter-selection ................................................................. 216
5.3.4 Characterisation of putative $M.\text{marinum} \Delta$DosR/S + USP strains and putative $M.\text{marinum}$ DosR/S AES double crossover strains by Southern Blotting ......................................................... 218
  5.3.4.1 Overview ........................................................................................................ 218
  5.3.4.2 Southern Blotting ......................................................................................... 219
5.4 Discussion ........................................................................................................... 223

6 General Discussion ............................................................................................... 228
6.1 Key findings and significance of this study ......................................................... 228
  6.1.1 Chapter 3 – Optimisation of techniques for the assessment of $M.\text{marinum}$ infection progression in THP-1 cells and the zebrafish infection model ......................................................... 228
  6.1.2 Chapter 4 – Investigating the effect of selected HDPs on $M.\text{marinum}$ infection progression in THP-1 cells and the zebrafish ................................................................. 229
  6.1.3 Chapter 5 – Generation of TCSs deletion mutants in $M.\text{marinum} ................................................................. 230
6.2 Recommendations for further work .................................................................. 230
  6.2.1 Investigating the potential of Bac5 as a novel therapy using THP-1 cells ........ 230
    6.2.1.1 Effect of Bac5 treatment on intracellular replication and intercellular spread of $M.\text{marinum}$ during infection ................................................................. 230
    6.2.1.2 Effect of Bac5 treatment on host gene expression ....................................... 231
    6.2.1.3 Investigation of the mechanism of Bac5 signalling ..................................... 231
  6.2.2 Investigating the potential of Bac5 as a novel therapy using zebrafish ........... 232
    6.2.2.1 Studies using the zebrafish embryo ............................................................ 232
    6.2.2.2 Studies using the zebrafish adult ............................................................... 234
    6.2.2.3 Studies using transgenic zebrafish ........................................................... 235
  6.2.3 Investigating the importance of selected TCSs on virulence ....................... 236
    6.2.3.1 Generation of TCSs mutants for further study ......................................... 236

References ................................................................................................................. 238

Appendices ................................................................................................................. 258
Appendix 1- Maps for plasmids used during this study ........................................... 258
Appendix 2 - Icy protocol scripts ............................................................................. 261
Appendix 3 - Zebrafish images from Bac5 sequential injection experiments............ 268
Appendix 4 - Protein sequence alignments and genomic views for TCSs ............... 274
Appendix 5 – Investigation of the growth and transformation efficiencies of Leicester and London M. marinum M strains................................................................. 284
Appendix 6 – Permissions information for reproduced published material .......... 285

List of Figures

Figure 1.1. The three potential outcomes of infection of the human host with M. tuberculosis................................................................................................................. 21
Figure 1.2. Structure and cellular constituents of the tuberculous granuloma. ........ 23
Figure 1.3. Examples of mycobacterial granulomas in various hosts................. 28
Figure 1.4. Progression of M. marinum infection of zebrafish embryos............... 30
Figure 1.5. Injection sites of zebrafish embryos................................................ 34
Figure 1.6. Structures diversity and classification of natural HDPs.................... 41
Figure 1.7. Immune functions of HDPs and IDRs............................................. 43
Figure 1.8. Genomic organisation and mechanism of action of TCSs.................. 57
Figure 3.1. Trial THP-1 cell infections with M. marinum in 24- and 96-well plates.... 101
Figure 3.2. Trial THP-1 cell infections with M. marinum at an MOI of 1............. 102
Figure 3.3. Resting and PMA-activated THP-1 cell cytokine response to infection with M. marinum................................................................................................................. 103
Figure 3.4. Interactions between zebrafish immune cells and M. marinum......... 105
Figure 3.5. Whole embryo bacterial load counts from dissociation plating 5 dpi with GFP M. marinum................................................................. 107
Figure 3.6. Icy fluorescent pixel count (FPC) protocol used to quantify fluorescence pixels above a user-defined threshold intensity................................................. 108
Figure 3.7. Validation of Icy Fluorescent Pixel Count (FPC) protocol.............. 109
Figure 3.8. FPC quantification of relative M. marinum burden in zebrafish........ 112
Figure 3.9. FPC as an accurate measure of relative M. marinum burden in zebrafish.. 114
Figure 3.10. FPC of relative M. marinum burden in zebrafish 6 hpi.................. 117
Figure 3.11. Quantification of leukocyte recruitment to the HBV region of zebrafish from fluorescence images using Icy Spot Detector plugin............................... 119
Figure 3.12. Leukocyte recruitment timings following HBV infection with *M. marinum*.

Figure 3.13. Verification of macrophage recruitment timings following HBV infection of zebrafish with *M. marinum*.

Figure 3.14. Verification of neutrophil recruitment timings and bacterial burden following HBV infection of zebrafish with *M. marinum*.

Figure 3.15. Icy protocol developed to quantify the number of bacterial clusters and their distance distribution throughout the zebrafish.

Figure 3.16. Quantification of *M. marinum*-zebrafish infection progression using the Icy Bacterial Distance Distribution protocol.

Figure 3.17. qRT-PCR analysis of *M. marinum*-infected zebrafish 24 hpi.

Figure 4.1. Direct bacteriocidal effects of HDPs on *M. marinum* and *E. coli*.

Figure 4.2. Effect of HDPs on THP-1 cell gene expression.

Figure 4.3. Schematic of co-injection experiments to determine the effects of bactenecin-5 (Bac5) on zebrafish macrophage recruitment or host immunity gene expression.

Figure 4.4. Effect of injection of bactenecin-5 (Bac5) to the HBV on zebrafish macrophage recruitment to the HBV region at 3 hpi.

Figure 4.5. Effect of injection of bactenecin-5 (Bac5) to the HBV on zebrafish macrophage recruitment to the HBV region at 24 hpi.

Figure 4.6. Injection of bactenecin-5 (Bac5) to the HBV increases zebrafish macrophage recruitment to the HBV region.

Figure 4.7. Injection of bactenecin-5 (Bac5) to the HBV does not affect zebrafish macrophage recruitment to the HBV region over time.

Figure 4.8. Injection of bactenecin-5 (Bac5) to the HBV does not affect cytokine gene expression levels of uninfected and *M. marinum*-infected zebrafish.

Figure 4.9. Schematic of co-injection experiments to determine the effects of bactenecin-5 (Bac5) on zebrafish neutrophil recruitment or *M. marinum* bacterial burden.

Figure 4.10. Effect of injection of bactenecin-5 (Bac5) to the HBV on zebrafish neutrophil recruitment to the HBV region at 6 hpi.

Figure 4.11. Effect of injection of bactenecin-5 (Bac5) to the HBV on zebrafish neutrophil recruitment to the HBV region at 24 hpi.
Figure 4.12. Effect of injection of bactenecin-5 (Bac5) to the HBV on zebrafish neutrophil recruitment to the HBV region at 72 hpi.

Figure 4.13. Injection of bactenecin-5 (Bac5) to the HBV does not affect zebrafish neutrophil recruitment to the HBV region.

Figure 4.14. Injection of bactenecin-5 (Bac5) to the HBV does not affect zebrafish neutrophil recruitment to the HBV region over time.

Figure 4.15. Injection of bactenecin-5 (Bac5) to the HBV does not affect bacterial burden of localised M. marinum infection.

Figure 4.16. Injection of bactenecin-5 (Bac5) to the HBV does not affect bacterial burden of localised M. marinum infection over time.

Figure 4.17. Schematic of sequential injection experiments to determine the effects of bactenecin-5 (Bac5) on zebrafish neutrophil recruitment or M. marinum bacterial burden.

Figure 4.18. Injection of bactenecin-5 (Bac5) to the HBV after 48 hours of M. marinum infection does not affect zebrafish neutrophil recruitment.

Figure 4.19. Injection of bactenecin-5 (Bac5) to the zebrafish HBV after 48 hours of M. marinum infection does not affect bacterial burden.

Figure 4.20. Effect of injection of bactenecin-5 (Bac5) to the HBV after 48 hours of M. marinum infection on zebrafish survival.

Figure 4.21. Schematic of sequential injection experiments to determine the effects of bactenecin-5 (Bac5) on zebrafish immunity gene expression.

Figure 4.22. Injection of bactenecin-5 (Bac5) to the HBV of M. marinum-infected zebrafish 72 hpi or 90 hpi does not affect zebrafish immunity gene expression.

Figure 5.1. Coding sequence amino acid alignments and genomic context views of DosR/S/T TCSs proteins in M. tuberculosis and M. marinum.

Figure 5.2. Schematic showing the recombineering method.

Figure 5.3. Schematic of putative TCSs deletion mutant characterisation PCRs.

Figure 5.4. Characterisation PCRs for putative TCSs deletion mutants.

Figure 5.5. Characterisation PCRs for putative M. marinum ΔDosR/S + USP mutants.

Figure 5.6. Characterisation PCRs for putative M. marinum ΔDosR/S mutants.

Figure 5.7. Growth of M. smegmatis (Msm) and M. marinum (Mmar) WT and recombineering strains during incubation with acetamide (ac).
| Figure 5.8 | Transformation efficiencies of *M. smegmatis* (Msm) and *M. marinum* (Mmar) strains. |
| Figure 5.9 | Characterisation colony PCRs to compare rate of loss of pJV53 through passaging from *M. smegmatis* and *M. marinum*. |
| Figure 5.10 | Schematic of cloning protocol and sucrose counter-selection. |
| Figure 5.11 | Characterisation PCRs for putative *M. marinum* DosR/S AES single crossover strains from sucrose counter-selection Round 5. |
| Figure 5.12 | Characterisation PCRs for putative *M. marinum* DosR/S AES double crossover strains from sucrose counter-selection Round 5. |
| Figure 5.13 | Optimisation of Southern Blotting. |
| Figure 5.14 | Characterisation by Southern Blotting of putative ΔDosR/S + USP and putative ΔDosR/S mutant strains. |

| Figure A1 | Mycobacteria-adapted recombineering vector pYUB854. |
| Figure A2 | Mycobacteria-adapted recombineering vector pJV53. |
| Figure A3 | Sucrose counter-selection vector pSMT100. |
| Figure A4 | Plasmid used to generate *M. marinum* DsRed strain, pMSP12::DsRed2. |
| Figure A5 | Icy FPC protocol Block 2 (Script Block v1). |
| Figure A6 | Icy FPC protocol Block 4 (Script Block v1). |
| Figure A7 | Bacterial Distance Distribution script used to generate the Icy protocol. |
| Figure A8 | Icy Bacterial Distance Distribution protocol Block 14 (Extract Fish/Yolk/Injection ROIs). |
| Figure A9 | Icy Bacterial Distance Distribution protocol Block 26 (Export to Excel). |
| Figure A10 | Icy Bacterial Distance Distribution protocol Block 27 (Create Detection Token). |
| Figure A11 | Icy Bacterial Distance Distribution protocol Block 29 (Export with ROIs & Detections). |
| Figure A12 | Effect of injection of bactenecin-5 (Bac5) to the hindbrain ventricle after 48 hours of *M. marinum* infection on zebrafish neutrophil recruitment at 72hpi. |
| Figure A13 | Effect of injection of bactenecin-5 (Bac5) to the hindbrain ventricle after 48 hours of *M. marinum* infection on zebrafish neutrophil recruitment at 120hpi. |
| Figure A14 | Coding sequence amino acid alignments and genomic context views of KdpD/E TCSs proteins in *M. tuberculosis* and *M. marinum*. |
Figure A15. Coding sequence amino acid alignments and genomic context views of MtrA/B TCSs proteins in *M. tuberculosis* and *M. marinum*. .......................................................... 275

Figure A16. Coding sequence amino acid alignments and genomic context views of MprA/B TCSs proteins in *M. tuberculosis* and *M. marinum*. .......................................................... 276

Figure A17. Coding sequence amino acid alignments and genomic context views of NarL/Rv0845 TCSs proteins in *M. tuberculosis* and *M. marinum*. ........................................... 277

Figure A18. Coding sequence amino acid alignments and genomic context views of PdtaR and PdtaS TCSs proteins in *M. tuberculosis* and *M. marinum*. ........................................... 278

Figure A19. Coding sequence amino acid alignments and genomic context views of PhoP/R TCSs proteins in *M. tuberculosis* and *M. marinum*. ....................................................... 279

Figure A20. Coding sequence amino acid alignments and genomic context views of PrrA/B TCSs proteins in *M. tuberculosis* and *M. marinum*. ....................................................... 280

Figure A21. Coding sequence amino acid alignments and genomic context views of RegX3/SenX3 TCSs proteins in *M. tuberculosis* and *M. marinum*. ............................................. 281

Figure A22. Coding sequence amino acid alignments and genomic context views of TcrX/Y TCSs proteins in *M. tuberculosis* and *M. marinum*. ..................................................... 282

Figure A23. Coding sequence amino acid alignments and genomic context views of TrcR/S TCSs proteins in *M. tuberculosis* and *M. marinum*. ..................................................... 283

Figure A24. Growth and transformation efficiency at 28.5°C and 32°C of London and Leicester *M. marinum* M strains. ................................................................................................. 284

Figure A25. License information for material reproduced on page 21 of this thesis. .......................................................... 288

Figure A26. License information for material reproduced on page 23 of this thesis. .......................................................... 289

Figure A27. License information for material reproduced on page 28 of this thesis. .......................................................... 290

Figure A28. License information for material reproduced on page 30 of this thesis. .......................................................... 291

Figure A29. License information for material reproduced on page 43 of this thesis. .......................................................... 292

List of Tables

Table 1.1. Type and features of TB granulomas. .................................................................................................................. 24

Table 1.2. General properties of HDPs as innate immune effectors. .................................................................................. 40

Table 1.3. Key immunomodulatory activities of the selected HDPs. .................................................................................. 44
Table 1.4. Summary of in vivo studies involving HDPs which are of relevance to this thesis.

Table 1.5. Environmental signals, regulons and virulence characteristics of deletion mutants for the selected TCSs.

Table 2.1. Bacterial strains used during this study.
Table 2.2. Plasmids used in this study.
Table 2.3. Plasmids generated during this study.
Table 2.4. Primers for PCR and plasmid generation.
Table 2.5. Restriction enzymes and their sites used in plasmid generation.
Table 2.6. Primers used for sequencing.
Table 2.7. Expected PCR products for two-component systems (TCSs) mutant construction verifications.
Table 2.8. Primers used in TCSS mutant verification.
Table 2.9. Taqman gene expression assay IDs used in qRT-PCRs during this study.
Table 2.10. Kits used during this study.
Table 2.11. Chemicals and reagents used during this study.
Table 2.12. Solutions used during this study.
Table 2.13. Enzymes used in this study.
Table 2.14. Zebrafish lines used during this study.
Table 4.1. Observations on zebrafish health following injections.

Table 5.1 Coding sequence amino acid identities of selected TCSs proteins between M. tuberculosis and M. marinum.
Table 5.2. Summary of TCSs genomic context comparisons between M. tuberculosis and M. marinum.
Table 5.3. Summary of recombineering rounds undertaken during this project.
Table 5.4. Summary of sucrose counter-selection rounds undertaken during this project.
Table 5.5. Summary of Southern Blotting optimisations.
Table A1. Summary of permissions information for all material reproduced from published works in this thesis.
1 Introduction

1.1 *Mycobacterium tuberculosis* and Tuberculosis (TB)

1.1.1 *M. tuberculosis*, TB, latent TB and multi-drug resistant (MDR) TB

Many mycobacterial species are pathogenic, including *M. tuberculosis* and the other members of the *Mycobacterium tuberculosis* complex (MTBC). The closely-related MTBC members are the causative agents of tuberculosis (TB) in humans and also some other organisms. Other mycobacteria are generally less pathogenic than those of the MTBC but are capable of causing disease in humans, particularly immunocompromised individuals. Pathogenic mycobacteria include the causative agent of leprosy, *Mycobacterium leprae*, and the members of the *Mycobacterium avium* complex, including *M. marinum* which causes TB in ectotherms and a granulomatous skin infection in humans.

Human TB cases, caused primarily by *M. tuberculosis*, are typically pulmonary infections. The majority of infections occur in the developing world\[^{360}\], where the HIV/AIDS epidemic works in synergy with TB, leading to even greater morbidity and mortality\[^{214}\]. TB remains the world's primary bacterial cause of death and also has a high prevalence, leading to an estimated 1.4 million deaths and 10.4 million new cases globally in 2015\[^{360}\]. There are many different factors, both biological and socioeconomic, which contribute to the spread of TB including HIV-related immunodeficiency, the prevalence of drug-resistant TB, the problems associated with treatment and reactivation of latent TB, poor sanitation and overcrowding\[^{288}\]. Furthermore, there is currently no effective vaccine for the prevention of TB in adults\[^{360}\]. The Bacille-Calmette-Guerin (BCG) vaccine, which utilises an attenuated strain of *M. bovis*, is widely used and protects young children from severe forms of TB, but provides variable protection of 0-80% against pulmonary TB in adults\[^{303}\].

A hallmark of *M. tuberculosis* pathogenesis is latent TB infection (LTBI). It is estimated that a third of the world’s population has LTBI, approximately 2-3 billion individuals\[^{360}\]. Asymptomatic LTBI is maintained throughout the host lifetime within
immune structures called granulomas and can be diagnosed by a positive reaction to the purified protein derivative skin test, also called the tuberculin skin test\cite{22, 94}. LTBI are estimated to account for over 95% of \textit{M. tuberculosis} infections globally\cite{367} and can reactivate with a probability of 10% during the lifetime of a person to active, transmissible disease by induction of a productive cough that facilitates transmission to new hosts\cite{142, 157}. Reactivation can occur in response to an individual’s immunity waning, which can be caused by ageing, HIV infection, diabetes, immunodeficiency, chemotherapy and treatment with immunosuppressants following transplant surgery\cite{22, 182}. LTBI therefore represents a reservoir of infection which maintains a worldwide epidemic\cite{367}.

1.1.2 \textit{M. tuberculosis} lung pathogenesis and granuloma formation

\textit{M. tuberculosis} is a facultative intracellular pathogen of macrophages and is highly adapted for lung infection such that a single bacterium is sufficient to cause infection\cite{285}. Conserved determinants of virulence and protection within mycobacterial pathogens and their respective natural hosts form the basis of reciprocal strategies to expand or contain mycobacterial infections\cite{326}. TB disease results from this series of complex interactions between the infecting bacilli and host immune system which have evolved over a long period of time. Following the establishment of an infection in the human host, several outcomes are possible\cite{157} (Figure 1.1). In the majority of human TB cases the host is able to contain the infection by cell-mediated immunity and avoid progression to clinical disease for the duration of their lifetime\cite{157, 367}.

At the onset of TB infection following inhalation of aerosols containing bacteria, the bacilli are taken up by macrophages and transported across the epithelium into the lung. The bacteria possess the ability to subvert host endocytic trafficking, thereby resisting the immune response and enabling them to replicate within macrophages\cite{103, 157}. The bacilli achieve this by arresting phagosome maturation and preventing phagosome fusion with the lysosome, which in turn prevents acidification and accumulation of hydrolytic enzymes\cite{12, 97, 130, 157, 271, 286}. In resting macrophages, the bacilli are retained in the early recycling endosome, protected from lytic lysosomal enzymes\cite{157}. Following macrophage activation by cytokines including interferon γ (IFN-γ), the bacilli are delivered to acidic lysosome-like vacuoles\cite{157}. In addition, activated
macrophages possess low oxygen levels and produce nitric oxide and other radicals. Therefore, activated macrophages provide conditions which are unfavourable for active replication and metabolism of *M. tuberculosis*.

Figure 1.1. The three potential outcomes of infection of the human host with *M. tuberculosis*. a) The frequency of spontaneous healing is unknown, but is assumed to be minute. b) In the immunocompromised host, disease can develop directly after infection. c) In most cases, mycobacteria are initially contained and disease develops later as a result of reactivation. The granuloma is the site of infection, persistence, pathology and protection. Adapted from Kaufmann (2001)\[^{157}\]; excerpt from Figure 1 reprinted by permission from Macmillan Publishers Ltd, from: Nature Reviews Immunology, 1 (1), Kaufmann, S.H., How can immunology contribute to the control of tuberculosis?, p. 20-30, DOI: (10.1038/35095558), Copyright 2001 Macmillan Magazines Ltd. https://www.nature.com/nri/index.html

Macrophage activation by interaction with cytokines such as IFN-γ and interleukin (IL) 1β, produced by Type 1 T-helper (Th1) polarised CD4+ T cells, is therefore crucial in the control of TB infections. TB bacilli are capable of modulating the host response to infection through multiple mechanisms, including increasing production of Th2-polarising IL-1β in dendritic cells (DC)\[^{85}\] and IL-10\[^{11}\] in other cell types, which would reduce production of Th1-associated cytokines including IFN-γ. As the Th2 response is associated with parasitic infections and allergic responses\[^{57, 101}\], it is not productive during TB infections and is associated with latency\[^{64}\]. In support of this, recruitment of
resting macrophages which have not been activated by the Th1 response has been shown to benefit the infecting bacilli during early stages of *M. marinum*-zebrafish infection\[41, 345].

Another hallmark of *M. tuberculosis* pathogenesis is the subsequent formation of highly organised structures of immune cells called granulomas\[4]. Infected macrophages recruit other immune cells (including macrophages, T lymphocytes, B lymphocytes, dendritic cells, neutrophils, fibroblasts and extracellular matrix components\[61, 268]), until the central structure is surrounded by lymphocytes (Figure 1.2). As granulomas mature, they induce angiogenesis. Human and rabbit TB granulomas show enhanced vascular endothelial growth factor (VEGF) expression and a functionally abnormal vasculature comprised of spatially and morphologically heterogeneous granuloma-associated vessels\[67]. In addition to providing a local environment in which cells of the immune system can interact, the granuloma environment exposes the bacilli to a number of stressful conditions (Figure 1.2). These are postulated to include oxygen and nutrient starvation, low pH and nitric oxide exposure\[288]. In response to these stresses, the bacilli can enter a state of non-replicating persistence (NRP) for survival leading to latent infection\[351-353]. NRP is distinct from dormancy and is characterised by lack of replication and reduced metabolic activity.

Granulomas were thought to be primarily static structures which physically wall off persisting mycobacteria while preserving the function of neighbouring tissue. Recent studies using the macaque model and marked isolates of *M. tuberculosis* have indicated that granulomas are a more dynamic structure with individual granulomas within the same host showing considerable differences in function and infection dynamics\[183, 198]. These studies suggest that the responses occurring in each individual lesion determine the clinical outcome of the infection. Some aspects of granuloma formation and function were found to be similar between individual lesions within a single host during active disease; with most lung lesions founded by a single bacterium and possessing similar bacterial burdens, as well as most dissemination events occurring locally, forming new lesions a short distance from the parent lesion\[183, 198]. However, infection progression between individual lesions varied considerably with some lesions sterilised (no recoverable CFU) whilst others progressed, which was partially attributed to differential bacterial killing between individual lesions\[183]. Furthermore, some
multifocal lung lesions (comprised of two or more granulomas) originated from joining of adjacent independent granulomas whilst others originated from localised spread to form adjacent new granulomas which subsequently joined together\[183\].

Several types of granuloma have been identified in human TB infections (Table 1.1)\[22\], suggesting that there is a spectrum of pathology during pulmonary TB infections\[285\]. M. tuberculosis exploits immune-mediated damage to spread within infected lungs, showing a gradation of stages in which calcified tuberculous granulomas with few viable bacteria typically develop a necrotic core called caseum which includes extracellular mycobacteria\[22\]. It is thought that the caseum is responsible for morbidity and contagion as transmission of pulmonary TB is suggested to require the rupture of caseating foci into bronchial tubes\[66\].

**Figure 1.2. Structure and cellular constituents of the tuberculous granuloma.** The most common form of granuloma found in human TB infection, a caseous/necrotic granuloma, is shown with the proposed stresses present indicated. The human tuberculous granuloma is a compact, organized aggregate of epithelioid cells - macrophages that have undergone a specialized transformation to have tightly interdigitated cell membranes that link adjacent cells. Granulomas also contain many other cell types, such as neutrophils, dendritic cells, B and T cells, natural killer cells, fibroblasts and cells that secrete extracellular matrix components. The epithelial cells surrounding the granuloma (not shown) are thought to participate in its formation. Bacteria are found within infected macrophages and the central necrotic area, which includes dead and dying macrophages. Adapted from Ramakrishnan (2012)\[268\]; adapted by permission from Macmillan Publishers Ltd, from Nature Reviews Immunology, 12 (5), Ramakrishnan, L., Revisiting the role of the granuloma in tuberculosis, p. 352-66, DOI: (10.1038/nri3211), Copyright 2012 Macmillan Publishers Ltd. https://www.nature.com/nri/index.html
Table 1.1. Type and features of TB granulomas.

<table>
<thead>
<tr>
<th>Type of granuloma</th>
<th>Composition</th>
<th>Primary location of <em>M. tuberculosis</em> bacilli</th>
<th>Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrotic granuloma</td>
<td>Mainly fibroblasts with few macrophages</td>
<td>Unknown, possibly in macrophages or the fibrotic area</td>
<td>Mostly latent TB, also seen in active TB</td>
</tr>
<tr>
<td>Caseous granuloma</td>
<td>Epithelioid macrophages and neutrophils, surrounded by lymphocytes; sometimes in turn surrounded by peripheral fibrosis; centre contains dead host cells and is caseous, necrotic and hypoxic</td>
<td>Macrophages and necrotic centre, and possibly in fibrotic rim</td>
<td>Active and latent TB</td>
</tr>
<tr>
<td>Non-necrotising granuloma</td>
<td>Mainly macrophages with some lymphocytes</td>
<td>Macrophages</td>
<td>Active TB</td>
</tr>
</tbody>
</table>

Information adapted from N. Keshavan’s PhD thesis[160] and Barry et al. (2009)[22].

1.1.3 Requirement for novel therapies and vaccines

Treating TB is made difficult by the exceptionally slow growth of the bacillus and latent state[55]. The slow grow requires extensive treatments for drug-susceptible TB with high concentrations of 4 first-line antibiotics for 6 months[149, 174, 360]. The long treatment duration provides problems with use of ineffective treatment formulations and patient compliance, which in turn leads to the selection of genetically drug-resistant variants, known as multidrug-resistant TB (MDR-TB)[360]. MDR-TB infections are rapidly increasing in numbers and require even longer treatments with more expensive and more toxic drugs[360], exacerbating the treatment problems and selecting for even more severe drug-resistant TB. This extensively drug-resistant TB has been reported in 117 countries worldwide[360]. In addition to the issues associated with treating MDR-TB, entry into the latent state provides phenotypic antibiotic resistance as current antibiotics target actively replicating bacteria[55]. The different treatment regimens of varying length recommended for LTBI are estimated to reduce the risk of developing active TB by at least 60%[360], but are not fully effective against this form of the disease. Furthermore, identification of which patients with LTBI will develop active disease and therefore should be targeted for treatment is not currently possible[9, 94]. Preventative treatment of all individuals with LTBI would result in the exposure of large numbers of people to anti-TB drug side effects for little therapeutic benefit and increases the risk of
anti-TB drug resistance[9]. Finally, there are currently no drugs for prophylaxis or effective vaccines to prevent the spread of TB infections in the human population.

The combination of these issues with the prevention and treatment of TB infections has been exacerbated by the ageing population leading to increased reactivations of LTBI and the contemporaneous decline in the development of new antibacterial therapies. Only one effective drug treatment (bedaquiline) has been developed against *M. tuberculosis* in the last 4 decades[276, 300], which unfortunately causes unwanted side-effects including arrhythmias and even mortality[54]. In light of these events, there is an urgent need to discover novel antimicrobial therapies and approaches for the treatment and prevention of TB infections. To achieve this, we require significant advances in several research areas if we are to fulfil the Stop TB Strategy goal of eradicating TB by 2050. Such advances include a greater understanding of the two main barriers to the successful treatment of TB – latency and multidrug-resistance. Increased understanding of these states would permit the development of new therapies which possess some of the properties described below.

The ability to cause clearance of both MDR and latent phase bacilli is particularly important due to the high prevalence of MDR-TB infections, LTBI and the 10% reactivation rate for LTBI, which combine to provide a large reservoir of potentially MDR-TB infection. As latent bacilli reside in granulomas, such treatments would involve the successful penetration of granulomas by either the therapeutic compound or mediators of its effects. This requirement is difficult to achieve for most drugs as it requires the drug or effectors to reach the granuloma from their administration site, pass the many different immune cell types surrounding the caseum (at which point they can reach infecting bacilli for the first time as some are extracellular in the caseum), translocate through the macrophage and phagosome membranes, and then act on the bacteria contained within[121]. Therefore, pressures are placed upon any therapies by the localisation of bacteria in many different environments during late stage infections.

In addition, new therapies are required which lead to reduced incidences of genetic resistance. This could be achieved by therapies with a diverse range of targets or whose targets are not readily mutated, as well as more effective and/or faster acting therapies, thus reducing dosing frequency and treatment durations rendering access to effective
treatments and compliance less of a problem. Therapies are also required which are able to treat infections with current MDR strains, or prevent these infections. Treatment of MDR strain infections could be achieved through the use of therapies which possess different targets to existing antibiotics, as any new therapies targeting actively replicating bacteria will also rapidly select for genetic resistance. Therapies with different targets to current antibiotics may also permit the treatment of LTBI. Prevention of MDR-TB infections could be achieved using prophylactic treatments or an effective vaccine to reduce the transmission of new infections.

All of the above therapeutic properties would significantly reduce the danger posed by the LTBI reservoir in the human population, as household contacts are exposed to *M. tuberculosis* for months or even years when living with a person infected with latent TB. The study of potential novel therapies for TB infections has focussed on several candidates. Naturally occurring host defence peptides (HDPs) and the identification of novel drug targets which would usually act to promote bacterial virulence have both generated attention. Such virulence molecules could involve regulatory determinants of infection, including two-component signal transduction systems (TCSs). These two potential therapies are discussed in Section 1.4 and Section 1.5.

### 1.2 TB infection models

#### 1.2.1 Established TB infection models

Established models of *M. tuberculosis* infection include the mouse, rabbits, guinea-pigs and non-human primates[203]. However, these models possess several limitations which restrict the advancement of our knowledge of TB infections. Firstly, these models do not allow the study of a natural host-pathogen system. As mycobacteria have a particularly long history of evolving alongside their hosts the host-pathogen interactions have become specialised, meaning that the immunomodulators produced by TB bacilli have evolved in the presence of the human immune system and their effects may be different, or even completely absent, in other hosts. In addition, *M. tuberculosis* itself poses challenges for the study of TB infections as it is slow growing, difficult to manipulate genetically and demands high level safety facilities and training[263]. An advantage of the
rabbit, guinea-pig and non-human primate models is that the granulomas they form during *M. tuberculosis* infection are caseating, hypoxic structures similar to those found in human TB\[202, 343\]. However, these organisms are difficult to maintain in large numbers, expensive to work with and the ethics of performing large-scale infection experiments are inhibitory. These limitations have led to the widespread use of the mouse model, which is inexpensive, easy to maintain in large numbers, genetically tractable and well-characterised.

However, the mouse model has one key disadvantage as a model for TB infections: it does not reflect human TB infections in several ways, perhaps because TB infection of mice is not a natural host-pathogen interaction. During infection of mice there is a continuously high bacterial burden and continued replication of infecting bacilli even during chronic infection, which is distinct from LTBI in humans in which *M. tuberculosis* bacilli are almost completely dormant\[288\]. In addition, Severe Combined Immunodeficiency (SCID) mice which lack a functional adaptive immune system are often used, and these animals cannot form mature granulomas for the effective containment of the infection. Furthermore, the granulomas formed in mice during TB infections are loose cellular aggregates that are histologically distinct from human caseous granulomas (Figure 1.3)\[22, 256, 326\]. Moreover, the lack of caseation and cavitation of granulomas results in a lack of hypoxia in the majority of the mouse models, with the exception of the more recently developed C3HeB/FeJ strain\[113, 346\]. The C3HeB/FeJ strain possesses an alternative limitation in that it is hypersusceptible to TB infection, resulting in far increased replication of the bacteria compared to other mouse models\[168, 346\]. C3HeB/FeJ mice are hypersusceptible to TB through possession of an allelic variant of the Intracellular pathogen resistance 1 gene (*Ipr1*), carried on the super-susceptibility to TB locus of mouse chromosome 1 (*sst1*), which leads to greater necrosis and reduced apoptosis of infected macrophages, reducing intracellular pathogen clearance in this mouse strain\[240\].

As a result of the above TB infection model limitations, our understanding of the adaptation of *M. tuberculosis* to the host, survival during latent infection and reactivation to transmissible disease has been hampered because of the lack of suitable laboratory hosts in which granulomas similar to those found in human TB form.
1.2.2 The M. marinum-zebrafish model

The zebrafish (Danio rerio) embryo infection model to study mycobacterial pathogenesis using the natural pathogen M. marinum has emerged as a leading model in TB research\[45, 77, 204, 263, 313, 326]\]. The model reflects the early stages of TB\[53]\] and develops granulomas that have a structure similar to those found in human TB infection\[326]\] (Figure 1.3). The system also avoids many of the aforementioned issues and has recently been proven to be a powerful tool for further understanding mycobacterial infections, with studies that have questioned the traditional belief that granuloma formation is beneficial to the host. The M. marinum-zebrafish system provides an experimentally tractable, natural infection model in which to interrogate the role of conserved mycobacterial regulatory pathways in infection or investigate drug treatment efficacy, which we believe will yield valuable information relevant to their role in or treatment of human TB.

1.2.2.1 M. marinum as a model for M. tuberculosis pathogenesis

It has been shown that M. marinum infection is a suitable model in TB research. Firstly, the genomes of M. tuberculosis and M. marinum have been sequenced, and revealed that they possess a high coding sequence amino acid identity between orthologous regions of the genomes of 85\%[326]. Secondly, M. marinum is the closest genetic relative of the
MTBC and is a natural pathogen of ectotherms, such as fish and frogs\textsuperscript{313}, causing tuberculous disease. Finally, the pathogenesis of \textit{M. marinum} and \textit{M. tuberculosis} have been studied and found to be similar, with both bacilli primarily infecting macrophages to cause TB disease\textsuperscript{151, 309, 326, 330}. Following infection of zebrafish embryos with \textit{M. marinum}, key early steps in mycobacterial pathogenesis can be seen. Firstly, zebrafish macrophages phagocytose the bacteria but are unable to eradicate them\textsuperscript{326}. Following this, infected macrophages migrate to the tissue and form aggregates within the first four days of infection\textsuperscript{326}; these aggregates are early granulomas. \textit{M. marinum} also provides one further advantage for study in that it is easier to work with than \textit{M. tuberculosis} due to its faster growth rate and lower safety classification.

\subsection{Zebrafish as a model organism for studying infections and drug treatments}

Zebrafish as a model organism have many general advantages, they: are easy and relatively inexpensive to maintain in large numbers; produce a large clutch-size with a short life cycle; are amenable to high-throughput genetic screening; are genetically tractable; express genes that have mammalian orthologues; and possess most, if not all, of the cell types found in the mammalian immune system\textsuperscript{204, 263}.

In addition, zebrafish embryos possess many advantages for the study of infections or drug treatment efficacy. Zebrafish are transparent during the first two weeks of life and several transgenic zebrafish lines are available which possess fluorescently labelled macrophages and neutrophils\textsuperscript{69, 119, 272}. These features, and zebrafish fecundity, permit easy monitoring through live-imaging of infection progression, the interactions between host cells and pathogens, and localisation of expression markers (Figure 1.4) in both medium-throughput and longitudinal studies. In addition, the study of changes in gene expression over the whole organism are possible in the zebrafish embryo model\textsuperscript{204}. Zebrafish develop an adaptive immune system at 4 weeks post-fertilisation\textsuperscript{204}. Therefore, the \textit{M. marinum}-zebrafish embryo infection model also permits the study of the initial stages of granuloma formation in a setting where the adaptive immune system is not yet functional\textsuperscript{328}, rendering the model a powerful tool for the assessment of the actions of the innate immune system in isolation.
Infected adult zebrafish develop caseating granulomas similar to those seen in human TB infection hosts (Figure 1.3)[203, 256], and an adult zebrafish infection system has also recently been used successfully as a model for studying latent mycobacterial infection[244]. Thus the zebrafish adult model of TB infection permits the study of later infection stages requiring adaptive immunity (such as mature granuloma formation, chronic infection and latent infection). The larvae and adult models can therefore be used to determine the importance of each arm of the immune system in fighting an infection, and can inform on the effects of the bacilli or drug treatments in both immunocompromised and immunocompetent individuals. The importance of this dual capability has been highlighted by a recent study assessing the virulence of infecting strains, as *M. marinum* deficient in the ESX-5 secretion system is moderately attenuated in the larval model but strikingly hypervirulent in the adult infection model[354].
1.2.2.3  Insights into mycobacterial pathogenesis and granuloma formation from the M. marinum-zebrafish model

Granulomas were traditionally thought to be host-beneficial due to their containment effects\[203\], though it was known that the formation of granulomas was only partially efficacious in protecting the host as they can harbour bacteria for decades, including latent bacteria before their reactivation to form active disease\[86\]. Recent studies using the M. marinum-zebrafish model of TB infection have demonstrated that granuloma formation is also beneficial to the infecting bacteria\[70\].

One pair of studies report that a mycobacterial virulence determinant, region of difference 1 (RD1), promotes granuloma formation in early zebrafish infection to enable the establishment of an infection via dissemination from the initial infection site\[344, 345\]. RD1 promotes intercellular bacterial spread by recruiting uninfected macrophages to the infection site and enhancing death of infected cells. The dead infected macrophage cells and bacteria they release are subsequently phagocytosed by the uninfected macrophages which have recently arrived at the site of infection. The newly arrived macrophages then themselves become infected and are able to migrate and form new sites of infection\[70\]. The early granuloma site thereby serves to facilitate the infection as this process is repeated. Consistent with this view, aggregation of macrophages during M. marinum infection has been shown to promote intercellular bacterial spread by the correlation of macrophage aggregation with dramatic increases in the number of infected macrophages and bacterial burden\[345\].

A second pair of studies report that induction of granuloma formation by mycobacteria in turn induces granuloma-associated angiogenesis which promotes mycobacterial growth and enables the establishment of an infection via dissemination to new sites\[228, 229\]. Studies in the larval zebrafish model demonstrated that bacteria-induced granuloma formation is associated with angiogenesis and vascular permeability. The host endothelial cell regulator angiopoietin-2 (ANG-2) was found to be expressed in and around human and zebrafish granulomas, and is expressed in human granulomas by macrophage and stromal cells. ANG-2 was identified as an important factor in vascular dysfunction during mycobacterial infection and functions to promote angiogenesis and vascular permeability. Inhibition of angiogenesis through blocking signalling mediated by VEGF receptor decreased bacterial burden in both adult and larval zebrafish as well
as limiting dissemination in the larval model\cite{229}. Similarly, inhibition of the granuloma-induced vascular remodelling mediated via ANG-2 signalling limited mycobacterial growth in both larval and adult zebrafish models\cite{228}. Therefore, angiogenesis during granuloma formation benefits the infecting mycobacteria.

Finally, a recent study demonstrated the role of “epithelioid” macrophages in the promotion of mycobacterial infection within the granuloma\cite{63}. Granuloma-associated macrophages undergo a series of morphological changes to become epithelioid macrophages, which display epithelial cell characteristics such as abundant cytoplasm and interdigitation with neighbouring cells. The nature of the transformation of epithelioid macrophages in TB granulomas was not well understood. The larval zebrafish model was used to demonstrate that macrophages induced expression of the epithelial cell marker E-cadherin during mycobacterial granuloma formation resulting in adherens junction formation and cell-cell adhesion. Adherens junctions were also formed between epithelioid macrophages within mycobacterial granulomas in the adult zebrafish model, and shown to form a scaffold within the granuloma. Transcriptomic analysis of granuloma macrophages isolated from adult zebrafish identified molecular factors involved in the transformation and function of epithelioid macrophages. Macrophage epithelial reprogramming was found to be conserved between mycobacterial granulomas in a mouse model and humans, suggesting that the findings in the zebrafish model are applicable to human TB infections. Inhibition of cadherin-mediated macrophage adhesion in the zebrafish adult resulted in disorganised granulomas which demonstrated increased immune cell access and both reduced bacterial burden and increased host survival. Therefore, the transformation of epithelioid macrophages benefits the infecting mycobacteria.

Collectively, these studies demonstrate that the \textit{M. marinum}-zebrafish model is a powerful tool for further understanding mycobacterial infections. These studies exploited the ability to visualise mycobacterial pathogenesis in transparent zebrafish larvae and investigate the role of the innate immune system in isolation and the adaptive immune system through use of both the larval and adult models.
1.3 The assessment of \( M. \) \( marinum \) infection progression in THP-1 cells and the zebrafish

1.3.1 Assessment of infection progression in THP-1 cells

The THP-1 cell line was derived from an acute monocytic leukaemia patient over thirty years ago and has since become one of the most widely used cell lines to investigate the function and regulation of monocytes\(^{[48, 264, 333]}\). THP-1 cells can be differentiated into macrophage-like cells using several compounds including: macrophage colony-stimulating factor with IFN\( \gamma \); 1\( \alpha \), 25-dihydroxyvitamin D3; and phorbol-12-myristate-13-acetate (PMA)\(^{[48, 264, 333]}\). It has been shown that THP-1 cells activated to the macrophage-like state by PMA treatment, followed by at least 24 hours of rest without activation, increased the degree of macrophage differentiation indicated by the increased expression of macrophage markers\(^{[65]}\).

\( M. \) \( marinum \), like \( M. \) \( tuberculosis \), is a macrophage pathogen. \( M. \) \( marinum \)-infection of THP-1 cells has been used to assess the virulence of different bacterial strains and aid in the identification of virulence factors in several studies\(^{[2, 49, 81, 87, 301, 317, 337]}\). Infections have been performed both with\(^{[2, 49, 81, 301, 317, 337]}\) and without\(^{[87]}\) PMA-activation to the macrophage-like state, and with\(^{[49, 81, 87, 337]}\) and without\(^{[2, 301, 317]}\) antibiotic treatment to kill extracellular bacteria following the infection step. Antibiotic treatment with gentamicin or amikacin for 2-4 hours is used to eliminate all extracellular bacteria which had not been phagocytosed during the infection incubation. A low dose of either antibiotic can also be added to the tissue culture medium for long term incubations. These antibiotic treatments permit the determination of the intracellular bacterial burden of the cells or the intracellular survival of the bacteria, respectively. Infection progression in THP-1 cells has been quantified by several methods including colony forming unit (CFU) plating of cell lysates to determine the intracellular bacterial burden\(^{[49, 81, 87, 317, 337]}\) and enzyme-linked immunosorbent assay (ELISA) or fluorescent bead immunoassay of the cell supernatant to assess the host immune response\(^{[2, 81]}\).
1.3.2 Quantification of infection progression in the *M. marinum*-zebrafish model

Three possible injection sites can be used to infect zebrafish larvae (Figure 1.5). In order of complexity, due to decreasing size of target region, these are the yolk sac, hindbrain ventricle (HBV) and caudal vein. Yolk sac injection is the most high-throughput method, can be automated, leads to systemic infection and is predominantly used for drug screening\cite{45,232,308}. HBV injection provides a defined infection site \cite{328}, whilst caudal vein injection is used predominantly for generating systemic infections.

![Figure 1.5. Injection sites of zebrafish embryos. Adapted from Volkman et al. (2004)\cite{344}; Figure 3, excerpt part A modified by addition of yolk sac injection site label and arrow, reproduced from PLOS Biology, 2 (11), Volkman, H. E., et al., Tuberculous granuloma formation is enhanced by a mycobacterium virulence determinant, p. e367, DOI: (10.1371/journal.pbio.0020367), © 2004 Volkman et al, under a Creative Commons Attribution License, CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/legalcode).](image)

The optical transparency of zebrafish embryos and larvae provide the opportunity to witness the steps of microbial pathogenesis in real-time during longitudinal studies by live-imaging. It is useful to be able to perform longitudinal studies, which involve the collection of data from the same specimen over time, in many biological experiments as this permits the identification of key time points and the mapping of changes, for example. To take full advantage of these unique opportunities provided by the zebrafish infection model, it is desirable to perform quantitative analysis of the infection parameters visualised during microscopy as well as qualitative analysis. Repeated live-imaging of the same zebrafish specimen during longitudinal studies presents a
challenge as stress during the imaging procedures may affect the results. Therefore, quantitative analysis would ideally be performed after image acquisition to minimise distress to the zebrafish.

The study of mycobacterial pathogenesis in the *M. marinum*-zebrafish model has involved the quantification of several infection progression parameters: zebrafish survival following infection\(^{[319, 338]}\), immune cell recruitment to the infection\(^ {\text{[53, 69, 134]}}\), bacterial burden over time\(^ {\text{[5, 88, 319, 328, 338, 344, 354]}}\), bacterial dissemination over time\(^ {\text{[5, 344, 345]}}\) and the host immune response\(^ {\text{[53, 338]}}\). The methods used to quantify some of these infection progression parameters and their limitations are discussed below. These methods often require fixed samples, manual counting and use in combination to provide information on infection progression. Whilst they enable the analysis of large numbers of zebrafish at a single time point, repeated live-imaging as well as the rapid collection of data and analysis of large numbers of zebrafish during longitudinal studies are prevented. There is therefore scope to improve techniques for imaging and quantifying infection progression in zebrafish for use in longitudinal studies.

1.3.2.1 Quantification of infected zebrafish bacterial burden

Enumerating the number of infecting bacteria at a given time point in the infection, referred to as the bacterial burden, was traditionally performed by determining the number of CFU recovered following the dissociation of zebrafish embryos or larvae and plating of the lysates\(^ {\text{[53, 62, 317, 327, 344, 354]}}\). However, the method has several limitations: it is unreliable and not very sensitive as a large proportion of the infecting bacteria are killed during the lysis process; further inaccuracies arise as different bacterial strains can be differentially affected by the lysis; and the method allows only the single, terminal collection of data\(^ {\text{[320]}}\).

To eliminate the issues associated with CFU plating, several groups developed an alternative method, called fluorescent pixel count (FPC), for quantifying the bacterial burden of infected zebrafish during the course of this PhD\(^ {\text{[5, 317]}}\). FPC utilises fluorescently labelled *M. marinum* strains, and involves the quantification of the number of pixels over a user defined threshold from images of zebrafish larvae taken using standardised imaging settings. The method allows for the rapid quantification of
bacterial burden in live zebrafish embryos and larvae, permitting the collection of data at multiple time points for longitudinal studies. FPC has become the standard method for comparing bacterial burdens of infected zebrafish.[5, 317, 327, 328, 345]

1.3.2.2 Quantification of leukocyte recruitment to the infection site

The HBV is a cavity which does not usually contain leukocytes, enabling quantification of zebrafish leukocyte recruitment to the HBV in response to the injection of *M. marinum*, drug compounds and chemokines[53, 69, 134, 328, 329, 363]. *M. marinum* bacterial burden following HBV injection has also been determined[328]. The field standard is to inject the desired solutions to the HBV of 30 hours post fertilisation (hpf) zebrafish embryos and quantify leukocyte recruitment 3-6 hours later. Zebrafish embryos have been both live-imaged and fixed in paraformaldehyde (PFA) for later immunostaining and imaging at these time points to determine leukocyte recruitment. The number of leukocytes recruited to the HBV are manually counted by scrolling through the z-plane of the zebrafish in the lateral view using a confocal microscope. A critical step to prevent bias is blinding to the injection groups during counting. PFA-fixation of specimen allows for their processing at a later stage, permitting the analysis of a greater number of zebrafish embryos. However, the process is destructive and the specimen cannot be further analysed, preventing longitudinal studies. By contrast, manual quantification of leukocytes in live zebrafish embryo samples is time consuming and does not permit the analysis of a large number of zebrafish embryos.

1.3.2.3 Quantification of bacterial dissemination in infected zebrafish

Dissemination of infection can be defined as the process of infection spread from the initial site to further locations and is an important part of infection progression. Manual quantification of the number of granulomas and changes in their size during live-imaging of *M. marinum*-infected zebrafish larvae has previously been performed as a measure of infecting strain virulence and the effect of drug treatment on individual lesions[5, 344, 345]. This is time consuming and does not permit the analysis of a large number of zebrafish embryos. FPC quantification from images of bacterial pixel count in different zebrafish larvae sections (head, trunk and tail) and of bacterial cluster size has
been used as a measure of bacterial dissemination in different zebrafish host strains\cite{328}, and provides a method for the rapid, sequential analysis of granuloma characteristics.

### 1.4 Host defence peptides (HDPs)

#### 1.4.1 Introduction to HDPs

The first line of defence against infecting pathogens is the broad innate immune response\cite{200, 249, 331}, which is responsible for recognising and eradicating invading microbes\cite{293}. An important part of this early response to infection is local inflammation\cite{203}, although excessive or prolonged inflammation can cause detrimental effects for the host, including sepsis\cite{226, 249, 331}. Local inflammation involves the co-ordinated response of numerous innate immune cells and molecules including host defence peptides (HDPs)\cite{293}. HDPs are evolutionarily conserved molecules of the innate immune system, found in all complex organisms including fish and mammals\cite{33, 50, 194, 195, 208, 293, 358}. Their widespread nature suggests that they are a crucial and an evolutionarily ancient host defence against infections\cite{156}. Their key role in innate immunity is indicated by their constitutive expression by cells likely to contact pathogens and inducible expression in response to pathogen contact or cytokines by many other cell types\cite{50, 156, 358}.

Consistent with a major role for HDPs in innate immunity, various studies have demonstrated that multiple HDPs provide protection against a wide range of infections in vivo, including bacterial, parasitic and viral infections\cite{3, 32, 276, 279, 297}. HDPs achieve host protection against infections through their direct antimicrobial and immunomodulatory functions. Their direct antimicrobial activities mean that HDPs are also known as antimicrobial peptides (AMPs). The following sections introduce the properties of HDPs, their direct antimicrobial and immunomodulatory functions, and their potential as novel therapies, with a focus on the HDPs to be studied, their role in TB infections and potential as novel therapies for TB infections. There are two excellent and thorough reviews on the immunomodulatory functions of HDPs by Choi et al. (2011)\cite{50} and Mansour et al. (2014)\cite{195}, each providing original references for specific immunomodulatory activities of the peptides of interest in this work (cathelicidins and
their synthetic derivatives), and these reviews have therefore influenced the content of Section 1.4.

### 1.4.2 Mammalian and teleost HDPs – cathelicidins, piscidins and synthetic innate defence regulator (IDR) peptides

Defensins and cathelicidins are the two major families of HDPs[220] and have been extensively studied, though other families do exist. Teleosts (a group comprising most ray-finned fish including zebrafish) possess homologues of both major groups of HDPs found in humans, as well as an additional group termed piscidins[96, 156]. Cathelicidins comprise a conserved proregion, called the cathelin domain, which is cleaved to release the active peptides of variable structures[372]. A review of the different functions of cathelicidins from different structural groups can be found in Zanetti (2004)[372]. Cathelicidins are constitutively expressed by neutrophils and also induced during inflammation in a variety of tissues[31, 163, 231, 359, 372]. The central role of cathelicidins in protection against infection is demonstrated by their differential expression in a variety of diseases, reviewed for LL-37 in Dürr et al. (2006)[83].

The single human cathelicidin LL-37 is 37-amino acids long and the α-helical, processed form of hCAP-18[195, 372]. Bovine cathelicidins are called bactenecins, indolicidin and bovine myeloid antimicrobial peptides (BMAP)[166]. Two proline-rich bactenecins were identified and characterised in the same studies[106, 114], the 5 kDa bactenecin-5 (Bac5) and 7 kDa bactenecin-7, which are 42 and 59 amino acids in length, respectively. As well as being proline-rich, Bac5 is also arginine rich[106]. We consider the structure of Bac5 as unknown - it has been reported to possess a β-hairpin[166], cyclic[106], and linear, non-α-helical structure[300]. Another bovine cathelicidin, bactenecin, is also arginine rich, possesses a cyclic structure and is the smallest known HDP at 12 amino acids in length, which has made it attractive in studies of HDPs[33, 361]. Teleost cathelicidins have not been widely studied; perhaps the most studied of the teleost HDPs is the piscidin epinecidin-1[236]. This HDP has an α-helical structure, is 21 amino acids in length and was isolated from grouper (Epinephelus coioides).

The study of the immunomodulatory effects of HDPs and whether HDPs are suitable for use as treatments for infections has led to the generation of small synthetic cationic
peptides termed innate defence regulator (IDR) peptides. IDR peptides are linear derivatives of the bovine cathelicidin bactenecin which display enhanced immunomodulatory activities and reduced cytotoxicities compared to HDPs\cite{221, 293, 334, 358}. The IDR peptides display similar protective effects as HDPs in murine models of infection, including with herpes simplex virus and drug resistant strains of bacteria including \textit{M. tuberculosis}\cite{3, 30, 195, 221, 276, 293, 297, 310}. IDR peptides of relevance to this work, and included in Table 1.3 and Table 1.4, are IDR-1\cite{293}, IDR-1002\cite{221}, IDR-1018\cite{3}, IDR-HH2\cite{165} and Bac2A\cite{33}. IDR-1, IDR-HH2 and Bac2A are proline-rich, arginine-rich and arginine-rich derivatives of bactenecin, respectively. The remaining peptides were generated by random modification of Bac2A and selection for improved chemokine stimulatory activities; they are all arginine rich. Therefore, whilst there is some broad sequence similarity between the peptides they are not closely related.

\subsection{1.4.3 General properties of HDPs}

The characteristics of HDPs as innate immune effectors have not been fully resolved, those which are known are detailed in Table 1.2. These properties facilitate their interactions with cell membranes\cite{10, 37, 82, 178, 180, 218, 365}, which are essential for both their direct antimicrobial and immunomodulatory functions\cite{195, 358}. Many HDPs are capable of penetrating host and bacterial cell membranes\cite{195}. HDPs possess diverse amphipathic structures which can be classified as $\alpha$-helices, $\beta$-sheets, extended structures and loops. Figure 1.6 shows the range of structures seen with different HDPs.

Characteristics which remain to be investigated include the identification of many of the cell surface receptors and/or intracellular partners which interact with HDPs during immunomodulation, and the mechanisms by which they mediate different immunomodulatory effects\cite{50}. In addition, knowledge of the molecular mechanisms of HDP gene regulation is limited\cite{372}. Furthermore, the relationship between HDP structures and their direct killing and immunomodulatory mechanisms requires further investigation\cite{50, 96}. A study of the structure-function relationships of human LL-37, one of the most widely studied HDPs, revealed that differential proteolytic processing at the skin may lead to different antimicrobial and immunostimulatory functions, with shorter processed forms incapable of immunomodulation\cite{35}. This study implicates proteolytic processing in control of the functions of HDPs, although whether this holds true for
other HDPs is yet to be investigated. In addition, the generation of synthetic peptides with improved activities provides the opportunity to study the structure activity relationships of the peptides for their direct effects and immunomodulatory effects.

Table 1.2. General properties of HDPs as innate immune effectors.

<table>
<thead>
<tr>
<th>Innate immune mediator characteristic</th>
<th>Details</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Production at exposed surfaces</td>
<td>Epithelial and innate immune cells (phagocytes), particularly neutrophils</td>
<td>[152, 295, 372]</td>
</tr>
<tr>
<td>Localisation at exposed surfaces</td>
<td>Skin, body fluids, secretions, and mucosal surfaces</td>
<td>[75, 82]</td>
</tr>
<tr>
<td>Tightly controlled expression by transcriptional and post-transcriptional regulation</td>
<td>(i) Produced as pre-pro-proteins (ii) Proteolytically processed to release mature, biologically active forms</td>
<td>[47, 126, 373]</td>
</tr>
<tr>
<td>Host and pathogen membrane interactions</td>
<td>(i) 12 to ~50 amino acids in length (ii) Cationic – net positive charge +2 to +9 (iii) ≥ 30% hydrophobic residues (iv) Diverse amphipathic structures - α-helices, β-sheets, extended structures and loops</td>
<td>[318]</td>
</tr>
<tr>
<td>Uptake by host cells&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Proposed mechanisms: (i) Cell-surface receptors, including Gi-protein coupled receptors and Extracellular Signal-Related Kinase (ERK) (ii) Atypical endocytic pathway&lt;sup&gt;b&lt;/sup&gt;</td>
<td>[16, 76, 176, 221, 324]</td>
</tr>
<tr>
<td>Receptor interactions for immunomodulation&lt;sup&gt;a&lt;/sup&gt;</td>
<td>(i) Cell-surface receptors, including Gi-protein coupled receptors (ii) Intracellular interaction partners, including Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)</td>
<td>[76, 176, 209, 221]</td>
</tr>
</tbody>
</table>

<sup>a</sup> = Mechanisms for most HDPs are unknown, examples provided for the human cathelicidin LL-37. <sup>b</sup> = Uptake by this mechanism would be similar to other cationic cell-penetrating peptides. Much of the information presented in this table was sourced from reviews by Choi et al. (2012), Katzenback (2015) and Mansour et al. (2014).
**Figure 1.6. Structures diversity and classification of natural HDPs.** Shown are examples for each of the four structural classifications of HDPs, indicated in the left bottom corner. Different names for the structural classifications exist, with both forms used in Figure 1.6 and Table 1.2. $\alpha = \alpha$-helices, $\beta = \beta$-sheets, $\alpha\beta = \alpha\beta$ = loops and non- $\alpha\beta = \alpha\beta$ = extended structures. Figure taken from Wang (2013)\[^{350}\]; unmodified Figure 4 reproduced from Pharmaceuticals, 6 (6), Wang, G., Database-Guided Discovery of Potent Peptides to Combat HIV-1 or Superbugs, p. 728, DOI: (10.3390/ph6060728), Copyright 2013, under a Creative Commons Attribution License, CC BY-NC-SA 3.0 (https://creativecommons.org/licenses/by-nc-sa/3.0/legalcode).

### 1.4.4 Mechanisms of action of HDPs

#### 1.4.4.1 Direct antimicrobial activities of HDPs

The direct antimicrobial activities of HDPs lead to their study initially\[^{310}\]. HDPs have been shown to act directly against a variety of different pathogens, including bacteria, viruses, fungi, and protozoans\[^{13, 27, 37, 122, 177-179, 187, 211, 267, 295, 366}\]. The direct antimicrobial mechanisms of action of HDPs against bacteria involve multiple different targets including: inhibition of replication via disruption of RNA, protein, and/or DNA synthesis as well as of cell division and peptidoglycan synthesis; and perturbing the physical integrity of the bacterial cell membrane by poration or thinning\[^{37, 102, 123, 178, 180, 195, 218, 235, 318, 358}\]. Targeting of bacteria by HDPs is achieved through exploiting differences in membrane composition between bacteria and eukaryotes, including the
greater numbers of anionic lipids, reduced cholesterol content and greater
electrochemical potential gradient of bacterial membranes\cite{96, 195}. Examples of
microorganisms directly targeted by the HDPs selected for study in this work are
provided below in Table 1.3.

The direct effects of mammalian HDPs are antagonized in many physiological
conditions\cite{195, 221}. For example, the direct antimicrobial activity of LL-37 is hindered by
physiological salt concentrations\cite{32, 195}. The aforementioned protection by HDPs against
various infections \textit{in vivo} has therefore been attributed by several groups, including
ourselves, to their immunomodulatory activities\cite{50, 127, 128, 195, 255, 276, 318}. Investigation of
these activities is therefore taking precedence.

\textbf{1.4.4.2 Immunomodulatory activities of HDPs}

HDPs possess broad immunomodulatory activities, including increasing leukocyte
recruitment and pathogen killing, control of dendritic cell (DC) maturation, wound
healing and angiogenesis, and modulation of inflammation\cite{50, 195, 221, 310, 358}. For example,
LL-37 was able to induce keratinocyte migration and increase matrix metalloproteinase
(MMP) activation \textit{in vitro}, and significantly improved tissue formation and re-
epithelialisation in a murine wounding model\cite{44}. Overall, HDPs influence both innate
and adaptive immunity to protect against pathogens by preventing excessive harmful
inflammation, whilst increasing host defence capabilities\cite{50, 195, 276, 334}. Many of the
known immunomodulatory activities of HDPs and IDR peptides are represented in
Figure 1.7. Examples of the specific immunomodulatory activities of the HDPs selected
for study are provided below in tabular form for clarity and ease of understanding in
Table 1.3. The immunomodulatory activities of Bac5 have not been investigated to our
knowledge; therefore, the activities of synthetic derivatives of the related peptide
bactenecin have been presented as these have been extensively studied. Although their
structures differ, these IDR peptides share sequence similarities with Bac5 as they are
either proline or arginine rich, leading to our hypothesis that Bac5 will display similar
immunomodulatory activities.
Figure 1.7. Immune functions of HDPs and IDRs. The different immune actions of HDPs and IDR peptides at high concentrations (1) and at lower concentrations (2-6). 1) Direct antimicrobial or anti-biofilm activity against invading bacteria. 2) Indirect (through chemokine or chemokine receptor expression increases) or direct promotion of the recruitment of immune cells such as neutrophils and monocytes. 3) Suppress the induced production of proinflammatory cytokines such as Tumor Necrosis Factor α (TNF-α), IL-6, and IL-8, and enhance the production of anti-inflammatory mediators including IL-10 and chemokines such as Macrophage Chemotactic Protein 1 (MCP-1). 4) Induce macrophage and dendritic cell differentiation and activation. 5) Actions in (4) lead to the modulation of adaptive immunity and induce the recruitment of T cells. 6) Regulate specific cell activities including autophagy, apoptosis and the formation of neutrophil extracellular traps (NETs). Taken from Mansour et al. (2014); reprinted from: Trends in Immunology, 35 (9), Mansour, S.C., O.M. Pena, and R.E. Hancock, Host defense peptides: front-line immunomodulators, p. 443-50, DOI: (10.1016/j.it.2014.07.004), Copyright (2014), with permission from Elsevier.

There are some aspects of the key immunomodulatory activities contained within the summary in Table 1.3 which are worthy of further discussion. Firstly, it is important to note that the sequences of the HDPs, even the cathelicidins of human LL-37 and bovine origin are not highly conserved, which explains the variations in immunomodulatory activities between them. Overall, the study of the synthetic derivatives of bactenecin has demonstrated that they display a subset of the immunomodulatory activities of LL-37\[33\].
Table 1.3. Key immunomodulatory activities of the selected HDPs.

<table>
<thead>
<tr>
<th>Host defence mechanism</th>
<th>HDP activity</th>
<th>LL-37</th>
<th>Bactenecins and synthetic derivatives</th>
<th>Epinecidin-1</th>
</tr>
</thead>
</table>
|                        | Direct antimicrobial effects | (i) Bacteriocidal: *M. tuberculosis*, *M. smegmatis*, *M. bovis*[^199, 307]  
(ii) Anti-biofilm: *P. aeruginosa*[^234] | (i) Bacteriocidal: *Burkholderia pseudomallei*[^191]  
| Increased pathogen killing | Direct leukocyte chemoattraction | Neutrophils, eosinophils, monocytes, mast cells, T-cells[^223, 325, 364] | Neutrophils, THP-1 cells, monocytes[^33, 189, 190, 341, 368] | - |
|                        | Indirect leukocyte chemoattraction | ↑ IL-8, MCP-1, IL-8RB[^207, 208, 292] | ↑ MCP-1, leukocyte recruitment *in vivo*[^221, 293] | ↑ MCP-1[^235] |
|                        | Enhancement of intracellular killing | (i) Synergistic effects with IL-1β[^369]  
(ii) ↓ Neutrophil and ↑ epithelial cell apoptosis[^18, 19, 215]  
(iii) ↑ Infected macrophage autophagy[^370]  
(iv) ↑ Macrophage intracellular *M. tuberculosis* killing[^206, 307] | ↑ Neutrophil intracellular bacterial killing[^224] | - |
|                        | Enhancement of extracellular killing | (i) Anti-biofilm effects[^234], in NETs and MCETs[^195]  
(ii) ↑ ROS, IL-8, α-defensin secretion by neutrophils[^375] | Anti-biofilm effects[^73, 74, 194, 270], in NETs and MCETs[^195] | - |
<p>| Connecting | Indirect effects on | (i) Activation[^16, 68] | - | - |</p>
<table>
<thead>
<tr>
<th>innate and adaptive immune systems</th>
<th>adaptive immune system – dendritic cell effects</th>
<th>(ii) ↑ Endocytic capacity(^{[68]}) (iii) Modulation of cytokine secretions – polarises to Th1 response(^{[68]})</th>
<th>Adjunctive effects</th>
<th>(i) ↑ CpG sensing by B cells(^{[150]}) (ii) ↑ IFN by monocytes and ppDCs (with DNA) ⇒ augments NK cell functions(^{[254]})</th>
<th>↑ TNF-( \alpha ), MCP-1 and IFN-( \gamma ) by PBMC (with CpG)(^{[368]})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control of inflammation</td>
<td>Modulation of cytokine secretion – both ↑ and ↓</td>
<td>TNF-( \alpha ), IL-12, IL-1( \beta ), IL-1RA and IL-10(^{[38, 208-210]})</td>
<td>Modulation of responses to other signals</td>
<td>(i) Alters TLR to NF-( \kappa )B signalling (response to LPS)(^{[208]}) (ii) ↑ IL-1( \beta ) by monocytes (response to LPS)(^{[93]}) (iii) ↓ Th1 polarisation by DCs and class-switching in splenic B-cells (response to IFN-( \gamma ))(^{[222]}) (iv) ↓ TNF-( \alpha ), ↓ IL-1( \beta ), ↑ IL-1RA by PBMC (response to IL-32)(^{[51]})</td>
<td>Alters TLR to NF-( \kappa )B signalling (response to LPS)(^{[148]})</td>
</tr>
</tbody>
</table>

Some of the information in this table was sourced from reviews by Choi \textit{et al.} (2012) and Mansour \textit{et al.} (2014)\(^{[50, 195]}\). ROS = Reactive Oxygen Species; MCETs = Mast Cell Derived Extracellular Traps; ppDC = precursor DC; NK = Natural Killer; PBMC = Peripheral Blood Mononuclear Cell, LPS = lipopolysaccharide, MRSA = Methicillin-resistant \textit{Staphylococcus aureus}; TLR = Toll-like receptor.
1.4.4.3 Studies of the protective effects of immunomodulation by HDPs against infections in vivo

Due to their protective effects against harmful inflammation, HDPs were initially investigated as immunomodulators with the ability to prevent sepsis\cite{52, 115, 293, 299}. The study of HDPs as novel immunotherapies for the treatment of infections is a growing field. A summary of in vivo infection studies with HDPs and IDR peptides which are of relevance to this work is provided in Table 1.4. The summary focuses on reports involving the three peptides to be studied or related synthetic IDR peptides, as well as studies involving the zebrafish, our chosen model organism. The immunomodulatory mechanism behind the observed protective effects was not determined in many of the studies. This information was included where possible and confirms that the immunomodulatory activities of HDPs and IDR peptides are beneficial during infections in vivo and these peptides are indeed potential novel therapeutics.

There are several findings from the in vivo studies which are significant for this work. The cross-species immunomodulation displayed by the studied peptides confirm the conservation of immunity mechanisms between vertebrates, and support the use of zebrafish in modelling mammalian inflammatory conditions and infections. The displayed cross-species immunomodulation included the ability of teleost HDPs to perform immunomodulation in a mammalian system\cite{147} and the ability of other vertebrate HDPs, such as chicken\cite{291} and bovine\cite{181}, to perform immunomodulation in the zebrafish. These studies indicate that immunomodulation of zebrafish by the exogenous peptide Bac5 is possible during our experiments. In addition, studies using transgenic zebrafish constitutively expressing HDPs demonstrated that continuous treatment with an exogenous peptide can be beneficial in the host defence against infection\cite{238, 252}, which is promising for further studies of the effects of HDPs using transgenic expression and for the use of HDPs in gene therapies. Furthermore, the studies indicated that HDPs can be used in effective prophylactic strategies\cite{153, 181, 221, 237, 239, 252, 291}, the importance of which in TB infections has been discussed previously in Section 1.1.3. In addition, the results of the study using adenovirus administration of DNA encoding LL-37 are promising for the use of HDPs in gene therapies\cite{155}. Finally, IDR-1018 and IDR-HH2 demonstrated protective effects in a murine model of TB infection\cite{276}, which is further discussed in Section 1.4.5.2.
Table 1.4. Summary of *in vivo* studies involving HDPs which are of relevance to this thesis.

<table>
<thead>
<tr>
<th>HDP/IDR peptide</th>
<th>Model organism</th>
<th>Infectious organism</th>
<th>Infection stage</th>
<th>Administration</th>
<th>Effects</th>
<th>Immunomodulation</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human LL-37</td>
<td>Mouse</td>
<td>S. aureus</td>
<td>Early</td>
<td>Intraperitoneal</td>
<td>Direct antimicrobial ↓ Bacterial burden</td>
<td>-</td>
<td>[32]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MRSA</td>
<td>Co-treatment</td>
<td>Intratracheal</td>
<td>↓ Pneumonia</td>
<td>↓ Pro-inflammatory genes</td>
<td>[143]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E. coli</td>
<td>Pre-treatment (adenovirus vector)</td>
<td>Intravenous</td>
<td>↑ Survival (anti-endotoxaemia effects)</td>
<td>-</td>
<td>[15]</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td></td>
<td></td>
<td>Intratracheal</td>
<td>↓ Bacterial burden</td>
<td>↓ Pro-inflammatory gene (TNF-α)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine-derived IDR-1</td>
<td>Mouse</td>
<td>MRSA</td>
<td>Co-treatment</td>
<td>Intratracheal</td>
<td>↓ Pneumonia</td>
<td>↓ Pro-inflammatory genes</td>
<td>[143]</td>
</tr>
<tr>
<td>Bovine-derived IMX00C1</td>
<td>Mouse</td>
<td>S. aureus</td>
<td>Early</td>
<td>Intraperitoneal</td>
<td>No direct antimicrobial ↓ Bacterial burden</td>
<td>-</td>
<td>[32]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S. enterica serovar Typhimurium</td>
<td>Co-treatment</td>
<td>Intraperitoneal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine-derived IDR-1018</td>
<td>Mouse</td>
<td>Plasmodium berghei ANKA</td>
<td>Established (with anti-malarials)</td>
<td>Intraperitoneal or intravenous</td>
<td>↑ Survival</td>
<td>↓ Inflammatory gene network</td>
<td>[3]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Drug-susceptible H37Rv and MDR M.</td>
<td>Co-treatment</td>
<td>Intravaginal</td>
<td>↓ Viral replication ↓ Disease development</td>
<td>-</td>
<td>[297]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Herpes simplex virus-2</td>
<td>Late progressive</td>
<td>Intratracheal</td>
<td>Direct antimicrobial ↓ Bacterial burden ↓ Pneumonia</td>
<td>-</td>
<td>[276]</td>
</tr>
<tr>
<td>Pathogen</td>
<td>Species</td>
<td>Route</td>
<td>Treatment</td>
<td>Effect on Bacterial Burden</td>
<td>Effect on Pro-inflammatory Genes</td>
<td>References</td>
<td></td>
</tr>
<tr>
<td>-------------------------------------------------------------------------</td>
<td>----------------</td>
<td>------------------------</td>
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<td>---------------------------</td>
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<td></td>
</tr>
<tr>
<td>Tuberculosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>[137]</td>
<td></td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td></td>
<td>Pre-treatment</td>
<td>Intravenous</td>
<td>↓ Bacterial burden</td>
<td>↓ Pro-inflammatory genes</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>Mouse</td>
<td>Pre-treatment</td>
<td>Intraperitoneal</td>
<td>No direct antimicrobial</td>
<td>↑ Chemokines</td>
<td>[221]</td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>Mouse</td>
<td>Pre-treatment</td>
<td>Feeding Tg</td>
<td>Direct antimicrobial</td>
<td>↑ Survival</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Edwardsiella tarda</em></td>
<td>Zebrafish adult</td>
<td>Pre-treatment</td>
<td>Feeding transgenic (Tg) zebrafish eggs</td>
<td>↑ Survival</td>
<td>↑ Immunity gene expression</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Japanese encephalitis virus (JEV)</em></td>
<td>Mouse</td>
<td>Co-treatment</td>
<td>Applied to wound</td>
<td>↑ Survival</td>
<td>↑ Wound closure and angiogenesis</td>
<td>[147]</td>
<td></td>
</tr>
<tr>
<td><em>MRSA</em></td>
<td>Zebrafish adult</td>
<td>Co-treatment</td>
<td></td>
<td></td>
<td></td>
<td>[146]</td>
<td></td>
</tr>
<tr>
<td><em>V. vulnificus</em></td>
<td>Zebrafish adult</td>
<td>Pre-treatment</td>
<td>Oral</td>
<td>↑ Survival</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>V. vulnificus</em></td>
<td>Zebrafish adult</td>
<td>Pre-treatment</td>
<td>Feeding Tg</td>
<td>↑ Survival</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>V. vulnificus</em></td>
<td>Zebrafish adult</td>
<td>Co-treatment</td>
<td>Tg expression</td>
<td>↑ Survival</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Streptococcus agalactiae</em></td>
<td>Zebrafish adult</td>
<td>Pre-treatment</td>
<td>Tg expression</td>
<td>↑ Survival</td>
<td>↑ Immunity gene expression</td>
<td>[144, 238]</td>
<td></td>
</tr>
<tr>
<td><em>S. enterica serovar enteriditis</em></td>
<td>Zebrafish embryo</td>
<td>Pre-treatment</td>
<td>Yolk sac injection</td>
<td>↓ Bacterial burden</td>
<td></td>
<td>[291]</td>
<td></td>
</tr>
</tbody>
</table>

Some of the information in this table was sourced from reviews by Choi et al. (2012) and Mansour et al. (2014) [50, 195].
1.4.5 HDPs as novel therapeutics for TB infections

1.4.5.1 Advantages and limitations of HDPs as novel therapeutics

A benefit of HDPs as therapies is that they are less likely to select for genetic resistance than traditional antibiotics\cite{96, 102, 195} and could provide broad protection against a range of pathogens\cite{219}. As immunomodulators the peptides act on the host, rendering them unlikely to select for genetic resistance and suitable for treating infections with existing MDR strains\cite{225}. As direct antimicrobials the peptides act on a diverse range of targets, providing a combination of antimicrobial mechanisms in the same molecule, and exploit the differences in membrane compositions between bacteria and eukaryotes\cite{300}. Acquisition of resistance to HDPs requires the mutation of multiple targets or membrane composition characteristics, which is more difficult to achieve than the singular molecular targets of traditional antibiotics\cite{96, 318}. This is evidenced by the existence of only a handful of species which possess resistance to the direct activities of HDPs\cite{318}, despite their evolutionary conservation and ubiquity in host defence. Furthermore, targeting of the bacterial membrane or the synthesis of key macromolecules required for general cell maintenance by HDPs can lead to activities against bacteria which display slow or no growth\cite{96}, unlike traditional antibiotics.

Due to their different mechanisms of action, HDPs could be used in combination therapies with antibiotics, adjuvants or other HDPs to further reduce the risk of bacteria developing genetic resistance, treat MDR strain infections, and lower the required doses for effective therapies. For example, the direct actions of the defensin human neutrophil peptide 1 (HNP-1) in permeabilising the bacterial membrane permitted the reduction of the effective therapeutic dose of anti-tubercular drugs by half in murine models of TB infection\cite{155}. Furthermore, IDR-1002 acted with CpG and polyphosphazene to provide a potent protective immune response in mice infected with *Bordetella pertussis*\cite{167}, and LL-37 and bovine indolicidin produced a greater combined effect on the reduction in LPS-induced TNF-α production in THP-1 cells than when used alone\cite{33}.

The diversity of HDP sequences throughout nature can be exploited through the design of synthetic cationic peptides with direct antimicrobial activity or IDR peptides with enhanced immunomodulatory activity to generate new antimicrobials\cite{72}. It is
possible to imagine a family of drugs with different mechanisms of action to current antibiotics and the ability to rapidly target MDR strains and slow growing bacteria which are recalcitrant to antibiotic treatments. As the generation of new synthetic peptides continues, more is known about which features of the structures and sequences of HDPs improve activity or stability, increasing our ability to design peptides with the desired therapeutic activities. For example, teleost HDPs are more resistant to ionic disruption, perhaps due to their high arginine content, enabling the generation of peptides with direct antimicrobial activity in physiological conditions\textsuperscript{[96]}. Furthermore, shorter side chains\textsuperscript{[14]} and proline-rich sequences\textsuperscript{[298]} (which applies to the peptide of interest in this study, Bac5) have been implicated in resistance to protease degradation. Moreover, two studies have recently identified increased direct antimycobacterial activity of peptides possessing tryptophan residues, with each also identifying one of increased hydrophobicity and arginine content as additional factors\textsuperscript{[139, 154]}. Examination of the sequence properties of IDR peptides which display enhanced immunomodulatory activities would be expected to provide similar insights into the desirable sequences.

The emerging field of rational peptide design has already generated some promising candidates, including those with direct antimicrobial activity against fungi\textsuperscript{[311]}, \textit{M. tuberculosis} (II-D, Section 1.4.5.2)\textsuperscript{[161]}, antibiotic resistant bacteria\textsuperscript{[125]}, \textit{P. aeruginosa}\textsuperscript{[322]} and its biofilms\textsuperscript{[175]}, and \textit{E. coli} and MRSA\textsuperscript{[257]}. For example, engineered cationic antimicrobial peptide (eCAP) was able to reduce \textit{P. aeruginosa} biofilm growth associated with cystic fibrosis airway epithelial cells \textit{in vitro}\textsuperscript{[175]}. The IDR peptides, which have already been described, were selected for their enhanced abilities to stimulate MCP-1 release compared to the parent peptide\textsuperscript{[221, 293]}. Furthermore, it is also possible to design synthetic peptides with enhanced immunomodulatory and direct antimicrobial activities, providing very powerful new therapies. For example, a novel high-throughput screening method to assess anti-biofilm and desirable immunomodulatory activity of synthetic peptides was used successfully to screen for IDR-2009, which has enhanced activities compared to the parent peptide IDR-1002\textsuperscript{[129]}. In addition to the above general benefits, HDPs are particularly attractive as novel immunotherapies against TB for several reasons. The peptides provide the possibility of treating the initial infection and controlling inflammation and the associated
damage\textsuperscript{[225]}, as evidenced by the successful use of LL-37 to prevent sepsis in adult\textsuperscript{[52]} and neonatal\textsuperscript{[109]} rats. For TB infections in particular, the host immune system is aware of the latent infection, maintained within granulomas and thus surrounded by immune cells, but does not appear able to clear the bacilli, which can be considered to be hiding in plain sight. The immunomodulatory activities of HDPs may activate effector cells of the immune system to target latent and MDR bacilli and also provide the possibility of prophylaxis. This could be achieved through polarising the immune response away from the TB-induced Th2 response to a productive Th1 response, capable of clearing the infection. For example, TB-infected mice treated with IDR-1018 or IDR-HH2 demonstrated reduced bacterial burden and pneumonia, with macrophage morphology in infection-induced inflammation similar to the early infection stages of untreated mice\textsuperscript{[276]}. The early infection stages display increased infection control associated with increased TNF-\(\alpha\) and inducible nitric oxide synthase (iNOS) expression in the Th1 response, demonstrating its importance protective immunity against TB infections and the benefit of immunomodulation in therapies for TB. In addition, HDPs may possess the ability to penetrate granulomas, or perform immunomodulation on accessible cells within granulomas, again raising the possibility of targeting LTBI.

All of these advantages to the use of HDPs and their derivatives in novel TB therapies provide the possibility of the much-needed treatments which are capable of clearance of latent and MDR bacilli and also prophylaxis (Section 1.1.3). Despite these advantages, only one HDP has reached pre-clinical trials as a therapy for TB infection\textsuperscript{[300]}, leading to further scope for investigating the potential of HDPs as novel TB therapies.

Though HDPs represent an attractive alternative therapy for treating infections, there are several potential barriers to their therapeutic applications, reviewed by Mansour et al (2014)\textsuperscript{[195]}. HDPs are prohibitively expensive to synthesise in sufficient quantities for treatments, they can be degraded by many host proteases and some bacterial secreted enzymes\textsuperscript{[50, 195]}, and their direct effects can be antagonised by the high concentrations of cations and anions in many physiological conditions\textsuperscript{[195, 221]}. Finally, whilst immunomodulation by HDPs may aid in the resolution of infections and inflammation, inappropriate stimulation of inflammation by administered HDPs can result in undesirable effects such as immunosuppression and toxicities involving inflammatory tissue damage, mast cell degranulation and apoptosis\textsuperscript{[50, 195, 219, 276, 293]}. 
Of particular relevance to this work are the deleterious effects of the cathelicidin LL-37. It was discovered that administration of high doses of LL-37 has adverse effects during treatment of sepsis in neonatal rats (lower doses were protective)[109] and led to inflammation typically associated with rosacea in mice[195, 362]. In addition, unusually high levels of LL-37 are found in humans with rosacea, which is exacerbated by a proteolytic processing abnormality resulting in longer forms than in healthy individuals[281, 362] thus increasing their pro-inflammatory activities as previously described (Section 1.4.3)[35]. It will therefore be an important step in the development of HDPs and IDR peptides as potential new therapies to prove that their use in doses suitable for therapy is safe. One option for reducing the toxic effects of treatment with these peptides is to avoid systemic dosing, which would be possible in the treatment of TB infections through intratracheal administration.

The generation of synthetic cationic or IDR peptides using D- or non-natural-amino acids[138] with improved stability[161], direct[161] and immunomodulatory activities[221, 293], and/or reduced cytotoxicities[221, 293] may provide a way forwards for each of the barriers outlined above[195, 305]. For example, synthetic peptides comprised of D-amino acids displayed increased resistance to trypsin digestion and one of these, II-D, displayed increased direct antimicrobial activity against multiple mycobacterial strains, including M. tuberculosis[161]. In addition, the effectiveness of D-forms of LL-37 have been assessed and shown to induce IL-8 release by keratinocytes[35] and successfully block IL-6 production by LPS-stimulated DCs[79], suggesting that use of the D-forms of HDPs in immunotherapies may also have potential.

1.4.5.2 Cathelicidins as novel therapeutics for TB infections

Previous studies carried out by two groups (Modlin and Rivas-Santiago), have suggested roles for cathelicidins and other HDPs in the response to TB infections and also recommended the cathelicidin family of HDPs as novel therapies for TB disease. Rivas-Santiago et al. demonstrated that LL-37 is induced in many human cell types upon stimulation with M. tuberculosis itself as well as lipoarabinomannan and M. tuberculosis DNA[277]. The Modlin group have demonstrated that LL-37 mediates the protective effects of Vitamin D in human TB infections. Firstly, they demonstrated that Vitamin D
treatment induced LL-37 expression in human monocytes\textsuperscript{[185]}, which was also demonstrated in a separate study\textsuperscript{[290]}. The antimicrobial effect of Vitamin D was shown to require LL-37 through siRNA knockdown, which resulted increased bacterial growth in human monocytes despite Vitamin D treatment\textsuperscript{[186]}. Finally these \textit{in vitro} data were shown to have significance \textit{in vivo} through the presence of reduced levels of Vitamin D and cathelicidin in the sera of African-American individuals, known to be more susceptible to TB infections\textsuperscript{[185]}. These findings were independently confirmed through the correlation of Vitamin D deficiency and reduced levels of LL-37 in lung granulomas following TB infections\textsuperscript{[265]}.

The expression of HDPs during TB infections was further investigated by Rivas-Santiago \textit{et al.} Initially, it was shown that susceptibility to TB infections in mice correlated with reduced expression of a different family of HDPs, the defensins\textsuperscript{[280]}. They went on to investigate the role of the other major family of HPDs in TB infections, analysing expression of the murine cathelicidin cathelin-related antimicrobial peptide (CRAMP) upon infection with \textit{M. tuberculosis}. The study revealed that CRAMP expression peaks three times during infection: early (day 1), at the peak of protective immunity (day 21), and also when progressive infection starts (day 28)\textsuperscript{[46]}. Analysis of CRAMP expression at day 60 during progressive infection demonstrated reduced mRNA levels for CRAMP, although significant quantities were still found inside infected macrophages. The timings of the expression peaks are significant as they suggest a role for cathelicidins in protection against TB infections or in pathology of the disease.

The lack of LL-37 expression during progressive and latent TB infections has been confirmed \textit{in vivo}. LL-37 expression and/or protein presence was found to be equivalent to healthy hosts in: tissue staining of caseous granulomatous samples taken from TB infected individuals\textsuperscript{[277]}; tissue staining and gene expression analysis of cutaneous tuberculosis lesions\textsuperscript{[374]}; and gene expression analysis of blood samples (notably blood samples from individuals with active infection did contain elevated levels of LL-37 compared to healthy individuals)\textsuperscript{[116]}. The combination of the cathelicidin expression data with that of the Modlin group suggests a protective role for cathelicidins in the early host defence against infection, but their role in later infection is unclear as they may act to control inflammation during progressive infection or as pro-inflammatory agents which exacerbate the disease.
The protective effects of cathelicidins may be due to their direct antimycobacterial activities, with LL-37 capable of killing *M. tuberculosis*[^279]. Electron micrographs of the bacilli during LL-37 treatment revealed cell wall thinning, disruption and/or budding, suggesting that the direct effects may be due to perturbation of bacterial membrane integrity[^279]. Furthermore, the many immunomodulatory activities of LL-37 (Table 1.3) may provide protective effects through enhancement of pathogen clearance and control of inflammation. Some of these activities have been shown during TB infections: LL-37 induced autophagy[^370] to reduce mycobacterial burden[^120] in macrophages infected with *M. tuberculosis*; LL-37 also increases production of pro-inflammatory cytokines (TNF-α and IL-17) and anti-inflammatory cytokines (IL-10 and Transforming Growth Factor β (TGF-β)) in macrophages infected with *M. tuberculosis*. These findings indicate that LL-37 controls cytokine expression and autophagy during TB infections to increase the host defence.

Evidencing that their role in the host defence against TB infections is relevant *in vivo*, studies have demonstrated effective treatment with endogenous and exogenous HDPs or IDR peptides of mice infected with *M. tuberculosis*[^276, 282]. Treatment with arginine-rich synthetic derivatives of the bovine cathelicidin bactenecin, IDR-HH2 or IDR-1018, led to a reduction in bacterial burden and pneumonia at the progressive infection stage in the murine pulmonary TB infection model for both the antibiotic-sensitive H37Rv and MDR strains of *M. tuberculosis*[^276]. In addition, prophylactic and therapeutic treatment of mice with β-defensins also demonstrated protective effects against TB infection[^278, 282], suggesting that HDPs may also be a suitable therapy. However, whilst treatment of mice with LL-37 at the progressive stage of infection, associated with pneumonia, lead to a decrease in bacterial burden, there was also an increase in pneumonia[^279]. This study would suggest caution in the use of cathelicidins, and LL-37 in particular, as a treatment for late stage TB infections and indicates that the high levels of murine CRAMP associated with infected macrophages at 60 days post infection[^46] may exacerbate disease.

Collectively these reports indicate a protective role in nature for cathelicidins at the early infection stages with TB and strongly recommend cathelicidins and IDR peptides

[^279]: Reference number
[^370]: Reference number
[^120]: Reference number
[^276]: Reference number
[^282]: Reference number
[^46]: Reference number
as promising new therapies for early and MDR TB disease as well as prophylaxis, following further investigation into dosage and treatment timings.

1.4.6 HDPs selected for study

The findings for the importance of cathelicidins in TB infections (Section 1.4.5.2) lead to our hypothesis that treatment with exogenous cathelicidins may be beneficial to the human host during TB infections. Our collaborator, Dr Sam Willcocks, performed some initial experiments to test the direct antibacterial and immunomodulatory capabilities of the bovine cathelicidin Bac5, which is the exogenous peptide of interest in this study. A Bac5-A549 human epithelial cell line which constitutively expresses Bac5 and exports it to the extracellular medium was generated. The initial results revealed that: the Bac5-A459 cell supernatant was capable of direct killing of E. coli but not mycobacteria (either M. smegmatis or M. bovis BCG); and the Bac5-A549 cell line produced slightly increased levels of IL-8 and MMP-9 in response to challenge with M. smegmatis and M. bovis BCG, and significantly increased expression of both cytokines in response to TNF-α (Sam Willcocks, personal communication). These data led us to wish to investigate the therapeutic potential of Bac5 on mycobacterial infections in vivo using the M. marinum-zebrafish model of TB infection as it may form a promising novel therapy for TB disease.

As a first step, we wished to confirm the lack of direct antimycobacterial activities of Bac5 and investigate its immunomodulatory effects in vitro during M. marinum infection. We selected two control HDPs, for use predominantly in the in vitro experiments. The first is the fish piscidin epinecidin-1 (EPI), which serves as a control for the effects of a HDP with no direct antimycobacterial activities and of a fish peptide with immunomodulatory activities. This peptide was selected as its immunomodulatory functions have been previously studied in zebrafish experiments (Table 1.4). The second control HDP is the human cathelicidin LL-37, which serves as a control for the effects of a HDP with direct antimycobacterial activities and of a human HDP with immunomodulatory activities. LL-37 was selected due to its involvement in the innate immune defence against M. tuberculosis infection (Section 1.4.5.2).
1.5 Bacterial two-component signal transduction systems (TCSs)

1.5.1 Introduction to bacterial TCSs and their general properties

During the course of infection infecting bacteria experience varied and changing environmental conditions\cite{288}, including stresses imposed upon them by the immune system during anti-microbial responses. Bacteria employ selective gene expression at different stages of an infection to neutralise, or at least decrease, these anti-microbial actions of the immune system\cite{171}. Such environmental adaptation in response to specific stimuli in bacteria, including chemical and physical signals, is mediated primarily through the expression of transcriptional regulators. These include extracytoplasmic function sigma factors, serine-threonine protein kinases and two-component signal transduction systems (TCSs)\cite{36}. TCSs are present in some archaea, plants, and lower eukaryotes, but are ubiquitous in bacteria\cite{162}. The complexity of the environment(s) in which a bacterium typically resides combined with its genome size generally determines the number of TCSs it possesses\cite{36,112}. Bacterial TCSs have been shown to regulate gene expression in response to external stimuli, such as pH, nutrient availability, and the presence of antibiotics\cite{36,170}. Through these actions TCSs control many physiological processes, including virulence, transition into stationary phase, and antibiotic resistance\cite{36,170}. There is an excellent review on the TCSs of \textit{M. tuberculosis} by Bretl \textit{et al.} (2011)\cite{36}, including their role in virulence, which has influenced the content of Section 1.5.

Prototypical TCSs comprise a membrane-localised histidine sensor kinase (SK) and a cytosolic response regulator (RR) which are normally genetically linked in operons\cite{36}. However, cytosolic SKs are also possible, for example the PdtaS sensor\cite{259} which is also unusual in that it is genetically separated from its RR in \textit{M. tuberculosis}\cite{36}. A second unusual system exists in \textit{M. tuberculosis}, that of the RR DosR (also called DevR) which is genetically linked to its first cognate SK, DosS (also called DevS), but has an additional cognate SK which is located elsewhere in the genome, DosT (also called DevT)\cite{36,141,283}. The normal genomic organisations and functions of TCSs are shown in Figure 1.8.

SKs and RRs have been identified by their conserved domains and mediate signal transduction events from the external environment to inside the bacterial cell\cite{36,259},
permitting adaptation to the environment. Binding of the specific stimuli by the SK external sensor domain results in signal transmission through one or more transmembrane domains and the flexible linker region to the cytoplasmic region (containing the conserved ATPase and kinase domains)\[36, 259\]. The SK then usually forms a homodimer, binding in the cytosolic regions, and autophosphorylates at the conserved phosphorylation site within the cytosolic regions. Transfer of the phosphate, either directly or via phosphodonor intermediates, to the conserved receiver domain of the cognate RR results in a conformational change in the second conserved RR domain, the DNA-binding domain\[36, 112, 136, 259, 314-316, 355\]. The DNA-binding activities of the RRs increase following activation by phosphorylation such that they bind promoter regions of their target genes, increasing or decreasing their expression. The group of genes whose expression are affected by an RR are collectively termed its regulon and the differential expression of these genes permits the necessary environmental adaptations.

![Diagram](image)

**Figure 1.8. Genomic organisation and mechanism of action of TCSs.** A) Genetically linked and transcriptionally coupled TCSs genomic organisation. B) Typical TCSs mechanism of action. The SK undergoes autophosphorylation in response to an external stimulus, this phosphate group is then transferred to the RR, leading to the response. Some SKs can also act as phosphatases, increasing the control of RR activity and allowing more precise control of gene expression in response to environmental stimuli. Some RRs are under the control of more than one SK.

1.5.1.1 *Regulation of TCSs*

The vast majority of RRs are active and exert their biological functions only when phosphorylated\[112, 140\], therefore regulation of TCSs activity is possible through control
of RR phosphorylation. Such control is possible through the presence and detection of the specific environmental signal, SK phosphorylation, RR phosphorylation and RR dephosphorylation\cite{36, 205}. These events can all be affected by TCSs components or additional entities, providing multiple targets for the regulation of TCSs activity. For example, certain SKs possess RR phosphatase activities, including DosS in the \textit{M. tuberculosis} DosR/S/T system, enabling regulation of the system activation levels and a system reset when the environmental signal is absent\cite{158}. In addition, TCSs connectors are a group of proteins which modulate the activity of SKs and RRs at the post-translational level\cite{205}. TCSs connectors are typically synthesised in response to signals which are distinct from those sensed by the SK and establish regulatory links between otherwise independent TCSs, leading to a certain degree of cross-talk between different TCSs of the same organism. Targeting of any of the control steps during treatments would affect the activity of a TCSs and bacterial virulence.

\subsection*{1.5.1.2 Specificity and cross-talk in TCSs}

The activation of different TCSs regulons enables bacterial survival in different conditions, with specificity and cross-talk within and between different TCSs crucial for survival. Specificity is achieved through many different mechanisms\cite{6}. These include spatial separation in the cell of different TCSs, preferential interactions between the cognate SKs and RRs, and the possession of phosphatase activity by an SK. As the SK and RR have co-evolved together, they have developed specific interactions which can lead to discrimination by the SK against other substrates and preferential activation of the cognate RR. Phosphatase activity of an SK serves to block non-cognate activation of the RR in the absence of the specific environmental signal.

In addition to specificity in the different TCSs system, some cross-talk to enable a coordinated response to multiple signals or a diverse response to one signal would also benefit bacteria when adapting to different environments or resisting stresses. Crosstalk can also be achieved through many different mechanisms\cite{6}. These include cross-regulation of one TCSs by another, RR-RR heterodimerisation leading to simultaneous gene regulation, and co-regulation of target genes. For example, the \textit{M. tuberculosis} PhoP/R TCSs has recently been shown to orchestrate adaptation to hypoxia indirectly through PhoP activation of DosR expression, leading to the control of the
DosR regulon\textsuperscript{[110, 117, 289]}. In addition, NarL and DosR of \textit{M. tuberculosis} have been shown to interact and co-regulate 16 genes which are common between the two regulons\textsuperscript{[192]}. Furthermore, PhoP and MprA of \textit{M. tuberculosis} co-regulate expression of the transcription factor EspR, which may itself mediate many of the regulatory effects of both TCSs\textsuperscript{[42]}. The cross-talk between different TCSs may explain why knockdown of either the SK or RR does not lead to complete ablation of the associated response\textsuperscript{[6]}.

In light of the capacity for cross-talk between different TCSs systems, effective treatments targeting TCSs may require the targeting of multiple systems or key systems which display little cross-talk to achieve the desired effect.

\subsection*{1.5.1.3 \textit{M. tuberculosis} TCSs}

As previously discussed, \textit{M. tuberculosis} is able to survive within the hostile environment provided by phagocytes and persist within the human host in granulomas and their hostile environment for decades. In order to fully understand LTBI, it is necessary to elucidate the mechanisms the bacilli use to adapt to their hosts during infection, particularly during granuloma survival. \textit{M. tuberculosis} possesses 12 complete TCSs, four orphaned RR\s and one orphaned SK\textsuperscript{[36]}. That \textit{M. tuberculosis} has evolved with a predominantly intracellular lifestyle and as a strict human pathogen may explain the relatively low number of TCSs in comparison to its genome size\textsuperscript{[36]}. Genomic analysis of the 11 genetically linked TCSs present in \textit{M. tuberculosis} revealed that homologues of these genes exist in other mycobacterial species\textsuperscript{[36]}. The obligate intracellular pathogen \textit{M. leprae} with its further restricted ecological niche possesses only five of these TCSs: \textit{RegX3/SenX3, PrrA/B, MprA/B, MtrA/B} and \textit{PdtaR/S}. The conservation of these TCSs between two of the more distantly related members of the mycobacterial family would suggest that they each regulate fundamental physiological or virulence processes and renders them of particular interest for future studies\textsuperscript{[36]}. The essentiality of \textit{MtrA/B} and \textit{PrrA/B} in \textit{M. tuberculosis} confirms the importance of these systems.

Many of the TCSs have been implicated in the virulence of \textit{M. tuberculosis} during studies of TCSs mutant strains, which are detailed in Section 1.5.3, confirming their importance during infections and the need to study these systems if we are to understand the adaptation of \textit{M. tuberculosis} to the human host environment. In addition, recent studies
have implicated TCSs in bacterial antibiotic resistance, further suggesting that they play a key role during infection and recommending their study. For example, a recent transcriptomics and proteomics study of 90 TB isolates revealed that DosR, MtrA and RegX3 are all upregulated in drug-resistant strains\[376]. However, it has not yet been established whether upregulation of these genes causes the drug resistance or results from adaptation to stresses due to drug exposure and longer-term survival within the host. Further study of the TCSs and their role in infections would provide valuable insights into their functions and inform on new therapies for TB infections targeting the adaptation of the bacteria to the host or current drug treatments.

1.5.1.4 The DosR/S/T TCSs of M. tuberculosis – hypoxia adaptation and latency

A specific example of how a TCSs can lead to environmental adaptation is provided here with the DosR/S/T system of M. tuberculosis. This system has been widely studied due to its role in adaptation to hypoxia and therefore the latent phase of the bacilli, as it is thought that survival of hypoxia and other stresses in the granuloma leads the bacteria to enter an NRP state and cause latent infection. In this unusual system the RR DosR is controlled by two different SKs, DosS and DosT, which both bind haem as a prosthetic group\[172]. DosS is a redox sensor whilst DosT responds to hypoxia\[172], and together they sequentially control the response of M. tuberculosis to hypoxia. As the hypoxia sensor, DosT orchestrates the early response to oxygen limitation, sensing hypoxia and initially inducing the DosR regulon, before losing functionality as oxygen becomes increasingly limited\[141]. The DosR regulon includes DosS, which initially sustains the response caused by DosT, and further induces the regulon, until DosS maintains the induction of the DosR regulon alone as hypoxia continues\[141].

Further studies have confirmed the role of this TCSs in hypoxia adaptation. A consensus binding motif was identified for DosR upstream of genes induced by hypoxia\[248]. In addition, a DosR deletion mutant in the model mycobacteria strain M. smegmatis loses culturability in hypoxic stationary phase and demonstrates reduced expression under hypoxic conditions of a gene (hspX) normally highly upregulated during hypoxia\[227]. Furthermore, DosR induces expression of a ribosome stabilising factor during hypoxia, which is thought to be responsible for maintaining ribosome integrity during NRP survival of hypoxia to enable reactivation once oxygen becomes available\[332].
The role of the DosR/S/T system in the adaptation to hypoxia and the latent state has also recently been confirmed in vivo. The DosR regulon is constitutively overexpressed in all members of the hypervirulent Beijing strain lineage, with a single nucleotide polymorphism in the promoter region for DosR decreasing its expression and the DosT sensor being non-functional\cite{80}. These features are presumed to lead to an advantage within the host. One possible mechanism is by the reduced metabolic rates of the strains, which in turn provide them with the ability to persist for long periods of time under antibiotic treatments\cite{71} and potentially also the stresses applied to infecting bacilli by the host. It has also been shown that specific antibodies and T-cell responses for members of the DosR regulon are some of the most frequently found in infected individuals as well as household contacts\cite{164,173}. Furthermore, a positive IFN-γ release assay test to Rv2626c, one member of the DosR regulon, has been shown to distinguish between actively and latently infected individuals, even in BCG-vaccinated individuals, providing the possibility of rapid screening for latent infections for the first time\cite{251}.

Finally, the importance of the DosR/S/T TCSs in the virulence of \textit{M. tuberculosis} has also been demonstrated in studies of mutants, although this has sometimes been controversial (Section 1.5.3). The studies involving DosR/S/T mutants are summarised in Table 1.5 along with those for the other \textit{M. tuberculosis} TCSs. Overall, the DosR/S/T TCSs controls the adaptation of \textit{M. tuberculosis} to hypoxia and plays a key role in latency and reactivation of TB disease. Understanding this system is therefore crucial in the fight against LTBI and in the development of novel therapies against TB disease.

### 1.5.2 TCSs as targets in novel therapies

TCSs act to promote infections or control them to prevent excessive host damage associated with rapid death and non-productive infection. Therefore, different TCSs could be targeted for inhibition or activation to treat infections. A general advantage of TCSs as drug targets is that they are ubiquitous among bacteria and possess conserved structural motifs; these motifs are absent from higher eukaryotes, allowing for selective and safe targeting by inhibitors\cite{20,21,36}. The conserved motifs also permit the development of drugs with broad specificities which are able to target multiple TCSs simultaneously\cite{196}. This would be expected to lead to reduced development of genetic
resistance by bacteria and may be a requirement for effective therapy due to the cross-talk between the different systems. A recent study has described the development of an inhibitor with the capacity to target the DNA-binding activity of multiple RRs of the OmpR family in *M. tuberculosis* through molecular docking studies using the conserved motif. The inhibitor, C2, would be expected to target multiple members of the OmpR family (PhoP, MtrA, MprA, RegX3, PrrA, TrcR and TcrX) and was proven to affect the expression of MtrA, MprA and RegX3. Treatment with C2 resulted in an altered immune response with increased autophagy and nitric oxide production and lead to reduced bacterial burden of infected macrophages, indicating that such targeting of multiple TCSs may prove to be a promising new therapy.

TCSs are particularly attractive as novel therapies for TB disease due to the treatment possibilities they enable, the benefits of which in treating TB infections have been previously discussed. Firstly, that they have been implicated in mycobacterial virulence and drug resistance by studies of *M. tuberculosis* mutant strains is promising for their use as novel therapeutics. In addition, two of the *M. tuberculosis* TCSs have proven to be essential, providing ideal targets for new therapies. Moreover, most TCSs are not essential for growth under normal conditions, and therefore targeting TCSs only places selection pressure on the bacteria during certain environmental conditions, reducing the risk of selection for genetic resistance. In addition, TCSs provide a different target to antibiotics as they are required for virulence, drug resistance and dormancy, permitting the treatment of MDR infections and LTBI, and reducing the risk of developing genetic resistance. Targeting of TCSs, and therefore drug resistance to traditional antibiotics, also provides the possibility of combination therapies which are more effective. Furthermore, targeting of the ability of the infecting bacilli to survive within granulomas may enable the treatment of LTBI either directly or by disabling of the NRP state, forcing the bacteria into an actively replicating state which can be effectively treated with current antibiotics.

As a result of their attractiveness as drug targets, studies have been undertaken to identify inhibitors of *M. tuberculosis* TCSs. Several studies have targeted the DNA-binding domain of the RR, including the example of C2 given above and one study using DosR as bait to screen a phage library. The resulting inhibitor was capable of inhibiting DosR-mediated transcription and survival during hypoxia, demonstrating the
efficacy of targeting DosR function to prevent the adaptation of *M. tuberculosis* to hypoxia in vitro. Another method for generating inhibitors of TCSs has been to target the binding of the SK to the RR. For example, two inhibitors with sequence similarity to the N-terminal domain of DosR were found by screening a phage library for binding activity against the DosR-binding domain of DosS[159]. Treatment with the DosR mimetics prevented DosS autophosphorylation, thereby preventing activation of the TCSs, and restricted DosR-mediated transcription and hypoxic survival of *M. tuberculosis* in vitro.

In addition to the investigation of TCSs targeting as a novel therapy for TB infections, TCSs deletion strains and TCSs associated antigens are being investigated as novel vaccines due to their transient survival in the human host[377] and potential to protect against latent infections, respectively. The presence of immune responses to DosR regulon proteins and the detection of the proteins themselves in the sera of individuals infected with LTBI has led to interest in vaccines targeting these proteins, or dormancy antigens as they are also known. Such vaccines would permit the targeting of LTBI and the prevention of reactivation, with two vaccines currently under clinical trials for boosting the BCG vaccination with DosR regulon antigens[241]. One recent study has also described successful protection of mice against a hypervirulent Beijing strain following vaccination with a DosR regulon antigen and an adjuvant for TLR4[173]. The study of TCSs deletion strains as live vaccine candidates has focussed on attenuated PhoP/R deletion mutant strains and these have provided promising results of protection against TB infection in mice, guinea pigs and goats[7, 29, 36, 197, 342]. One potential vaccine was identified, initially called strain SO2 (*phoP* deletion only) and later MTBVAC (double mutant), and is described further here.

MTBVAC combines deletion of *phoP* and *fad26*, which encodes a protein required for the synthesis of phtiocerol dimycocerosates (mycobacterial cell envelope components)[233]. This satisfies the second Geneva Consensus on live TB vaccines which requires “two non-reverting independent mutations”[348], each leading to attenuation and without antibiotic resistance markers, for a strain to be suitable and avoid reversion to wild-type. MTBVAC is intended for use as a TB vaccine for neonates and has entered clinical trials in adults[8]. Initial safety and efficacy studies were conducted in mice and guinea pigs, with the strain proving safe in both models and providing increased protection against TB infection of mice when compared to BCG vaccination[111]. Further safety and
efficacy studies in neonatal mice revealed that the strain is safe, showing no effect on organ development and growth, and provided enhanced immunogenicity and better protection against *M. tuberculosis* than BCG vaccination\[^8\]. A potential mechanism for the increased protection is the increased expansion and prolonged presence of *M. tuberculosis*-specific CD4\(^+\) T-cells in mice after vaccination with the SO2 strain compared to BCG vaccination, with these increased responses correlating with better recall responses upon challenge with *M. tuberculosis*\[^216\].

A recent study has described the development and initial testing of a further attenuated form of MTBVAC\[^306\]. A third deletion, that of the secreted virulence factor *erp*, was added to the strain to provide a potential vaccine for high-risk populations of immunosuppressed individuals (BCG vaccination is not recommended for these groups). The MTBVAC erp(-) strain was attenuated in SCID mice (which lack a functional adaptive immune system) compared to BCG and the parent strain MTBVAC, and also provided protection against infection with *M. tuberculosis*. Such a vaccine is of particular importance for TB infection prevention as it would permit vaccination of HIV-infected individuals, countering the growing threat of synergy between the two pathogens as HIV infections increase.

These studies into novel TB vaccines are especially significant for TB infection prevention as there is currently no effective vaccine which protects adults against pulmonary tuberculosis, the most common form of the disease. The successes with the use of TCSs deletion mutant strains as TB vaccines recommends the further investigation of the remaining TCSs to determine whether there are any further vaccine candidates amongst them.

1.5.3 TCSs selected for study

Previous studies have suggested TCSs targeting as a novel therapy for TB disease through identification of their involvement in mycobacterial growth and virulence using *M. tuberculosis* mutant strains in tissue culture and also in animal infection models. The studies of *M. tuberculosis* mutant strains for the 11 complete TCSs selected for study are summarised in Table 1.5. The selected systems are those to which *M. marinum* possesses homologues. The presence of homologues to so many of the TCSs confirms *M.
*marinum* as a suitable model for the study of mycobacterial TCSs and is expected as *M. marinum* is the closest genetic relative of the MTBC and possesses similar pathology to *M. tuberculosis*, as previously discussed. The summary highlights that some of the selected TCSs have been more widely studied than others, leading to the functional roles played by many of these TCSs, as well as the regulon genes and environmental signals they respond to, being poorly understood. For example, little is known about the TcrX/Y system but the DosR/S/T system has been extensively studied and is very well characterised. This provides the opportunity to significantly enhance the knowledge of some of these systems.

The functional roles of TCSs are often elucidated through studies of mutant strains in these systems, through which the environmental signal(s) the TCSs responds to and fitness advantage they confer can be identified. Studies of several TCSs have recently demonstrated that their roles in mycobacterial virulence may be due to control of ESX secretion systems. There are five Type VII ESX secretion systems in *M. tuberculosis* which function to export factors which counteract the host defences and enable bacterial survival during infections, for example factors to prevent phagosome-lysosome fusion\(^{[91]}\). RegX3/SenX3 has recently been shown to control expression of a subset of genes in the ESX-5 locus, controlling the secretion system\(^{[92]}\). In addition, MprA and PhoP both regulate EspR expression, and this transcription factor in turn controls ESX-1 secretion system expression\(^{[42]}\). Therefore, EspR may in fact mediate the effects of MprA/B and PhoP/R on virulence\(^{[42, 242]}\) and the severe attenuation upon deletion of *phoP* which is being exploited in novel vaccines may be due to reduced ESX-1 activity\(^{[289]}\). There is further evidence that the lack of ESX-1 system activity may be crucial for attenuation of these strains from the fact that BCG and H37Ra, the attenuated strain of *M. tuberculosis*, both lack ESAT-6 secretion (which is a key factor exported by ESX-1) and providing ESX-1 or PhoP rescues their ability to control autophagy of infected macrophages\(^{[108, 284]}\). These studies are promising for the identification of further targets for novel therapies or further vaccine candidates through the study of the other TCSs of *M. tuberculosis*.

There are several other findings from the *in vivo* studies of *M. tuberculosis* TCSs mutants which are also significant for this work. That 8 of the 11 systems displayed differential virulence upon targeting is promising for TCSs targeting in novel therapies. For
example, RegX3/SenX3 and PhoP/R deletion mutants displayed consistent attenuation in different murine infection models whilst KdpD/E and TcrX/Y deletion mutants displayed hypervirulence in single murine infection models. However, in some cases the use of multiple mouse strains to study independent mutations in the same TCSs has led to conflicting results, such as for TrcR/S. In addition, the use of different hosts in studies of DosR/ST mutants led to the presentation of the full range of virulence phenotypes (attenuation, no phenotype and hypervirulence) for the targeting of a single TCSs.

These differences in the TCSs mutant virulence phenotypes between hosts can be explained by the limitations of the laboratory hosts used in studies of *M. tuberculosis* infections, which have been previously discussed (Section 1.2.1) and were reviewed for the studies of DosR/S/T by Voskuil (2015)[346]. As the DosR/S/T system is involved in adaptation to hypoxia, the use of a model with caseating and hypoxic granulomas would be key to its study. The majority of the studies of DosR/S/T mutants in mice displayed the expected result of no effect on virulence. The studies of these mutants in models which display caseating, hypoxic granulomas and therefore inhibited the growth and replication of *M. tuberculosis* (including the key study using macaques which was published by Mehra et al.[202] during this PhD), led to consistent attenuation following deletion of DosR/S/T. It can therefore be concluded both that the TCS is required for *M. tuberculosis* adaptation to hypoxia and virulence as well as that the use of suitable models for TB infection is critical in studies of the roles of TCSs.

The conflicting data on TCSs and their role in *M. tuberculosis* virulence leads to a need for greater clarity with respect to the roles of these TCSs in mycobacterial infections, which I argue can be obtained from the study of a natural infection pairing, that of *M. marinum* infection of zebrafish. As zebrafish embryos provide a higher throughput model for mycobacterial infections, deletion mutants for each TCSs could practicably be screened in the same model.
Table 1.5. Environmental signals, regulons and virulence characteristics of deletion mutants for the selected TCSs.

<table>
<thead>
<tr>
<th>TCSs</th>
<th>Environmental signal</th>
<th>Regulon gene number</th>
<th>Mutation(s)</th>
<th>Strain</th>
<th>In vitro model(s)</th>
<th>In vitro phenotypes</th>
<th>In vivo model(s)</th>
<th>In vivo phenotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>RegX3/</td>
<td>P[323], oxidative</td>
<td>~100</td>
<td>(\Delta (\text{senX3/\text{regX3}})) ([246])</td>
<td>H37Rv</td>
<td>mBMDM &amp; hTHP-1</td>
<td>Attenuated</td>
<td>DBA/2 &amp; SCID mice</td>
<td>DBA/2: attenuated; SCID: delayed time to death</td>
</tr>
<tr>
<td>SenX3</td>
<td>stress?[273]</td>
<td></td>
<td>(\Delta \text{senX3} \text{ or } \Delta \text{regX3}[273])</td>
<td>H37Rv</td>
<td>ND</td>
<td>ND</td>
<td>BALB/c mice</td>
<td>Attenuated</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(\text{regX3::Tn}[275])</td>
<td>CDC1551</td>
<td>ND</td>
<td>ND</td>
<td>BALB/c mice &amp; guinea pigs</td>
<td>Attenuated</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(\text{regX3::Tn or senX3::Tn}[274])</td>
<td>CDC1551</td>
<td>ND</td>
<td>ND</td>
<td>BALB/c mice</td>
<td>Attenuated (significantly greater effect for \text{regX3::Tn})</td>
</tr>
<tr>
<td>PhoP/R</td>
<td>Acidic pH, Cl[321]</td>
<td>&gt;150</td>
<td>(\text{phoP::Km}[253])</td>
<td>MT103</td>
<td>mBMDM</td>
<td>Attenuated</td>
<td>BALB/c mice</td>
<td>Attenuated</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(\text{phoP::Km}[197])</td>
<td>MT103</td>
<td>ND</td>
<td>ND</td>
<td>SCID mice</td>
<td>No death</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(\text{phoP::Km}[349])</td>
<td>H37Rv</td>
<td>hTHP-1, mAMJ2-C11, &amp; mJ74A.1</td>
<td>Attenuated</td>
<td>C57BL/6 mice</td>
<td>Attenuated</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(\Delta \text{phoPR::HygR})</td>
<td>1237</td>
<td>hTHP-1</td>
<td>Attenuated</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(\Delta \text{phoP::HygR}[118])</td>
<td>MT103</td>
<td>NRK-49F, &amp; HL, &amp; MRC-5</td>
<td>NRK-49F: no attenuation; HL &amp; MRC-5: decreased cytotoxicity</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(\text{phoP::Km}[99])</td>
<td>MT103</td>
<td>Human monocytes</td>
<td>Attenuated - increased late endosome trafficking</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(\text{phoP::Km}[98])</td>
<td>MT103</td>
<td>Human monocytes</td>
<td>Attenuated - increased late endosome trafficking</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Gene</td>
<td>Condition</td>
<td>Description</td>
<td>Δ Gene</td>
<td>Strain</td>
<td>Environment</td>
<td>Virulence</td>
<td>Animal</td>
<td>Notes</td>
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<tr>
<td>NarL/Rv</td>
<td>Nitrate (E. coli, P. aeruginosa); ND (Mtb)</td>
<td></td>
<td>ΔnarL&lt;sup&gt;[245]&lt;/sup&gt;</td>
<td>H37Rv</td>
<td>mBMDM</td>
<td>No phenotype</td>
<td>SCID mice</td>
<td>No phenotype</td>
</tr>
<tr>
<td>PrrA/B</td>
<td>Nitrogen-limiting conditions&lt;sup&gt;[131]&lt;/sup&gt;</td>
<td></td>
<td>prrA::Tn9563&lt;sup&gt;[95]&lt;/sup&gt;</td>
<td>MT103</td>
<td>mBMDM</td>
<td>Attenuated</td>
<td>BALB/c mice</td>
<td>No phenotype</td>
</tr>
<tr>
<td>MprA/B</td>
<td>SDS, alkaline pH, nutrient limitation&lt;sup&gt;[36, 243]&lt;/sup&gt;</td>
<td>&gt;200</td>
<td>ΔmprAB&lt;sup&gt;[243]&lt;/sup&gt;</td>
<td>H37Rv</td>
<td>Human monocytes</td>
<td>Hypervirulent</td>
<td>BALB/c mice</td>
<td>Lungs &amp; spleen: attenuated; liver: no phenotype</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ΔmprAB&lt;sup&gt;[242]&lt;/sup&gt;</td>
<td>H37Rv</td>
<td>Human monocytes</td>
<td></td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>K&lt;sup&gt;+&lt;/sup&gt;, ATP, osmolarity (E. coli); ND (Mtb)</td>
<td>&gt;4&lt;sup&gt;[377]&lt;/sup&gt;</td>
<td>ΔkdpDE&lt;sup&gt;[245]&lt;/sup&gt;</td>
<td>H37Rv</td>
<td>ND</td>
<td>ND</td>
<td>SCID mice</td>
<td>Enhanced time to death</td>
</tr>
<tr>
<td>TrcR/S</td>
<td>ND</td>
<td>~50</td>
<td>trcS::Tn&lt;sup&gt;[95]&lt;/sup&gt;</td>
<td>MT103</td>
<td>mBMDM</td>
<td>No phenotype</td>
<td>C57BL/6 mice</td>
<td>No phenotype</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ΔtrcS&lt;sup&gt;[245]&lt;/sup&gt;</td>
<td>H37Rv</td>
<td>ND</td>
<td>ND</td>
<td>SCID mice</td>
<td>Enhanced time to death</td>
</tr>
<tr>
<td>DosR/S/T</td>
<td>Low O&lt;sub&gt;2&lt;/sub&gt;, NO, CO, ascorbate&lt;sup&gt;[36]&lt;/sup&gt;</td>
<td>~100</td>
<td>dosR::Km&lt;sup&gt;[193]&lt;/sup&gt;</td>
<td>H37Rv</td>
<td>Human monocytes</td>
<td>No phenotype</td>
<td>Guinea pigs</td>
<td>Attenuated</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ΔdosRT&lt;sup&gt;[56]&lt;/sup&gt;</td>
<td>H37Rv</td>
<td>Mouse hollow-fibre granuloma model</td>
<td>No phenotype</td>
<td>C57BL/6 &amp; BALB/c mice, guinea pigs, &amp; rabbits</td>
<td>Mice &amp; guinea pigs: attenuated; rabbits: slight virulence defect in lungs</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ΔdosR&lt;sup&gt;[245]&lt;/sup&gt;</td>
<td>H37Rv</td>
<td>mBMDM</td>
<td>Hypervirulent</td>
<td>SCID &amp; DBA/2 mice</td>
<td>SCID: enhanced time to death, DBA/2: hypervirulent</td>
</tr>
<tr>
<td>Strain</td>
<td>Tn</td>
<td>MtrA/B</td>
<td>TcrX/Y</td>
<td>PdtaR/S</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>ΔRv3134-dosS</td>
<td>H37Rv, ND, ND</td>
<td>C57BL/6 mice</td>
<td>Spleen: slight hypervirulence; lungs: no phenotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dosR::Km</td>
<td>H37Rv, ND, ND</td>
<td>C57BL/6, C3HeB/FeJ, &amp; DBA/2J mice</td>
<td>No phenotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔdosR, ΔdosS, ΔdosT</td>
<td>H37Rv, mBMDM</td>
<td>ΔdosS attenuated; ΔdosR &amp; ΔdosT ND</td>
<td>C3HeB/FeJ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔdosS, ΔdosT</td>
<td>H37Rv, ND, ND</td>
<td>Macaques</td>
<td>ΔdosS attenuated and altered immunity gene expression; ΔdosR &amp; ΔdosT no phenotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MtrA/B</td>
<td>ND &gt;4</td>
<td>Attenuated late</td>
<td>Attenuated late</td>
<td>Attenuated late</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mtrA overexpression</td>
<td>H37Rv, mJ774A.1 &amp; hPBM</td>
<td>C57BL/6 mice</td>
<td>C57BL/6 mice</td>
<td>C57BL/6 mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mtrA overexpression</td>
<td>H37Rv, hMDM</td>
<td>Attenuated</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mtrA overexpression</td>
<td>H37Rv, Human monocytes</td>
<td>Increased late endosome trafficking</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔtcrXY::HygR</td>
<td>H37Rv, ND, ND</td>
<td>SCID mice</td>
<td>Enhanced time to death</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Δpdtas</td>
<td>H37Rv, ND, ND</td>
<td>SCID mice</td>
<td>No phenotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Tn = transposon insertion; Km = kanamycin resistance gene; HygR = hygromycin resistance gene; m or h before cell line denotes murine or human origin, respectively. Table adapted and updated from Bretl et al. (2011).
1.6 Aims of this study

We aimed to assess the potential of the HDP Bac5 and targeting of bacterial TCSs as novel therapies against mycobacterial infections, as well as investigate *M. marinum* adaptation during zebrafish infection, focusing on the role of TCSs genes. To achieve this, techniques to quantify *M. marinum* infection progression in THP-1 cells and in the zebrafish embryo model, primarily from images acquired through live-imaging of zebrafish, were developed and/or optimised.

These techniques would be used to test the hypotheses that:

1) Bovine Bac5 would be capable of immunomodulation in human THP-1 cells and the zebrafish

2) Treatment with Bac5 would reduce bacterial burden and increase survival of infected zebrafish embryos and larvae

3) *M. marinum* TCSs deletion mutant strains can be screened for virulence in the zebrafish embryo model
2 Materials and methods

2.1 General materials and methods

2.1.1 Bacterial strains and plasmids

Bacterial strains used in this study are detailed in Table 2.1. Plasmids used in this study, with antibiotic selection, are detailed in Table 2.2 and Appendix 1. Plasmids generated in this study are detailed in Table 2.3.

Table 2.1. Bacterial strains used during this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description/genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli HB101</td>
<td>$F^-, thi-1, hsdS20 (rb-, mb-), supE44, recA13, ara-14, leuB6, proA2, lacY1, galK2, rpsL20 (strr), xyl-5, mtl-1$. High transformation efficiency $10^8$ CFU/μg; employed as an intermediate cloning strain and used in Bac5 direct bacteriocidal effects experiments</td>
<td>Promega</td>
</tr>
<tr>
<td>M. smegmatis mc²-155</td>
<td>Wild-type (WT) M. smegmatis</td>
<td>Laboratory strain</td>
</tr>
<tr>
<td>M. marinum strain M (a) &quot;London&quot;</td>
<td>WT M. marinum</td>
<td>ATCC® BAA-535™</td>
</tr>
<tr>
<td>M. marinum strain M (b) &quot;Leicester&quot;</td>
<td>Used in parallel with strain (a) in mutant generation experiments</td>
<td>Dr Galina Mukomalova (Leicester University)</td>
</tr>
<tr>
<td>GFP M. marinum strain M⁰</td>
<td>WT M. marinum expressing GFP from pGFPHYG2</td>
<td>Ramakrishnan group, via B. Robertson</td>
</tr>
<tr>
<td>DsRed M. marinum strain M⁰</td>
<td>WT M. marinum expressing DsRed2 from pMSP12:DsRed2</td>
<td>Ramakrishnan group, via B. Robertson</td>
</tr>
</tbody>
</table>

a = Used for recombineering experiments described in Chapter 5 only.
Table 2.2. **Plasmids used in this study.** Plasmid maps are shown in Appendix 1, Figures A1-4.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Selection in media</th>
<th>Strains</th>
<th>Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCR™-Blunt II-TOPO® (Invitrogen)</td>
<td>Kanamycin</td>
<td><em>E. coli</em> HB101</td>
<td>Amplification of cloning inserts.</td>
</tr>
<tr>
<td>pJV53ApR</td>
<td>Apramycin</td>
<td><em>E. coli</em> HB101 <em>M. marinum</em> M (a)</td>
<td>Amplified in <em>E. coli</em>. Mycobacteria-adapted recombineering for <em>M. marinum</em> strains labelled with DsRed2 and GFP.</td>
</tr>
<tr>
<td>pSMT100</td>
<td>Hygromycin</td>
<td><em>E. coli</em> HB101 <em>M. marinum</em> M (a)</td>
<td>Sucrose counter-selection. Amplified in <em>E. coli</em>. Suicide plasmid containing the <em>sacB</em> gene used in sucrose counter-selection.</td>
</tr>
<tr>
<td>pSMT3[^a]</td>
<td>Hygromycin</td>
<td><em>E. coli</em> HB101 <em>M. marinum</em> M (a)</td>
<td>Amplified in <em>E. coli</em>. Expression vector which transforms well into mycobacterial strains. Used to determine <em>M. marinum</em> strain transformation efficiency.</td>
</tr>
<tr>
<td>pMSP12::DsRed2[^b]</td>
<td>Kanamycin</td>
<td><em>E. coli</em> HB101 <em>M. marinum</em> M (a)</td>
<td>Amplified in <em>E. coli</em>. Used to generate <em>M. marinum</em> strains expressing DsRed2 which were used in all zebrafish infection experiments.</td>
</tr>
<tr>
<td>pGFPHYG2[^c]</td>
<td>Hygromycin</td>
<td><em>E. coli</em> HB101 <em>M. marinum</em> M (a)</td>
<td>Amplified in <em>E. coli</em>. Used to generate <em>M. marinum</em> strains expressing GFPmut3 (enhanced brightness over GFP) which were used in all zebrafish infection experiments.</td>
</tr>
</tbody>
</table>

[^a]: Description of pSMT3 available in Addgene pSMT3-M details (plasmid #26589).
[^b]: pMSP12::DsRed2 was a gift from Lalita Ramakrishnan (Addgene plasmid # 30171).
[^c]: pGFPHYG2 was a gift from Lalita Ramakrishnan (Addgene plasmid # 30173).
### Table 2.3. Plasmids generated during this study.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>pYUB854::DosR/S+USP_AES*</td>
<td>Mycobacteria-adapted recombineering</td>
</tr>
<tr>
<td>pYUB854::DosR/S_AES</td>
<td></td>
</tr>
<tr>
<td>pYUB854::TcrX/Y_AES</td>
<td></td>
</tr>
<tr>
<td>pYUB854::RegX3/SenX3_AES</td>
<td></td>
</tr>
<tr>
<td>pYUB854::MtrA/B_AES</td>
<td></td>
</tr>
<tr>
<td>pYUB854::MprA/B_AES</td>
<td></td>
</tr>
<tr>
<td>pYUB854::PrrA/B_AES</td>
<td></td>
</tr>
<tr>
<td>pYUB854::PhoP/R_AES</td>
<td></td>
</tr>
<tr>
<td>pYUB854::KdpD/E_AES</td>
<td></td>
</tr>
<tr>
<td>pYUB854::NarL/Rv0845_AES</td>
<td></td>
</tr>
<tr>
<td>pYUB854::PdtaR_AES</td>
<td></td>
</tr>
<tr>
<td>pYUB854::PdtaS_AES</td>
<td></td>
</tr>
<tr>
<td>pSMT100::DosR/S+USP_AES</td>
<td>Sucrose counter-selection</td>
</tr>
<tr>
<td>pSMT100::DosR/S_AES</td>
<td></td>
</tr>
<tr>
<td>pSMT100::TcrX/Y_AES</td>
<td></td>
</tr>
<tr>
<td>pSMT100::RegX3/SenX3_AES</td>
<td></td>
</tr>
<tr>
<td>pSMT100::MtrA/B_AES</td>
<td></td>
</tr>
<tr>
<td>pSMT100::MprA/B_AES</td>
<td></td>
</tr>
<tr>
<td>pSMT100::PrrA/B_AES</td>
<td></td>
</tr>
<tr>
<td>pSMT100::PhoP/R_AES</td>
<td></td>
</tr>
<tr>
<td>pSMT100::KdpD/E_AES</td>
<td></td>
</tr>
<tr>
<td>pSMT100::NarL/Rv0845_AES</td>
<td></td>
</tr>
<tr>
<td>pSMT100::PdtaR_AES</td>
<td></td>
</tr>
<tr>
<td>pSMT100::PdtaS_AES</td>
<td></td>
</tr>
</tbody>
</table>

* = This plasmid had been constructed previously in the Williams Group.

#### 2.1.2 Primers

Primers used for PCR reactions and in the generation of plasmids are detailed in Table 2.4. Restriction enzymes and their sites used in the generation of plasmids are detailed in Table 2.5. Primers used for sequence verification are detailed in Table 2.6. Expected PCR products in TCSs mutant construction verification experiments are detailed in Table 2.7, primers used are shown in Table 2.8. All primers were designed based on sequences from the MarinoList website, checked for specificity using NCBI’s BLAST function and synthesised by Sigma Aldrich (UK).
Table 2.4. Primers for PCR and plasmid generation. Restriction enzyme sites shown in red.

<table>
<thead>
<tr>
<th>Primer</th>
<th>5’-3’ sequence</th>
<th>Target region</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gp60F&lt;sup&gt;a&lt;/sup&gt;</td>
<td>GCACCGGTTTCGTCGACGGG</td>
<td>gp60 gene</td>
<td>883</td>
</tr>
<tr>
<td>Gp60R&lt;sup&gt;a&lt;/sup&gt;</td>
<td>CTCTTCGACGGGCGGGTGGTCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MmarF&lt;sup&gt;a&lt;/sup&gt;</td>
<td>CGGCTACTGGAACGGACG</td>
<td>MMAR_2865</td>
<td>540</td>
</tr>
<tr>
<td>MmarR&lt;sup&gt;a&lt;/sup&gt;</td>
<td>GTTTGGCGTGCTCCGCAGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NewF5Down&lt;sup&gt;b&lt;/sup&gt;</td>
<td>AAAACTAGTGGCGGTGATCATCGGCG</td>
<td>DosR/S+USP</td>
<td>1000</td>
</tr>
<tr>
<td>NewR5Down&lt;sup&gt;b&lt;/sup&gt;</td>
<td>AAAAGCTTTGGGCAATCGGCTGCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NewF3Up&lt;sup&gt;b&lt;/sup&gt;</td>
<td>AAACTAGATGCGGTGTCAGGCCCAG</td>
<td>DosR/S (+USP)</td>
<td>1001</td>
</tr>
<tr>
<td>NewR3Up&lt;sup&gt;b&lt;/sup&gt;</td>
<td>AAAAGTTCCCGGACATCCGAGGAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RP1</td>
<td>AAATCTCGACAGACTCAAAAAATACCTA</td>
<td>RegX3/SenX3</td>
<td>999</td>
</tr>
<tr>
<td>RP2</td>
<td>AAATCTCAAGAGACTGCAAAAAATACCTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RP3</td>
<td>AAAAGCTTTGGGCAATCGGCTGCG</td>
<td>RegX3/SenX3</td>
<td>1000</td>
</tr>
<tr>
<td>RP4</td>
<td>AAAACTAGTGGGACGGAACGGACG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RP5</td>
<td>AAAAGTTCCCGGCAATCGGCTGCG</td>
<td>PhoP/R</td>
<td>1002</td>
</tr>
<tr>
<td>RP6</td>
<td>AAAATCTAGGTTGCTGCTAGGACAT</td>
<td>PhoP/R</td>
<td>1011</td>
</tr>
<tr>
<td>RP7</td>
<td>AAAAGCTTTGGGCAATCGGCTGCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RP8</td>
<td>AAAACTAGTGGGACGGAACGGACG</td>
<td>PhoP/R</td>
<td>1011</td>
</tr>
<tr>
<td>RP9</td>
<td>AAAAGCTTTGGGCAATCGGCTGCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RP10</td>
<td>AAAATCTAGGTTGCTGCTAGGACAT</td>
<td>PhoP/R</td>
<td>1011</td>
</tr>
<tr>
<td>RP11</td>
<td>AAAAGCTTTGGGCAATCGGCTGCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RP12</td>
<td>AAAACTAGTGGGACGGAACGGACG</td>
<td>PhoP/R</td>
<td>1011</td>
</tr>
<tr>
<td>RP13</td>
<td>AAAAGCTTTGGGCAATCGGCTGCG</td>
<td>PhoP/R</td>
<td>1011</td>
</tr>
<tr>
<td>RP14</td>
<td>AAAAGCTTTGGGCAATCGGCTGCG</td>
<td>PhoP/R</td>
<td>1011</td>
</tr>
<tr>
<td>RP15</td>
<td>AAAAGCTTTGGGCAATCGGCTGCG</td>
<td>PhoP/R</td>
<td>1011</td>
</tr>
<tr>
<td>RP16</td>
<td>AAAAGCTTTGGGCAATCGGCTGCG</td>
<td>PhoP/R</td>
<td>1011</td>
</tr>
<tr>
<td>RP17</td>
<td>AAAAGCTTTGGGCAATCGGCTGCG</td>
<td>PhoP/R</td>
<td>1011</td>
</tr>
<tr>
<td>RP18</td>
<td>AAAAGCTTTGGGCAATCGGCTGCG</td>
<td>PhoP/R</td>
<td>1011</td>
</tr>
<tr>
<td>RP19</td>
<td>AAAAGCTTTGGGCAATCGGCTGCG</td>
<td>PhoP/R</td>
<td>1011</td>
</tr>
<tr>
<td>RP20</td>
<td>AAAAGCTTTGGGCAATCGGCTGCG</td>
<td>PhoP/R</td>
<td>1011</td>
</tr>
<tr>
<td>RP21</td>
<td>AAAAGCTTTGGGCAATCGGCTGCG</td>
<td>PhoP/R</td>
<td>1011</td>
</tr>
<tr>
<td>RP22</td>
<td>AAAAGCTTTGGGCAATCGGCTGCG</td>
<td>PhoP/R</td>
<td>1011</td>
</tr>
<tr>
<td>RP23</td>
<td>AAAAGCTTTGGGCAATCGGCTGCG</td>
<td>PhoP/R</td>
<td>1011</td>
</tr>
<tr>
<td>RP24</td>
<td>AAAAGCTTTGGGCAATCGGCTGCG</td>
<td>PhoP/R</td>
<td>1011</td>
</tr>
<tr>
<td>RP60</td>
<td>AAAAGCTTTGGGCAATCGGCTGCG</td>
<td>TrcR/S</td>
<td>351</td>
</tr>
<tr>
<td>RP61</td>
<td>AAAAGCTTTGGGCAATCGGCTGCG</td>
<td>TrcR/S</td>
<td>1005</td>
</tr>
<tr>
<td>RP27</td>
<td>AAAAGCTTTGGGCAATCGGCTGCG</td>
<td>TrcR/S</td>
<td>1005</td>
</tr>
</tbody>
</table>
Table 2.5. Restriction enzyme sites and their sites used in plasmid generation.

<table>
<thead>
<tr>
<th>Restriction enzyme site</th>
<th>Restriction enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCTAGC</td>
<td>NheI</td>
</tr>
<tr>
<td>ACTAGT</td>
<td>SpeI</td>
</tr>
<tr>
<td>AGGCCT</td>
<td>StuI</td>
</tr>
<tr>
<td>TCTAGA</td>
<td>XbaI</td>
</tr>
<tr>
<td>AAGCTT</td>
<td>HindIII</td>
</tr>
<tr>
<td>GTTACCG</td>
<td>KpnI</td>
</tr>
<tr>
<td>ACCGCT</td>
<td>AgeI</td>
</tr>
<tr>
<td>GATTCC</td>
<td>BamHI</td>
</tr>
<tr>
<td>GAATTC</td>
<td>EcoRI</td>
</tr>
</tbody>
</table>

a = These primers were designed during my MRes rotation.
b = These primers had been designed previously in the Williams Group.
Table 2.6. Primers used for sequencing.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence 5’-3’</th>
<th>Plasmids sequenced</th>
<th>Site sequenced</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13 forward(-21)*</td>
<td>TGTAAAACGACGGCCAGT</td>
<td>pCR Blunt</td>
<td>Cloning site</td>
</tr>
<tr>
<td>M13 reverse*</td>
<td>CAGGAAACAGCTATGAC</td>
<td>pCR Blunt</td>
<td>Cloning site</td>
</tr>
<tr>
<td>RP62</td>
<td>GCAAGCAGCAGATTACGCGC</td>
<td>pYUB854::AES</td>
<td>Cloning site 1</td>
</tr>
<tr>
<td>RP63</td>
<td>CAACCGGGGTAAATCAATCT</td>
<td>pYUB854::AES</td>
<td>Cloning site 2</td>
</tr>
</tbody>
</table>

* = Beckman Coulter Genomics universal primers for sequencing.

Table 2.7. Expected PCR products for two-component systems (TCSs) mutant construction verifications.

<table>
<thead>
<tr>
<th>ΔTCSs mutant</th>
<th>Whole TCSs locus</th>
<th>Hyg cassette upstream&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Hyg cassette downstream&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mutant</td>
<td>WT</td>
<td></td>
</tr>
<tr>
<td>DosR/S+USP</td>
<td>4653</td>
<td>5931</td>
<td>1649</td>
</tr>
<tr>
<td>DosR/S</td>
<td>4916</td>
<td>5379</td>
<td>1744</td>
</tr>
<tr>
<td>KdpD/E</td>
<td>4891</td>
<td>6158</td>
<td>1715</td>
</tr>
<tr>
<td>MprA/B</td>
<td>4946</td>
<td>5238</td>
<td>1742</td>
</tr>
<tr>
<td>MtrA/B</td>
<td>4844</td>
<td>5312</td>
<td>1613</td>
</tr>
<tr>
<td>NarL/Rv0845</td>
<td>4915</td>
<td>4993</td>
<td>1595</td>
</tr>
<tr>
<td>PdtaR</td>
<td>4936</td>
<td>3603</td>
<td>1645</td>
</tr>
<tr>
<td>PdtaS</td>
<td>4880</td>
<td>4441</td>
<td>1735</td>
</tr>
<tr>
<td>PhoP/R</td>
<td>4972</td>
<td>5236</td>
<td>1640</td>
</tr>
<tr>
<td>PrrA/B</td>
<td>4887</td>
<td>5014</td>
<td>1674</td>
</tr>
<tr>
<td>RegX3/SenX3</td>
<td>4800</td>
<td>4878</td>
<td>1585</td>
</tr>
<tr>
<td>TcrX/Y</td>
<td>4945</td>
<td>5209</td>
<td>1724</td>
</tr>
<tr>
<td>TrcR/S</td>
<td>4352</td>
<td>4656</td>
<td>1125</td>
</tr>
</tbody>
</table>

<sup>a</sup> = Primers RP93/94 (Table 2.4) were also used, expected product size 521bp.

<sup>b</sup> = Product sizes given for mutants only as WT M. marinum would provide no products.
Table 2.8. Primers used in TCSS mutant verification.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Location</th>
<th>Primer sequence 5’-3’</th>
<th>Primer number in Figure 5.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>RP50</td>
<td>Hyg cassette upstream</td>
<td>CTGGATCTCTCCGGCTTCAC</td>
<td>4</td>
</tr>
<tr>
<td>RP51</td>
<td>Hyg cassette downstream</td>
<td>CCTCAGTCAGACCCGAGGA</td>
<td>5</td>
</tr>
<tr>
<td>RP49</td>
<td>DosR/S+USP Upstream</td>
<td>GTGGATATTGGCCGCGGT</td>
<td>3</td>
</tr>
<tr>
<td>RP52</td>
<td>DosR/S+USP Downstream</td>
<td>GACACCAGTCATGCTGCTC</td>
<td>6</td>
</tr>
<tr>
<td>RP64</td>
<td>DosR/S Upstream</td>
<td>GCACGTGAGGATAGCTTAG</td>
<td>3</td>
</tr>
<tr>
<td>RP65</td>
<td>DosR/S Downstream</td>
<td>CGGTGCAGGCATCATC</td>
<td>6</td>
</tr>
<tr>
<td>RP66</td>
<td>KdpD/E Upstream</td>
<td>GGACAGCTGGCGATGATCA</td>
<td>3</td>
</tr>
<tr>
<td>RP67</td>
<td>KdpD/E Downstream</td>
<td>GTGGTCCCCAGCGCGATA</td>
<td>6</td>
</tr>
<tr>
<td>RP68</td>
<td>MprA/B Upstream</td>
<td>GCGGCTGATATCTGAGGCA</td>
<td>3</td>
</tr>
<tr>
<td>RP69</td>
<td>MprA/B Downstream</td>
<td>GTGACTTGCACGGTGAGG</td>
<td>6</td>
</tr>
<tr>
<td>RP70</td>
<td>MtrA/B Upstream</td>
<td>GTGGATCTACCGGCAGCA</td>
<td>3</td>
</tr>
<tr>
<td>RP71</td>
<td>MtrA/B Downstream</td>
<td>GTTTACCGGTCACAGGAT</td>
<td>6</td>
</tr>
<tr>
<td>RP72</td>
<td>NarL/Rv0845 Upstream</td>
<td>CGACTCGCATATTGTCCGG</td>
<td>3</td>
</tr>
<tr>
<td>RP73</td>
<td>NarL/Rv0845 Downstream</td>
<td>CACGGTGCGGGTTGGCA</td>
<td>6</td>
</tr>
<tr>
<td>RP74</td>
<td>PdtaR Upstream</td>
<td>CTGGATACCTATCCGGCA</td>
<td>3</td>
</tr>
<tr>
<td>RP75</td>
<td>PdtaR Downstream</td>
<td>GTGGTGGACCTACACAGGA</td>
<td>6</td>
</tr>
<tr>
<td>RP76</td>
<td>PdtaS Upstream</td>
<td>CGATCCCCTGTTGGACAG</td>
<td>3</td>
</tr>
<tr>
<td>RP77</td>
<td>PdtaS Downstream</td>
<td>CACCAACATCGTGCGCCAG</td>
<td>6</td>
</tr>
<tr>
<td>RP78</td>
<td>PhoP/R Upstream</td>
<td>CTGACCACCTGCTGATGA</td>
<td>3</td>
</tr>
<tr>
<td>RP79</td>
<td>PhoP/R Downstream</td>
<td>CTGACCACCCAGATCGCAA</td>
<td>6</td>
</tr>
<tr>
<td>RP80</td>
<td>PrrA/B Upstream</td>
<td>GCAACTCCGTCTCCGGTTTC</td>
<td>3</td>
</tr>
<tr>
<td>RP81</td>
<td>PrrA/B Downstream</td>
<td>CAGATCGATCCCAAGGCTCC</td>
<td>6</td>
</tr>
<tr>
<td>RP82</td>
<td>RegX3/SenX3 Upstream</td>
<td>CAGTCCGTGAAGCGTGAT</td>
<td>3</td>
</tr>
<tr>
<td>RP83</td>
<td>RegX3/SenX3 Downstream</td>
<td>GCACGTGCGTGCTGGAGGA</td>
<td>6</td>
</tr>
<tr>
<td>RP84</td>
<td>TcrX/Y Upstream</td>
<td>CTGGAGCACCGGCTGACT</td>
<td>3</td>
</tr>
<tr>
<td>RP85</td>
<td>TcrX/Y Downstream</td>
<td>GGAATTCCATCCACGGCG</td>
<td>6</td>
</tr>
<tr>
<td>RP86</td>
<td>TrcR/S Upstream</td>
<td>CAAGATGTGGCGGTCTCG</td>
<td>3</td>
</tr>
<tr>
<td>RP87</td>
<td>TrcR/S Downstream</td>
<td>CCTGGCCACAACATCGATG</td>
<td>6</td>
</tr>
<tr>
<td>KOCheck3F</td>
<td>DosR/S+USP locus between flanking regions</td>
<td>GTATTGACTACGCGACCGGC</td>
<td>7</td>
</tr>
<tr>
<td>KOCheck3R*</td>
<td>DosR/S+USP locus between flanking regions</td>
<td>GTGGTGCAATGCACGCGCAACA</td>
<td>8</td>
</tr>
</tbody>
</table>

*a* = Primers RP93/94 (Table 2.4) were also used, these are primers 1 and 2 in Figure 5.4, respectively.  
* = These primers had been designed previously in the Williams Group by Faye Rodgers.
2.1.3 qRT-PCR probes

The Taqman gene expression assay IDs (Applied Biosystems) for qRT-PCR primers and probes are detailed in Table 2.9.

Table 2.9. Taqman gene expression assay IDs used in qRT-PCRs during this study.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Taqman assay ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S rRNA</td>
<td>4319413E</td>
</tr>
<tr>
<td>il-1β</td>
<td>Dr03114368_m1</td>
</tr>
<tr>
<td>mmp-9</td>
<td>Dr03139882_m1</td>
</tr>
<tr>
<td>tnfα</td>
<td>Dr03126850_m1</td>
</tr>
<tr>
<td>cxcl-c1c</td>
<td>Dr03436643_m1</td>
</tr>
<tr>
<td>ifn-phi1</td>
<td>Dr03100938_m1</td>
</tr>
</tbody>
</table>

2.1.4 Kits

Kits used in this study are detailed in Table 2.10.

Table 2.10. Kits used during this study.

<table>
<thead>
<tr>
<th>Kit</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotin Chromogenic Detection</td>
<td>Thermo Scientific</td>
</tr>
<tr>
<td>Biotin DecaLabel DNA Labelling</td>
<td>Thermo Scientific</td>
</tr>
<tr>
<td>High Capacity cDNA Reverse Transcription</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>MagMAX™-96 Total RNA Isolation</td>
<td>Ambion</td>
</tr>
<tr>
<td>QIAprep Spin Mini-Prep</td>
<td>QIagen</td>
</tr>
<tr>
<td>QIagen Gel Extraction</td>
<td>QIagen</td>
</tr>
<tr>
<td>QIagen Plasmid Maxi</td>
<td>QIagen</td>
</tr>
<tr>
<td>Rapid Ligation</td>
<td>Fermentas</td>
</tr>
<tr>
<td>Taqman® Fast Universal PCR Master mix</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>(2X), no AmpErase® UNG</td>
<td></td>
</tr>
<tr>
<td>Zero Blunt® TOPO® PCR Cloning</td>
<td>Thermo Scientific</td>
</tr>
</tbody>
</table>
2.1.5 Chemicals and reagents

Chemicals and reagents used in this study are detailed in Table 2.11, solutions are detailed in Table 2.12.

### Table 2.11. Chemicals and reagents used during this study.

<table>
<thead>
<tr>
<th>Chemical/reagent</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose</td>
<td>Sigma</td>
</tr>
<tr>
<td>Amikacin disulfate</td>
<td>Sigma</td>
</tr>
<tr>
<td>Apramycin</td>
<td>Sigma</td>
</tr>
<tr>
<td>Bactenecin 5 (Bac5, bovine, sequence: RFRPPIRRPPPPFYPFPRPPPIRPFP)</td>
<td>Biomatik</td>
</tr>
<tr>
<td>Cathelicidin/LL-37 (human, sequence: LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTE)</td>
<td>Biomatik</td>
</tr>
<tr>
<td>Chloroform</td>
<td>BDH, VWR</td>
</tr>
<tr>
<td>CTAB (cetyltrimethyl ammonium bromide)</td>
<td>Sigma</td>
</tr>
<tr>
<td>Difco Middlebrook 7H9</td>
<td>BD</td>
</tr>
<tr>
<td>Difco Middlebrook 7H10</td>
<td>BD</td>
</tr>
<tr>
<td>Difco Middlebrook 7H11</td>
<td>BD</td>
</tr>
<tr>
<td>DMSO (Dimethyl sulfoxide)</td>
<td>Sigma</td>
</tr>
<tr>
<td>DNA loading dye (6X)</td>
<td>Thermo Scientific</td>
</tr>
<tr>
<td>dNTP Set (10mM each)</td>
<td>Thermo Scientific</td>
</tr>
<tr>
<td>EDTA (ethylene-diamene-tetraacetate)</td>
<td>Sigma</td>
</tr>
<tr>
<td>Epinecidin-1 (EPL, fish, sequence: GFIFHIKGLFHAHMKIHLV)</td>
<td>Biomatik</td>
</tr>
<tr>
<td>Ethanol</td>
<td>BDH, VWR</td>
</tr>
<tr>
<td>FBS (foetal bovine serum), heat-inactivated</td>
<td>Sigma</td>
</tr>
<tr>
<td>GeneRuler 1Kb DNA ladder</td>
<td>Thermo Scientific</td>
</tr>
<tr>
<td>Glycerol</td>
<td>Sigma</td>
</tr>
<tr>
<td>Hydrochloric acid</td>
<td>Sigma</td>
</tr>
<tr>
<td>Hygromycin B</td>
<td>Sigma</td>
</tr>
<tr>
<td>Isoamyl alcohol</td>
<td>Sigma</td>
</tr>
<tr>
<td>Kanamycin sulphate</td>
<td>Sigma</td>
</tr>
<tr>
<td>TopVision low melting point agarose</td>
<td>Thermo Scientific</td>
</tr>
<tr>
<td>Methylene blue</td>
<td>Sigma</td>
</tr>
<tr>
<td>Middlebrook OADC supplement</td>
<td>BD</td>
</tr>
<tr>
<td>Middlebrook 7H10 OADC growth supplement (powder)</td>
<td>Stratech</td>
</tr>
<tr>
<td>MS-222 (tricaine methanesulfonate)</td>
<td>Sigma</td>
</tr>
<tr>
<td>PBS (phosphate-buffered saline)</td>
<td>Sigma</td>
</tr>
</tbody>
</table>
Table 2.12. Solutions used during this study.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>1X PBS (tablets)</td>
<td>10 mM phosphate buffer, 137 mM NaCl, 2.7 mM KCl at pH 7.4</td>
</tr>
<tr>
<td>CTAB (Cetyl trimethylammonium bromide)</td>
<td>0.1 M Tris HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA, 20 g/L CTAB</td>
</tr>
<tr>
<td>Complete RPMI</td>
<td>RPMI 1640 Medium, GlutaMAX™ supplement containing 10% FBS, 50 units/ml penicillin and 50 µg/ml streptomycin</td>
</tr>
<tr>
<td>Complete RPMI NPS</td>
<td>RPMI 1640 Medium, GlutaMAX™ supplement containing 10% FBS</td>
</tr>
<tr>
<td>0.5X E2*</td>
<td>7.5 mM NaCl, 0.25 mM KCl, 0.5 mM MgSO₄, 75 µm KH₂PO₄, 25 µm Na₂HPO₄, 0.5 mM CaCl₂, 0.35 mM NaHCO₃, 0.5 mg/L methylene blue</td>
</tr>
</tbody>
</table>

* = 0.5X E2 media is made fresh for each experiment from stock solutions E2A, E2A Buffer, E2B and E2C as described in The Zebrafish Book[356].

2.1.6 Enzymes

Enzymes used in this study are detailed in Table 2.13.
Table 2.13. Enzymes used in this study.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>FastDigest™ Series Restriction Enzymes</td>
<td>Fisher Scientific</td>
</tr>
<tr>
<td>Pfu DNA polymerase</td>
<td>Thermo Scientific</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>Thermo Scientific</td>
</tr>
</tbody>
</table>

2.1.7 Zebrafish lines

Zebrafish lines used in this study are detailed in Table 2.14.

Table 2.14. Zebrafish lines used during this study.

<table>
<thead>
<tr>
<th>Zebrafish line</th>
<th>Description</th>
<th>Full name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tranac</td>
<td>Transparent line *(nacre and transparent double mutant)*².</td>
<td>mitfa²/w²; mpv17b18/b18</td>
</tr>
<tr>
<td>Tg(mpeg-1:mCherry)</td>
<td>AB (WT) background. mCherry expressed in macrophages. Express the transcription factor Gal4-VP16 under the control of the mpeg-1 (macrophage expressed gene 1) promoter with the Gal4 binding site upstream of an mCherry-nitroreductase fusion protein[^90].</td>
<td>Tg(mpeg-1:mCherry)i23</td>
</tr>
<tr>
<td>Casper Tg(mpx:GFP)</td>
<td>Transparent casper background. GFP expressed in neutrophil cells. Contains a modified bacterial artificial chromosome (BAC) containing the entire myeloperoxidase (mpx) regulatory region. Carries enhanced GFP with an SV40 polyadenylation site at the mpx gene ATG start site within the BAC[^201,272].</td>
<td>mitfa²/w²; roy²/a²; Tg(mpx:GFP)i114</td>
</tr>
<tr>
<td>Casper Tg(LysC:GFP)</td>
<td>Transparent casper background. GFP expressed in macrophage and neutrophil cells in early development, expression in neutrophils only from 2 dpf[^363]. Express enhanced GFP under the control of the lysC (lysozyme C, alternative name lyz) promoter[^124].</td>
<td>mitfa²/w²; roy²/a²; Tg(LysC:GFP)nz117</td>
</tr>
</tbody>
</table>

² = The transparent mutant carries a mutation in mpv17 causing a complete lack of iridophores and a disruption of melanocyte patterning[^169,302,347] leading to partial transparency through a lack of light reflection. The nacre mutant carries a mutation in the microphthalmia-associated transcription factor a (mitfa) gene causing a complete lack of melanocytes[^184] and partial transparency through a lack of light absorption[^357]. The TraNac line combines homozygous tra and nacre mutations and is therefore completely transparent though a lack of light absorption and reflection (phenotypically resembles casper line)[^258].

b = The roy orbison mutant carries a mutation in an unknown gene causing a complete lack of iridophores and a disruption of melanocyte patterning leading to partial transparency through a lack of light reflection[^357]. The casper line combines homozygous nacre and roy orbison mutations and is therefore completely transparent though a lack of light absorption and reflection[^357].
2.2 Microbiology methods

2.2.1 Bacterial culture conditions and antibiotics

*E. coli* and *M. smegmatis* strains were cultured at 37°C in Luria broth (LB) media or on LB agar plates. LB components per L: 5 g NaCl (Sigma), 5 g yeast extract (Fisher Scientific), 10 g Tryptone (Fisher Scientific), 15 g agar (LB agar only, Merck Millipore). *M. marinum* strains were maintained at 28.5°C in 7H9 media or on 7H10/7H11 plates, both supplemented with 10% OADC. 7H11 agar was used initially in this study to culture *M. marinum*. 7H10 agar was used to culture *M. marinum* for: all THP-1 infection experiments; zebrafish infection experiments presented in Chapter 4 and Sections 3.3.4.4, 3.3.4.5, 3.3.4.6 and 3.3.5 in Chapter 3; round 8 of recombineering and rounds 5-8 of sucrose counter-selection in Chapter 5.

All liquid cultures were incubated with shaking at 150 rotations per minute (rpm) and liquid cultures of mycobacteria also contained 0.05% Tween-80. Overnight starter cultures of mycobacteria were inoculated in 5 ml appropriate medium in 30 ml universal containers (Thermo Scientific) and incubated at the appropriate temperature. Starter cultures of *E. coli* were inoculated on the morning of the experiment in 5 ml LB in 30 ml universal containers. Logarithmic-phase starter cultures were used to inoculate (at OD\(_{600}\) 0.1 for mycobacterial cultures and 0.5 for *E. coli* cultures) larger cultures of 50 ml appropriate media in 250 ml glass conical flasks with sponge bungs.

Strains were stored in 20% glycerol at -80°C. Where appropriate, growth media were supplemented with 25 μg/ml kanamycin, 40 μg/ml apramycin and/or 50 μg/ml hygromycin B. Antibiotics (Sigma) were prepared by dissolving in distilled water and filter-sterilising using a 0.2 μm pore syringe filter (Nalgene), with the exception of Hygromycin B which had been provided in sterile liquid form by Sigma.

2.2.2 Optical density (OD) measurement

The OD\(_{600}\)nm (OD\(_{600}\)) of bacterial cultures was measured in a spectrophotometer (Eppendorf BioPhotometer) using 1.5 ml plastic cuvettes (BRAND). The appropriate media was used as a blank. Samples of 1 ml were taken and samples diluted in the appropriate media if the OD\(_{600}\) reading measured greater than 1.0.
2.2.3 Growth curves

Large 50 ml cultures were prepared as described, incubated at the appropriate temperature for the strain used before sampling for OD\(_{600}\) measurement at the indicated time points. For initial growth measurements, starter cultures were re-inoculated to 5 ml volumes, cultures incubated and samples taken at the indicated time points for OD\(_{600}\) measurement.

2.2.4 Passaging to investigate rate of pJV53 loss

Mycobacterial starter cultures (containing antibiotic) were used at an OD\(_{600}\) 1.0 to inoculate 5 ml 7H9 10% OADC media containing no antibiotic in universal containers to OD\(_{600}\) 0.1, and cultures re-incubated at the appropriate temperature. The first inoculation from the starter cultures is passage 1. Once the cultures had reached stationary phase (OD\(_{600}\) 9.0 for \(M.\) marinum and 3.5 for \(M.\) smegmatis), 1 ml of each culture was harvested by centrifugation at 16,000 \(\times\) g and the pellets stored at -20°C. The cultures were also used to inoculate fresh 5 ml media volumes containing no antibiotic as before (passage 2). Cell pellets were then used in colony PCRs with gp60 primers and results analysed by gel electrophoresis to determine the presence or absence of pJV53 in strains.

2.2.5 96-well plate host defence peptides (HDP) treatments assay

\(M.\) marinum starter cultures were used at OD\(_{600}\) 1.0 and \(E.\) coli starter cultures at OD\(_{600}\) 0.5 (ensuring the bacteria were in log phase) before dilution in a solution of 1:4 (v:v) RPMI:water to a concentration of 1.5x10\(^6\) CFU per ml. 10X working stocks of the HDPs were prepared fresh for each repeat experiment in 1:4 (v:v) RPMI:water immediately prior to use. Working stocks for each peptide ranged from 0 µg/ml to 600 µg/ml. 100 µl of bacterial solution (1.5x10\(^5\) CFU/well) was added to each well with 10 µl of appropriate 10X working HDP stock and the 96-well plate incubated for 24 hours at 28.5°C. For \(M.\) marinum experiments, serial dilutions in 1X PBS were plated on 7H10 agar for each well and CFU counts taken after 5 days of incubation at 28.5°C. For \(E.\) coli experiments, serial dilutions in 1X PBS were plated on LB agar for each well and CFU counts taken after overnight incubation at 28.5°C.
2.3 Molecular biology methods

2.3.1 Competent bacteria

*M. marinum* 50 ml cultures were grown to mid log phase (OD$_{600}$ of approximately 1). The cultures were centrifuged at 20,800 x g for 15 minutes, and the cells resuspended in 40 ml of 10% glycerol. Cells were washed in this way a total of three times, with the final pellet being resuspended in 2.5 ml or 0.25 ml of 10% glycerol. Final resuspensions could be frozen, in aliquots, at -80°C. For the preparation of competent *pJV53*-containing *M. marinum* cells, a 50 ml culture was grown to mid log phase (OD$_{600}$ of 0.4-0.8) before incubation for a further five hours after the addition of acetamide to induce expression of the recombination proteins. The cells were then prepared as above.

*M. smegmatis* competent cells were prepared as above with cells kept on ice for all steps, using ice-cold 10% glycerol, and with all centrifugation steps performed at 4°C.

*E. coli* 400 ml cultures were grown to mid log phase (OD$_{600}$ 0.5-1.0). Cultures were centrifuged at 20,800 x g for 20 minutes at 4°C. Cells were kept on ice from now on. Cells were gently resuspended in 200 ml cold sterile 0.1 M MgCl$_2$, incubated for 20 minutes and pelleted as above. Cells were then gently resuspended in 100 ml cold sterile 0.1 M CaCl$_2$, incubated for 20 minutes and pelleted as above. Cells were gently resuspended in 5 ml 0.1 M CaCl$_2$ 14% glycerol and frozen, in aliquots, at -80°C.

2.3.2 Transformations

Competent mycobacterial cells were transformed by electroporation with a Gene Pulser II Electroporator (BioRad) and electroporation cuvettes (0.2 cm, BioRad) as follows. Two hundred microlitres of cells were added to up to 5 μl (≤10 μg) of plasmid DNA solution at room temperature for *M. marinum* and on ice for *M. smegmatis*. Cells were electroporated with the following conditions: capacitance at 25 μF, resistance at 1000 Ω, voltage at 2.5 kV. The cells were recovered in 3 ml 7H9 medium at with no shaking and no antibiotic selection at 28.5°C for 5 hours for *M. marinum* and 37°C for 3 hours for *M. smegmatis*.
Competent *E. coli* cells were transformed by heat shock as follows: 50 µl cells were added to up to 5 µl plasmid DNA (≤5 µg) and incubated on ice for 30 minutes; the cells were then transferred to 42°C for 45 seconds before being incubated on ice for a further 2 minutes. Cells were recovered in 500 µl LB medium at 37°C for 1 hour, with shaking at 150 rpm and no antibiotic selection.

2.3.3 Preparation of mycobacterial genomic DNA (gDNA) – CTAB method

Steps were performed at room temperature unless otherwise specified. 5 ml bacterial cultures were grown to late log-phase (OD$_{600}$ 5.0 for *M. marinum* and 2.0 for *M. smegmatis*) and harvested by centrifugation at 20,800 x g for 10 minutes. The cell pellets were resuspended in 500 µl distilled H$_2$O with 50 µl 10 mg/ml lysozyme, vortexed and incubated at 37°C for 1 hour. 6 µl 10 mg/ml proteinase K and 70 µl 10% SDS were added and the mixture vortexed and incubated at 65°C for 10 minutes. 100 µl 5 M NaCl and 80 µl 10% CTAB (in 0.7 M NaCl) was added, vortexed and incubated at 65°C for 10 minutes. 700 µl chloroform:iso-amylalcohol (24:1) was added, the mixture vortexed and then centrifuged for 5 minutes at 16,000 x g. 550 µl of the top phase was recovered and an equal volume of isopropanol gently mixed with this, and then incubated at -20°C overnight. The DNA was pelleted by centrifugation at 16,000 x g for 20 minutes and the pellet washed with 200 µl 70% ethanol. The DNA was air dried for 5-10 minutes and resuspended in 50 µl distilled H$_2$O.

2.3.4 PCR

PCR reactions were carried out with a DNA Engine Dyad Thermal Cycler (Bio-Rad) using Pfu or Taq polymerase with the dNTP Set according to the manufacturer’s recommendations. 2.5 µl DMSO was added to each 50 µl reaction to aid in the denaturation of GC-rich mycobacterial DNA sequences. Template DNA consisted of 100 ng gDNA or plasmid. For colony PCR (cPCR), bacterial cultures of OD$_{600}$ 1.0-3.0 were pelleted by centrifugation at 13,000rpm for 2 minutes and the pellet sampled for the PCR reaction using a pipette tip. Gradient PCRs were performed to optimise primer annealing temperatures where required. Asymmetric PCR was performed where stated, involving addition of only one primer for 20 cycles, followed by addition of the second primer to the existing reaction and running for the full 35 cycles.
2.3.5 Restriction enzyme digests

FastDigest® restriction enzymes were used according to the manufacturer's recommendations. Digested fragments were purified from agarose gels using the QIAGen Gel Extraction Kit according to the manufacturer's recommendations.

2.3.6 Agarose gel electrophoresis

Analysis of PCR products, restriction enzyme digests and the separation of DNA for Southern Blotting was carried out using gel electrophoresis. Agarose gels were composed of 1% (w/v) agarose in distilled H₂O and SYBR® Safe DNA Gel Stain in a 1:20,000 dilution. The GeneRuler 1Kb DNA ladder and 6x loading dye were routinely used during gel electrophoresis. Gels were run at 95 V for 1 hour (except separation gels for Southern blotting which were run at 50 V for 2 hours) in 1X TAE buffer using a PowerPac Basic (Bio-Rad). Gel images were obtained using a Syngene InGenius (Syngene Bioimaging).

2.3.7 Cloning

Initial cloning of pYUB854::AES constructs for sequence-verification was performed using the Zero Blunt® Cloning Kit according to the manufacturer's recommendations. In brief, PCR reactions were performed and products gel extracted; products were inserted into pCR-Blunt® and transformed to competent E. coli HB101; successfully transformed HB101 colonies were selected using kanamycin resistance and then used to inoculate starter cultures; plasmids were purified from cultures using the QIAGen Spin Miniprep Kit and sent for sequencing of the inserts with M13 primers by Beckman Coulter Genomics; sequence-verified inserts were then extracted from pCR-Blunt® by appropriate restriction enzyme digest and inserted into the destination vectors as described below.

All destination vector ligations were carried out using the Rapid DNA Ligation Kit according to the manufacturer’s recommendations, using a 3:1 molar ratio of insert to vector. E. coli HB101 transformations were carried out using the recommended volume of ligation reactions to provide strains for the amplification of the desired constructs. Constructs were purified from E. coli starter cultures for use in further experiments using the QIAGen Spin Miniprep Kit.
2.3.7.1 Construction of pYUB854::AES and pJV53ApR for mycobacteria-adapted recombineering

The AES (allelic exchange substrate) consists of a hygromycin resistance cassette flanked by ~1 Kb upstream and ~1 Kb downstream of the region to be knocked out of the *M. marinum* genome. As outlined above, pYUB854::AES was generated by amplifying 1 Kb regions upstream and downstream of the TCSs operons or genes by PCR from WT *M. marinum* gDNA, amplifying and verifying individual regions using the Zero Blunt® Cloning Kit, before sequence-verified homology regions were sequentially cloned in pairs into the vector pYUB854 to flank the hygromycin resistance cassette. PCRs to generate the flanking regions used the primers detailed in Table 2.4.

pYUB854::AES was transformed into competent *E. coli* as above and transformed cells selected by hygromycin resistance following plating to LB agar supplemented with 50 µg/ml hygromycin. Plasmid DNA was isolated as above and the correct insertion of the flank verified by sequencing using the primers detailed in Table 2.6. To minimise the effects of the deletion on the transcription of the surrounding genes, the flanks were cloned into pYUB854 such that the hygromycin cassette would be transcribed in the opposite orientation to the surrounding genes following insertion into the genome, preventing read-through and increased expression of surrounding genes affecting the phenotype of the generated mutants.

To use pJV53 with the kanamycin-resistant DsRed and GFP *M. marinum* strains, it was necessary to exchange the kanamycin resistance gene within the vector for an apramycin resistance gene, creating plasmid pJV53ApR.

2.3.7.2 Construction of pSMT100:AES for sucrose counter selection

The AES regions were transferred from the pYUB854::AES constructs to pSMT100 for each TCSs as described above. AES were extracted from pYUB854 using the SpeI and Stul sites and gel extraction before insertion into pSMT100 using the SpeI and EcoRV sites.
2.3.8 Mycobacteria-adapted recombineering

Phage-encoded recombination proteins, Gp60 and Gp61, were introduced into competent host \textit{M. marinum} cells by transformation of plasmid pJV53ApR, which carries \textit{gp60} and \textit{gp61} under the control of an acetamide-inducible promoter. Transformants were selected by apramycin resistance for pJV53ApR, and mid-log phase (OD$_{600}$ 0.5) cultures incubated with 0.2% acetamide for 5 or 12 hours to induce \textit{gp60} and \textit{gp61} expression prior to competent cells being made. pYUB854::AES DNA was isolated using the QIAprep Spin Miniprep Kit or QIAgen Plasmid Maxi Kit before digestion with FastDigest$^\text{TM}$ SpeI and KpnI to generate the linear AES. The linear AES was purified from the plasmid backbone by gel extraction. The linear AES was then transformed into competent pJV53ApR-containing \textit{M. marinum} cells and transformants selected for using hygromycin resistance only to allow loss of the pJV53ApR plasmid.

2.3.9 Sucrose counter-selection

The suicide plasmid pSMT100, which contains \textit{sacB} as a counter-selectable marker, was selected for these experiments. The pSMT100:AES plasmids were isolated using QIAgen Plasmid Maxi Kit and transformed into competent \textit{M. marinum} strains, with single-crossover transformants selected for using hygromycin resistance by plating to 7H10 supplemented with 50 µg/ml hygromycin. Single colonies were picked to 5 ml 7H9 media containing 50 µg/ml hygromycin and incubated at 28.5°C for three days. Cells were harvested from these cultures by centrifugation prior to selection of double-crossover mutants by plating onto 7H10 supplemented with 50 µg/ml hygromycin and 10% or 20% sucrose.

2.3.10 Southern Blotting

2.3.10.1 Generation of probe for Southern Blotting

A probe was selected which would bind to the upstream homology region (UR) in the DosR/S + USP AES. Attempts were made to generate a ~500 bp probe for the UR by PCR, however, the PCRs failed to provide a product. A 370 bp probe for the UR was therefore generated by restriction enzyme digest from pYUB854:DosR/S+USP_AES. Firstly, pYUB854:DosR/S+USP_AES was digested with FastDigest$^\text{TM}$ SpeI and HindIII,
and the 1 Kb UR product purified by gel extraction. The UR product was then digested with FastDigest™ SmaI and the smaller 370 bp product purified by gel extraction.

2.3.10.2 Labelling of probes for Southern Blotting

The Biotin DecaLabel DNA Labelling Kit was used according to manufacturer's recommendations to label 1 µg probe and 250 ng control template in 20 hour reactions. The Biotin Chromogenic Detection Kit was used according to the manufacturer's recommendations to determine the probe labelling efficiency prior to use in Southern Blotting. The labelling efficiency was determined as 10-fold less effective than was expected, and therefore 10x probe was used in hybridisation steps.

2.3.10.3 Southern Blotting procedure

For initial Southern Blotting trials (Blots 1 and 2) 5 µg *M. marinum* gDNA or 2 µg pYUB854::DosR/S+USP_AES was used per well, for all other experiments 10 µg gDNA, 8.9 ng pYUB854::DosR/S+USP_AES (copy number matched to 10 µg gDNA) or 250 ng pYUB854::DosR/S+USP_AES was loaded per well. gDNA was digested using FastDigest™ AcuI or NcoI according to manufacturer's recommendations for 5 hours. Plasmid DNA was digested using FastDigest™ SpeI and XbaI according to manufacturer's recommendations for 1 hour. DNA was separated using gel electrophoresis (1% agarose, 50 V, 2 hours). Images of gels were taken with a ruler alongside the well edge and one long edge for reference to position of any detected bands on the final membrane. For initial Southern Blotting trials (Blots 1 and 2), I proceeded directly to electroblotting. For all other experiments DNA transfer was improved by treating the gel at room temperature on a rotary shaker with: 0.2 M HCl for 10 minutes; 1.5 M NaCl, 0.5 M NaOH for 15 minutes and then repeated; 1.5 M NaCl, 0.5 M Tris-Cl pH7.5 for 15 minutes and then repeated. The pH of the solution was confirmed as 7.5 before proceeding, if the pH was higher the final treatment steps were repeated. Electroblotting to a BrightStar®-Plus Positively Charged Nylon Membrane (Ambion) was performed in 1X TAE buffer at 15 V for 12-16 hours at 4°C using a Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad). DNA was fixed to the membrane by UV crosslinking for 5 minutes at energy 900,000 µJ/cm² using a CL-1000 Ultraviolet Crosslinker (UVP). Hybridisation was performed using UltraHyb® Ultrasensitive Hybridisation Buffer according to the manufacturer's recommendations. Initial trials
optimised hybridisation conditions: pre-hybridisation length was increased from 30 minutes to 1 hour; hybridisation temperatures were increased from 42°C to 55°C; wash step temperatures were increased from 42°C to 55°C to 60°C; additional washes to minimise ionic interactions were also trialled (troubleshooting, manufacturer’s guide). The conditions selected for the final blots were: pre-hybridisation for 1 hour at 55°C; hybridisation for 24 hours at 55°C; standard washes at 60°C. Probe concentration during hybridisation was 1.2 ng/ml. Detection was performed using the Biotin Chromogenic Detection Kit according to manufacturer’s recommendations. Initial detection incubations were performed for the maximum 14 hours (overnight). Following hybridisation optimisations, optimal detection incubation was determined to be 15-30 minutes for final blots.

2.3.11 qRT-PCR
Samples were taken as described in Section 2.4.5 and Section 2.5.5. Samples were processed for RNA using the MagMAX™-96 Total RNA Isolation Kit according to the manufacturer's instructions. RNA quantity and quality was determined using a NanoDrop™ 1000 (Thermo Scientific). The High-Capacity cDNA Reverse Transcription Kit was used according to manufacturer’s instructions with 125 ng of total RNA sample per reaction. qRT-PCR was performed with 2% of the generated cDNA per reaction using the Taqman® Fast Universal PCR Master mix (2X), no AmpErase® UNG and Taqman primer and probes assays. All reactions were performed in duplicate using a 7500 Fast Real-Time PCR System (Applied Biosystems). Obtained cycle thresholds were normalised to 18S and expressed relative to one control sample for each experiment.

2.4 THP-1 cells investigations

2.4.1 THP-1 cells maintenance
The THP-1 acute monocytic leukaemia cell line (ATCC® TIB-202™) was maintained in RPMI complete media (Table 2.11) at a density of 2x10^5 - 1x10^6 cells in sterile plastic ware (75 cm² vented flasks, BD falcon) at 37°C and 5% CO₂ in a humidified chamber. All procedures were carried out using cells between passages 9 and 12 aseptically in a laminar flow cabinet, except preparation of CFU counting lysates which was performed
on the bench by a flame. Cells were stored in RMPI 1640 Medium, GlutaMAX™ supplement containing 10% DMSO at -80°C. Viability staining was carried out using trypan blue according to the manufacturer’s recommendations.

2.4.2 THP-1 cells infection

Media is described in Table 2.11. THP-1 cells were plated in RPMI complete media to 24-well and 96-well plates (1x10^6 cells/well 24-well plate and 5x10^5 cells/well 96-well plate except where otherwise stated), incubated for 24 hours at 37°C 5% CO2 with and without 20 ng/ml PMA, cell media changed to RPMI complete NPS media and all cells incubated for a further 24 hours without PMA. M. marinum was cultured to mid-logarithmic phase (OD600 1.0), cells harvested by centrifugation, cells resuspended in RPMI complete NPS media at 10X the appropriate MOI and appropriate volumes of 10X cell suspension added to each infection well (100 µl per well for 24-well plates and 10 µl per well for 96-well plates). Infection was allowed to proceed for 3 hours at 33°C 5% CO2 before cells were washed 3X with tissue culture media in wash step 1. Cell media was replaced with RPMI complete NPS media containing no amikacin or with 200 ng/ml amikacin added and cells incubated for a further 3h at 33°C 5% CO2. For initial experiments, cells were again washed 3X with tissue culture media in wash step 2 before media was replaced with RPMI complete NPS media containing no amikacin or 20 ng/ml amikacin and cells incubated for the indicated times at 33°C 5% CO2. Time 0h in initial assays was defined as after the final washes (wash step 2). Washing media was RPMI complete NPS media for all steps. Samples were processed for CFU plating and/or qRT-PCR as described below.

2.4.3 HDP treatment 96-well plate assay

THP-1 cells were plated, incubated with and without PMA, rested and infected as described above. Following wash step 1, cell media was replaced with RPMI 1640 Medium, GlutaMAX™ supplement (no additives). Working solutions of 10X the appropriate HDP concentration were made fresh for each HDP in RPMI 1640 Medium, GlutaMAX™ supplement (no additives). Working solutions were added to each well (10 µl per well) and cells incubated for the indicated times at 33°C 5% CO2. Time 0h in HDP assays was defined as after the first washes (wash step 1) as cells were not treated with amikacin. Samples were processed for qRT-PCR as described below.
2.4.4 CFU plating

CFU plating was performed at the indicated time points by removing cell media, washing cells 3x with 1x PBS, lysing cells for 5 minutes in 0.1% Triton-X-100 in 1x PBS (500 µl for 24-well plates and 100 µl for 96-well plates), dilution plating the lysates to 7H10 plates and counting the number of colonies formed after 5 days of incubation at 28.5°C.

2.4.5 qRT-PCR sampling

THP-1 cells were sampled for qRT-PCR at the indicated time points following treatments by adding 100 µl lysis buffer (MagMAX™-96 Total RNA Isolation Kit) per sample, and proceeding with sample processing as described in Section 2.3.11. For the initial experiment detailed in Section 3.3.1.4, one, two and three wells were pooled for each sample, for all other experiments pools of two wells were used. Samples were pooled by transferring the 100 µl lysis buffer from the first well to the second, and on to the third where appropriate, before processing the sample.

2.5 Zebrafish work

2.5.1 Zebrafish maintenance

All zebrafish embryos and larvae were maintained at 28.5°C in 0.5X E2, including infected zebrafish. Survival was monitored daily and dead zebrafish embryos and larvae removed. Zebrafish life stages are determined by the following ages: embryo 0-3 days, larva 3-30 days, juvenile 30-90 days and adult >90days post fertilisation. Where required (for injection or imaging procedures), zebrafish embryos and larvae were anaesthetised in E2 containing 4.2% MS-222, followed by recovery in 0.5X E2 media containing no anaesthetic. Where euthanasia was performed, zebrafish embryos and larvae were terminally anaesthetised in 0.5X E2 containing 15% MS-222 and death verified after overnight incubation. To prevent melanisation and aid imaging, Tg(mpeg-1: mCherry) zebrafish were maintained from 24 hours post-fertilisation (hpf) in 0.5X E2 containing 30 mg/L PTU.
2.5.2 Zebrafish injections

Embryos were dechorionated manually prior to injections using fine forceps. Yolk sac injections were performed at 1 day post-fertilisation (dpf, 30 hours post-fertilisation) and HBV injections at 2 dpf (50-54 hours post-fertilisation) with an IM 300 microinjector. Injection volumes were approximately 1 nl and the needles used were pulled borosilicate capillaries of 0.58 mm inner diameter (Harvard Apparatus, 30-0019). Embryos were injected with one of four possible injection solutions during this study, as specified in the text and figure legends: 1X PBS (Mock); 1X PBS containing \textit{M. marinum}; 10 mg/ml Bac5 (in nuclease-free H$_2$O); or 10 mg/ml Bac5 containing \textit{M. marinum}. The solution of 1X PBS was chosen as an osmotically-balanced non-inflammatory control (Mock) injection. To aid visualisation of the injection process, injection solutions all contained phenol red dye at a final concentration of 0.1% for experiments which did not involve Bac5 or at 0.0005% for experiments testing the effects of Bac5. A reduced phenol red dye concentration was used to prevent the precipitation of Bac5 seen with 0.1% phenol red. Embryos were positioned for injection using a 2% agarose channel mold\cite{356}. Infected zebrafish were monitored daily such that any zebrafish displaying signs of distress could be euthanized – signs of distress were not seen until 120 hpi, and so all experiments were terminated by this time point.

2.5.2.1 Culture preparation for injection

\textit{M. marinum} DsRed2 and GFP strains were grown to mid-logarithmic phase (OD$_{600}$ 1.0) in 5 ml starter cultures. Cells were harvested by centrifugation, washed 3X in 1X PBS, and resuspended at the appropriate dilution for injections in 1X PBS for \textit{M. marinum} only injections. Co-injection solutions were first resuspended at the appropriate dilution in 100 µl 1X PBS to provide the Mock/\textit{M. marinum} injection solution, with 10 µl of this preparation then being harvested by centrifugation and resuspended in 10 µl of 10 mg/ml Bac5 to provide the Bac5/\textit{M. marinum} injection solution. Growth curves were initially performed for each strain to determine the appropriate dilutions, and cultures were concentrated 5-fold for medium dose injections described in Chapter 3 and all work described in Chapter 4. Injection doses were verified by injecting bacterial solutions directly into a 10 µl drop of sterile 1X PBS and plating the drop to 7H10 for CFU counting after 5 days of incubation. Three 1X PBS drop injections were plated for each experiment.
2.5.3 CFU plating to determine zebrafish bacterial burden

Individual larvae were placed in microcentrifuge tubes containing 100 µl of 0.5X E2 containing 25 µg/ml kanamycin and 15 µg/ml gentamycin for 1 hour at room temperature. This medium was removed by aspiration and replaced with 150 µl of 0.25% Trypsin-EDTA. After incubation for 6 hours at 30°C, Triton X-100 in 1X PBS was added to a 0.1% final concentration, and the tubes were vortexed for 30 seconds. The sample from each tube was dilution plated onto individual 7H11 solid media plates containing 25 µg/ml kanamycin. Colony counts were performed after 5 days of incubation at 28.5°C.

The BBL® MycoPrep™ Specimen Digestion/Decontamination Kit (BD) was also used. Larvae were injected as described above, with individual larva euthanized and placed in microcentrifuge tubes containing 100 µl of 0.5X E2. The kit was then used according to the manufacturer’s recommendations, with vortexing to dissociate the larvae. Samples were dilution plated as above.

2.5.4 Microscopy and image quantification

Live zebrafish embryos and larvae were imaged using a Leica M205 FA fluorescence stereomicroscope. To enable quantification of infection progression parameters from images of zebrafish, transgenic zebrafish lines expressing fluorescently labelled immune cells and fluorescently-labelled *M. marinum* strains were used. Single z-plane images of whole zebrafish for total bacterial burden or bacterial dissemination (distance distribution) quantification were acquired in the lateral view. Z-stack images of the zebrafish HBV region for infection dose determination as well as leukocyte recruitment and HBV region bacterial burden quantification were acquired in the dorsal view with the z-stack range capturing the entire head region from the dorsal to ventral sides. All images were acquired using standardised settings for the parameter to be quantified such that repeat experiments could be compared and/or data pooled. In addition, all zebrafish were manually positioned in the described orientation to ensure the accuracy of parameter quantification from images. Data for zebrafish which died or suffered from pericardial and/or yolk sac edema during the course of longitudinal experiments presented in Chapter 4 was excluded from the analyses presented for all time points.
Brightness and contrast of images presented in this thesis were adjusted in Icy. Open-source image analysis software used in image quantification:

ImageJ  http://imagej.net/
Icy  http://icy.bioimageanalysis.org/

Icy has been created by the Quantitative Image Analysis Unit at Institut Pasteur (http://www.bioimageanalysis.org/). Copyright 2011 Institut Pasteur.

2.5.4.1 Quantification of bacterial burden (FPC protocol)

An Icy protocol to perform Fluorescent Pixel Count (FPC) was validated for use on images of infected zebrafish and then utilised throughout this study. This Pixel Density (Batch Mode) protocol (referred to as Icy FPC protocol throughout this thesis) was developed by Fabrice de Chaumont and made available for use in the open-source Icy platform (http://icy.bioimageanalysis.org/protocol/Pixel_density_(batch_mode)). The protocol uses the Icy Thresholded Pixel Density plugin (developed by Fabrice de Chaumont, http://icy.bioimageanalysis.org/plugin/Thresholded_pixel_density) to select pixels above a user-defined threshold in an image and combines this with desired outputs: an excel file detailing the selected pixels for each image and a binary image output displaying the selected pixels. Images of infected zebrafish and uninfected controls are acquired using standardised settings, and the Icy FPC protocol used to integrate the number of pixels in each image of infected zebrafish with values above a background threshold, as determined by the matched images of uninfected animals. In this study, the background intensity value was defined as the highest intensity pixel in the uninfected control images found outside of the yolk sac regions. Where stated, the standard definition of the background intensity value - the value for the highest intensity pixel in the uninfected control images - was also used. The FPC tool developed by the Ramakrishnan group in the ImageJ open platform for scientific image analysis is described in Adams et al. (2011)\textsuperscript{[5]} and provided with instructions in Takaki et al. (2012)\textsuperscript{[319]}. To validate the Icy FPC protocol, the same images were processed using the two different FPC methods and the values compared.

To counter the issue of high intensity yolk sac autofluorescence interfering with bacterial pixel counts for images acquired of whole zebrafish, regions of interest (ROI) were added to images of whole zebrafish. The first ROI covered the entire zebrafish
image and the second used a polygon to outline the yolk sac region. The Icy FPC protocol output provides FPC values for both ROIs, and pixels counted due to the yolk sac region autofluorescence were subtracted from the pixel count provided for the whole zebrafish. Yolk sac exclusion was used for initial FPC experiments described in Section 3.3.4.2.2 and injection dose determinations described in Section 3.3.4.3. Yolk sac exclusion was not required (and not performed) for all other experiments.

2.5.4.2 Quantification of HBV region bacterial burden and leukocyte recruitment

To permit the quantification of HBV region leukocyte recruitment and bacterial burden following injections images were first processed. Z-stack images of the whole head region of injected zebrafish had been acquired in the dorsal view. The in-focus z-slice was extracted for analysis. The selected z-slice was defined as the depth of slice from the top of the head in which bacterial fluorescence was seen in the majority of infected zebrafish groups; slices at that depth were then extracted and processed for all zebrafish in the experiment. To rapidly and reproducibly define a standardised HBV region for analysis, selected z-slice images were cropped at the otic vesicle, such that quantification was performed for the zebrafish head region from the tip to the otic vesicle - referred to as the “HBV region” throughout this thesis. Cropping facilitates the removal of the yolk sac and lymph nodes from the regions to be processed, eliminating issues with autofluorescence and large collections of immune cells in images respectively.

The processed z-slice was analysed using both the developed Icy FPC protocol to determine the bacterial burden, as described above, and the Icy Spot Detector plugin to determine leukocyte recruitment to the HBV region. For neutrophil counting, Scale 4 and Sensitivity 20% were used. For macrophage counting, Scale 3 and Sensitivity 20% were used. The Icy Spot Detector plugin was developed by Fabrice de Chaumont and Jean-Christophe Olivo-Marin\cite{230}, and made available for use in the open-source Icy platform (http://icy.bioimageanalysis.org/plugin/Spot_Detector).

2.5.4.3 Quantification of bacterial dissemination (bacterial distance distribution)

A protocol to quantify the bacterial distance distribution was developed in Icy with the assistance of Alexandre Dufour and Matthew Martin. The Bacterial Distance
Distribution protocol is provided in Figure 3.14, with scripts provided in Appendix 2. The protocol developed utilises the Icy Wavelet Spot Detector block to determine the number of bacterial clusters, their distance from the injection site in pixels and their position in a frequency histogram with a bin size of 10 pixels. The protocol requires the user to define three regions of interest for the analysis: one outlining the whole fish, one outlining the fish yolk sac and one point for distance determination (example here is the injection site, but could be any point). The user can define the image channel, ROIs, and the scale and sensitivity parameters for the analysis. The protocol outputs are: a binary image showing all detections; an output image showing all detections outside of the yolk sac region (utilises Icy Spot Detector plugin code); and an excel file containing the distance distribution data and the histogram bin data for each cluster identified outside of the yolk sac region.

To counter the issue of high intensity yolk sac autofluorescence interfering with bacterial pixel data for images acquired of whole zebrafish, regions of interest (ROI) were added to images of whole zebrafish. The first ROI covered the entire zebrafish image and the second used a polygon to outline the yolk sac region. The protocol output provides spot detection values for the whole zebrafish minus the yolk sac region. The protocol could be used without subtraction of the yolk sac region if the scale and sensitivity parameters were set to exclude the region and yolk sac subtraction blocks were removed from the protocol.

2.5.5 qRT-PCR sampling
Zebrafish were sampled for qRT-PCR at the indicated time points following injections by euthanizing the zebrafish, homogenising the samples using a pestle (Kimble-Chase) in lysis buffer (MagMAX™-96 Total RNA Isolation Kit), and proceeding with sample processing as described in Section 2.3.11. For the experiment detailed in Section 3.3.5, qRT-PCR was performed for pools of 4 whole zebrafish and zebrafish head samples for uninfected and infected embryo groups at 24 hpi. Samples of zebrafish embryo heads were taken following euthanasia by cutting with a scalpel between the head and trunk sections (Figure 3.16A). For all other experiments, qRT-PCR was performed for pools of 3 whole zebrafish embryos at 6 hpi and 24 hpi, or individual zebrafish larvae at 96 hpi.
2.6 Statistical analysis

All statistical analyses were carried out using GraphPad Prism 4.0 software (GraphPad Software, La Jolla California USA, www.graphpad.com). The D’Agostino-Pearson omnibus test was used to confirm a normal distribution of data (parametric data). Pearson’s correlation coefficient was used to assess the degree of correlation. To compare two groups, unpaired two-tailed t-tests or Mann-Whitney tests were used for parametric or non-parametric datasets, respectively. To compare more than two groups, one-way ANOVA followed by Bonferroni’s multiple comparison test or Kruskal-Wallis test followed by Dunn’s multiple comparison test were used for parametric or non-parametric data, respectively. To compare more than two groups during time-course experiments, repeated measures two-way or one-way ANOVA followed by Bonferroni’s multiple comparison test were used. P values of less than 0.05 were deemed statistically significant with ***p<0.001, **p<0.01 and *p<0.05.
3 Optimisation of techniques for the assessment of \textit{M. marinum} infection progression in THP-1 cells and the zebrafish infection model

3.1 Introduction

The optical transparency of zebrafish embryos and larvae permits the visualisation of pathogenesis during \textit{M. marinum} infections in real-time by live-imaging. Quantification of several infection progression parameters visualised during microscopy has been performed, including zebrafish survival following infection\textsuperscript{[319, 338]}, immune cell recruitment to the infection\textsuperscript{[53, 69, 134]}, bacterial burden over time\textsuperscript{[5, 88, 319, 328, 338, 344, 354]}, bacterial dissemination over time\textsuperscript{[5, 344, 345]} and the host immune response\textsuperscript{[53, 338]}. The methods previously used to quantify these infection progression parameters have several limitations including that they often require fixed samples, manual counting and use in combination to provide information on infection progression. Whilst they enable the analysis of large numbers of zebrafish at a single time point, repeated live-imaging as well as the rapid collection of data and analysis of large numbers of zebrafish during longitudinal studies are prevented. There is therefore scope to improve techniques for imaging and quantifying infection progression in zebrafish for use in longitudinal studies. To minimise distress to the zebrafish during microscopy, quantitative analysis of data from acquired images is preferable over analysis during imaging.

3.2 Aims

The aim of the initial THP-1 cell infection experiments presented in this chapter was to determine the optimal infection conditions for our assays and the optimal sampling for the quantification of gene expression by qRT-PCR as a measure of infection progression. The overall aim of the initial zebrafish infection work presented in this chapter was to set up the \textit{M. marinum}-zebrafish infection model at Imperial College London as this work had not been undertaken there previously, and then to develop and optimise methods to quantify \textit{M. marinum}-zebrafish infection progression parameters for use during longitudinal studies. The parameters to be quantified were zebrafish leukocyte recruitment to the HBV injection site, zebrafish immunity gene expression and infected zebrafish bacterial burden and bacterial dissemination.
3.3 Results

3.3.1 Optimisation of THP-1 cell infection with *M. marinum* and quantification of infection progression

3.3.1.1 Overview
HDP samples for testing during this study were limited, necessitating 96-well plate assays with THP-1 cells. Infections of THP-1 cells with *M. marinum* have been described in a 96-well format previously\[^{81, 301}\], though these infection experiments were performed under different conditions (Section 1.3.1). Therefore, we optimised infection conditions and qRT-PCR sampling to quantify host gene expression changes in response to the infection as this has not been previously described.

3.3.1.2 Optimisation of THP-1 cell seeding density for 96-well plate assays
To determine the optimal cell seeding density for 96-well and 24-well plate infections in our hands, several cell densities were trialled and the viability after 72 hours determined using trypan blue staining. To ensure that cells achieved confluency prior to the commencement of treatment, we selected THP-1 cell seeding densities of 1x10\(^6\) cells per well for 24-well plates and 5x10\(^5\) cells per well for 96-well plates.

3.3.1.3 Optimisation of multiplicity of infection (MOI) for *M. marinum* infection of THP-1 cells
We initially trialled infection experiments at an MOI of 10:1 (bacteria:THP-1 cells) to ensure that a reduction in bacterial burden could be visualised. Initial infection trials were performed using no, short and continuous antibiotic treatments to optimise the protocols for measurement of low and high stringency intracellular bacterial burden, and intracellular survival of the infecting bacilli, respectively.

The THP-1 cell infection results demonstrated variability in the infection burdens obtained from the procedures at an MOI of 10, seen by comparing Figure 3.1.A,B and
Figure 3.1.C,D. The majority of cells were seen to have lysed, indicated by floating cell debris, when samples were taken at 72 hours post-injection (hpi) in infected PMA-activated cell samples. For some experiments, the lysis was seen at 48 hpi and no samples taken 72 hpi as very few cells per well remained. This would indicate that the infection had progressed as far as the assay would permit before the termination of the experiments and therefore the lower MOI of 1 was next trialled. The MOI 10 infection results also showed that continuous amikacin treatment prevented the expansion of the infection as expected, enabling the determination of intracellular survival. Intracellular survival is a useful measure in experiments to determine the virulence of infecting bacterial strains. At this point in the PhD project it had become clear that such experiments would no longer be performed and therefore continuous amikacin treatments were not undertaken in further trials (Section 5.3.3.2).

Figure 3.1. Trial THP-1 cell infections with *M. marinum* in 24- and 96-well plates. (A-B) CFU counts from one experiment in a 24-well plate (A) and 96-well plate (B). (C-D) CFU counts from a second experiment in a 24-well plate (C) and 96-well plate (D). THP-1 cells were activated with 20 ng/ml PMA for 24 h, and incubated for a further 24 h without PMA. Cells were infected for 3 h with logarithmic-phase *M. marinum* at an MOI of 10, followed by incubation without amikacin (-Amikacin), with 200 µg/ml amikacin for 3 h (+Amikacin 3 h) or with 200 µg/ml amikacin continuously (+Amikacin continuous). Samples were taken after 0 h, 24 h, 48 h and 72 h following infection and 3 h of incubation with and without amikacin. The number of CFUs was determined by lysing infected THP-1 cells and dilution plating lysates. CFUs are plotted on logarithmic scale. Each data point represents three biological replicates, error bars represent S.E.M. Experiment was performed 5 times.

The THP-1 cell infection results demonstrated consistent infection burdens at an MOI of 1, with no cell lysis at 48 hpi (Figure 3.2). The results of the infection trials also confirm
that there is no difference in the growth rate between 24 and 48 hpi of *M. marinum* in THP-1 cells with and without 3 h of amikacin treatment as expected. The overall infection values and apparent initial growth rate seen are higher for those cells which were not treated with amikacin due to the continued infection with associated extracellular bacteria.

An MOI of 1 was therefore selected for use in future experiments.

![Figure 3.2. Trial THP-1 cell infections with *M. marinum* at an MOI of 1. (A-B) CFU counts from one representative experiment in a 96-well plate, experiment performed in duplicate. THP-1 cells were activated with 20 ng/ml PMA for 24 h, and incubated for a further 24 h without PMA. Cells were infected for 3 h with log-phase *M. marinum* at an MOI of 1, followed by incubation without amikacin (-Amikacin) or with 200 µg/ml amikacin for 3 h (+Amikacin 3 h). Cells were then incubated without amikacin and samples taken after 0 h, 24 h, 48 h and 72 h. The number of CFUs was determined by lysing infected THP-1 cells and dilution plating lysates. CFUs are shown on logarithmic scale (A) for ease of comparison to Figure 3.1 and on linear scale (B) for ease of comparison with Figure 4.2 (C-D). Each data point represents three biological replicates, error bars represent S.E.M. Experiment was performed twice.](image)

### 3.3.1.4 Optimisation of qRT-PCR sampling of THP-1 cells for 96-well plate assays

Firstly, we determined the number of wells per sample which are required to provide sufficient RNA of the desired quality for qRT-PCR analysis following 96-well plate assays. Nanodrop values following RNA extraction showed that sampling of single wells did not provide sufficient RNA for analysis and/or RNA of the required quality for most samples, whilst sampling of duplicate wells or of triplicate wells did (data not shown).

Secondly, we determined the time point for qRT-PCR sampling of THP-1 cells following infection (Figure 3.3). The results show that there is a detectable increase in cytokine production following *M. marinum* infection for both PMA-activated and non-activated THP-1 cells at each time point: *IL-1β* production can be seen to increase by >15-fold throughout the experiment; *TNF-α* production can be seen to show a greater fold change initially at 12 hpi of 7-fold, before gradually falling to approximately 2-fold at 48
hpi; and MMP-9 production shows a gradual increase in fold change throughout the experiment from 1.5-fold at 12 hpi to over ten-fold at 48 hpi. As expected, THP-1 cell cytokine production following infection with *M. marinum* demonstrated a larger increase for PMA-activated cells than resting cells at the early time points, consistent with reports that PMA-activated cells are more similar to macrophages than non-activated cells\(^{65}\). The observed differences in cytokine production between infected and uninfected cells are not statistically significant until 48 hpi, and then only for non-activated cells.

![Figure 3.3. Resting and PMA-activated THP-1 cell cytokine response to infection with *M. marinum*.](image)

**Figure 3.3. Resting and PMA-activated THP-1 cell cytokine response to infection with *M. marinum*.**

(A-C) Fold change of IL-1β, TNF-α and MMP-9 mRNA levels in resting and PMA-activated THP-1 cells following infection with *M. marinum* for 12 h (A), 24 h (B) and 48 h (C). THP-1 cells were incubated with or without 20 ng/ml PMA for 24 h, and then all cells incubated for a further 24 h without PMA. Cells were infected for 3 h with log-phase *M. marinum* at an MOI of 1, and samples taken after 12 h, 24 h, and 48 h. Relative expression levels were assessed by qRT-PCR, samples normalised to 18S and expressed relative to one control sample. Data from one representative experiment is shown (n=3), experiment was performed in duplicate. Each data point represents two treatment wells. Error bars represent S.E.M. Kruskal-Wallis with Dunn’s post-test. *p<0.05.
We selected sampling of duplicate wells at 24 hpi for qRT-PCR analysis of gene expression during future experiments as this time point provides detectable increases in the production of all three tested cytokines following infection with *M. marinum*, balancing the opposing trends seen for *TNF-α* and *MMP*-9. It was noted that the small fold changes involved will require the use of large sample sizes to resolve any effects seen during future experiments.

#### 3.3.2 Summary of zebrafish infection model optimisations performed during MRes rotation

We optimised zebrafish yolk sac infection protocols, infection doses for short term and long term infection, virulence assessment by larval survival studies, as well as time-lapse microscopy to study granuloma formation and initial interactions between infecting bacilli and the zebrafish immune system. In addition, initial zebrafish bacterial burden analyses using CFU plating after yolk sac infections revealed that the bacterial burden could not be determined from all individuals, as part of the zebrafish population harboured fast-growing commensal bacteria that grew on the antibiotic selection plates. Bleach treatment of embryos prior to infections to reduce the population of microbes associated with the zebrafish[^356] did not eliminate contamination with commensal bacteria upon CFU plating.

#### 3.3.3 Visualisation of initial interactions between infecting bacilli and the zebrafish immune system following HBV injection

The aim of these preliminary experiments was to demonstrate the interaction and recruitment of zebrafish immune cells with and to *M. marinum* aggregates in the HBV following HBV injections. At 72 hpi, initial interactions between the immune cells and bacteria can be seen (Figure 3.4) as well as initial granuloma formation (an aggregate of macrophages, consistent with the field opinion of initial granuloma formation[^69]) in two cases (Figure 3.4B,C and F,I). Macrophages were observed to be recruited to both primary and secondary bacterial aggregates, but not the site of injection, indicating that they are attracted to the bacteria themselves and not the site of an injury (Figure 3.4B,C). These results also reproduce some of the observations with yolk sac injections undertaken during the MRes rotation: that some bacterial aggregates are visible using
the brightfield channel alone (Figure 3.4A) and that there is variability of infection progression and severity seen between fish, as some fish can show very little evidence of infection at the injection site (Figure 3.4D,G), whilst others show very high levels of dissemination (Figure 3.4A-C) from the same infection procedure.

We concluded that the HBV injection procedure had been mastered and further experiments could be performed.

**Figure 3.4. Interactions between zebrafish immune cells and *M. marinum*.** Zebrafish injected into their HBV at two days post-fertilisation (dpf) with 100 CFU *M. marinum* expressing GFP. Images taken at 72 hpi using a fluorescence stereomicroscope. **(A-C)** Brightfield (A), brightfield and fluorescence channels overlay (B) and merged fluorescence channels (C) images of a zebrafish showing the initial bacterial aggregate and two secondary aggregates (arrowed) and initial interactions between zebrafish macrophages and *M. marinum*. The interactions indicated by the rightmost arrow appear to be the initial stages of granuloma formation as the bacilli are surrounded by macrophages. Bacterial aggregates are visible as lesions in the brightfield image shown in (A). **(D-F)** Brightfield and fluorescence channels overlay images of three zebrafish. **(G-I)** Merged fluorescence images of the boxed areas shown in (D-F) in the corresponding order. The initial stages of granuloma formation can be seen in (I) as the bacilli are surrounded by macrophages.
3.3.4 Quantification of zebrafish infection progression from fluorescence images using the bioimage informatics platform Icy

3.3.4.1 Overview

We developed and/or optimised techniques for the quantification of infection parameters from images of infected zebrafish embryos and larvae to permit the rapid, serial quantification of several parameters simultaneously in longitudinal studies of zebrafish infection. The quantified infection progression parameters were bacterial burden of infected zebrafish, leukocyte recruitment to the infection site and bacterial dissemination from the infection site. We chose to use the open-source bioimage informatics platform Icy to perform these quantifications for the particular analyses, described below, this platform enables over other software such as ImageJ.

3.3.4.2 Quantification of infected zebrafish bacterial burden

3.3.4.2.1 Summary of quantification of infected zebrafish bacterial burden by CFU plating attempts

During my MRes rotation, bleach treatment of zebrafish embryos prior to infections did not eliminate CFU plating issues with contamination by commensal bacteria and an alternative was trialled during the PhD. Attempts with the BBL® MycoPrep™ Specimen Digestion/Decontamination Kit\[317\], which involves the use of the decontaminating agent NaOH, were initially unsuccessful in that no bacteria were recovered following the procedure. Optimisation of decontaminating agent treatment length was performed and CFU plating successfully attempted. The data revealed large variations in the CFU counts between individual fish samples, with no bacterial CFU again recovered from the decontamination procedure for some of the samples, which suggests that samples may have been differentially affected by the decontamination and lysis procedures (Figure 3.5). Therefore, quantification of infected zebrafish bacterial burden by CFU plating was found to be an unreliable method and alternatives were sought.
Whole embryo bacterial load counts from dissociation plating 5 dpi with GFP *M. marinum*. Ten embryos per dose were injected into the yolk sac at 1 dpf with approximately 10, 100, 167, 250, 500 or 1,000 CFU of GFP *M. marinum*. Ten embryos were also injected with Mock as a control. Embryos had been bleach treated prior to removal from the chorion to minimise contamination with other bacterial strains. At 5dpi embryos were dissociation plated using the MycoPrep Kit for CFU counts of the bacterial load. Bacterial load counts were successful for ten embryos injected with 0, 10 and 1,000 CFUs, for six embryos injected with 250 CFUs and for five embryos injected with 100, 167 and 500 CFUs. Error bars represent S.E.M. Figure adapted from R. Price’s MRes Dissertation[260].

3.3.4.2.2 Quantification of infected zebrafish bacterial burden by validation and optimisation of an Icy fluorescent pixel count (FPC) protocol

3.3.4.2.2.1 Selection of the Icy FPC protocol

We decided to use FPC to determine the bacterial burden of infected zebrafish, as performed by other groups[5, 317], to eliminate the difficulties associated with CFU plating. An FPC protocol using the Icy bioimage informatics platform was validated for use in the zebrafish (developed by Fabrice de Chaumont, Figure 3.6). During application of the Adams *et al.* FPC tool[5], the requirement for a binary output image output to enable rapid visualisation of the results of FPC thresholding was discovered, which necessitated the use of the Icy FPC protocol. The Icy FPC protocol uses the same principles as those used by other research groups - that all pixels with an intensity value over a threshold set by the user are counted and that image acquisition settings must be standardised – but also provides a binary output image showing all of the counted pixels (Figure 3.7C-H).
Icy FPC protocol validation Experiment 1 – comparing the results obtained using the Icy FPC protocol and published ImageJ FPC tool to analyse the same images

To validate the Icy FPC protocol for use in the *M. marinum*-zebrafish infection model, Experiment 1 was performed in which the same images were processed using the Icy FPC protocol and a published tool followed by comparison of the results. The FPC tool developed by the Ramakrishnan group in the ImageJ open platform for scientific image analysis is described in Adams *et al.* (2011)[5] and provided with instructions in Takaki *et al.* (2012)[319]. Representative images from Experiment 1 show the range of bacterial burdens at 6 dpi (Figure 3.7A-D), with the zebrafish shown in Figure 3.7A displaying a very high bacterial burden in the head region whilst the zebrafish shown in Figure 3.7C displays almost no visible infection in the head region. All of the zebrafish larvae demonstrated dissemination of bacteria out of the HBV into the rest of the brain and other head regions by 6 dpi, with one of the ten zebrafish larvae demonstrating
bacterial dissemination to the tail (Figure 3.16A,C). Two different thresholds were used in the FPC analyses, a high threshold which excluded yolk sac autofluorescence (Figure 3.7I) and a low threshold which did not (Figure 3.7J). The use of two different thresholds is explained below. The FPC data using both thresholds shows that the ImageJ and Icy methods provide similar results for the analysis of the same images (Figure 3.7I-J).

Figure 3.7. Validation of Icy Fluorescent Pixel Count (FPC) protocol. Ten 2 dpf casper Tg(mpx:GFP) zebrafish embryos were injected into the HBV with a medium dose of M. marinum expressing DsRed2. Larvae were imaged in the lateral orientation at 6 dpi (8 dpf). Experiment was performed once. (A-D) Representative images of four out of ten fish. Overlay of brightfield and bacterial fluorescence channel is shown. White polygons outline the yolk sac region of autofluorescence which can be subtracted from FPC values. White boxes indicate the regions shown as FPC binary image outputs in (E-H). Scale bar (black) 200 µm. (E-H) Binary images showing the output of the Icy FPC protocol in the white boxed area of (C) in (E-F) and (D) in (G-H). Image outputs at the high threshold (E, G) and the low threshold (F, H) are shown. Blue arrows indicate regions of yolk sac autofluorescence. Blue arrowheads indicate regions of bacterial fluorescence. (I) Comparison of the FPC values provided for the same 10 images analysed at the high threshold using the ImageJ tool and Icy FPC protocol. Both sets of values are shown without yolk sac autofluorescence subtraction. (J) Comparison of the FPC values provided for the same 10 images analysed at the low threshold using the ImageJ tool without yolk sac autofluorescence region subtraction, Icy FPC protocol without yolk sac autofluorescence region subtraction and Icy FPC protocol with yolk sac autofluorescence region subtraction. Subtracted yolk sac regions are indicated in (A-D) by white polygons. (K) Comparison of the FPC values provided for the same 10 images analysed at high (72) and low (14) intensity thresholds using the Icy FPC protocol. The values shown for both thresholds are those with yolk sac autofluorescence subtracted.
We concluded that the validation of the Icy FPC protocol was successful due to the comparable results provided during Experiment 1.

3.3.4.2.2.3  Development of a yolk sac autofluorescence subtraction method

The field-standard threshold is defined as the highest intensity pixel in matched images of uninfected larvae and thus mostly excludes yolk sac autofluorescence. The yolk sac region shows significant autofluorescence in images, seen as the red colour within the black polygons in Figure 3.7A-D, the intensity of which varies between larvae. As a result, one of the infected larvae demonstrated higher yolk sac autofluorescence intensity than any of the uninfected larvae used as controls to set the background intensity threshold. Therefore, during initial analysis of Experiment 1 using the ImageJ FPC tool developed by the Ramakrishnan group, yolk sac autofluorescence was included in the FPC value for that larvae (Figure 3.7G, arrow). As a result, we decided to use the Icy FPC protocol which includes a binary output image that displays the counted pixels for each image analysed. The binary output images for each zebrafish larvae can then be used to confirm that the FPC method has been performed satisfactorily.

Application of the field standard threshold in the Icy FPC protocol to the Experiment 1 images results in the counting of a far reduced region of bacterial fluorescence in the binary output images than is seen in the original fluorescence images (Figure 3.7E,G), indicating a loss of bacterial pixel data. Conversely, use of a lower threshold - defined as the highest intensity pixel in the uninfected control images found outside of the yolk sac regions - includes yolk sac autofluorescence in the bacterial pixel count (Figure 3.7F,H). As a result, a method for excluding yolk sac autofluorescence but including bacterial pixel data was sought.

The output of Icy protocols focuses only on the user-defined region(s) of interest (ROI(s)) within an image which are added prior to analysis. We used one ROI to outline the entire zebrafish and another to outline the yolk sac region (Figure 3.7A-D, black polygons indicate yolk sac regions), such that pixels counted due to the yolk sac region autofluorescence can be subtracted from the pixel count provided for the whole zebrafish. FPC values obtained using the lower threshold from the ImageJ tool, Icy FPC protocol and Icy FPC protocol with subsequent yolk sac region subtraction show that
yolk sac region subtraction does not affect the overall data trend and can be used to improve the accuracy of comparisons of different infection conditions (Figure 3.7J). FPC values obtained using both low and high thresholds using the Icy FPC protocol with subsequent yolk sac region subtraction are shown in Figure 3.7K.

A disadvantage of subsequent yolk sac region subtraction from the FPC values is that any bacterial presence in the yolk sac due to dissemination to that site is excluded. However, it can be seen that a great deal of bacterial infection data is lost by excluding all pixels of the same intensity as the yolk sac autofluorescence (Figure 3.7E,G), which would also apply to bacteria present in the yolk sac. Using the Icy FPC protocol with yolk sac region subtraction each zebrafish image in the batch is treated in the same way, providing valid comparisons over the rest of the zebrafish which includes a greater proportion of the bacterial infection data, permitting the detection of small changes in bacterial burden between experimental groups.

3.3.4.2.2.4 Icy FPC protocol validation Experiment 2 – comparing the results obtained from analysis of infected zebrafish images showing varying bacterial burdens

We next assessed whether the Icy FPC protocol and post-processing method to subtract the yolk sac region could accurately distinguish between zebrafish showing high and low bacterial burdens in Experiment 2. For Experiment 2, zebrafish were infected with different starting doses to provide varying bacterial burdens for analysis at 72 hpi. Representative images taken at 72 hpi of larvae from the three infection groups are shown in Figure 3.8A-C. As expected, zebrafish larvae infected with the low dose showed the lowest bacterial burdens (Figure 3.8A) and those infected with the high dose the highest bacterial burdens (Figure 3.8C) at 72 hpi. The bacterial burdens obtained at 72 hpi for each starting dose showed little overlap between groups. As for Experiment 1, high yolk sac autofluorescence was seen and all zebrafish demonstrated bacterial dissemination outside of the HBV to other areas of the head but very few zebrafish demonstrated bacterial dissemination out of the head region. FPC values for individual zebrafish larvae accurately reflected the observed differences in bacterial burden from images (Figure 3.8D).
Figure 3.8. FPC quantification of relative *M. marinum* burden in zebrafish. (A-D) Twenty 2 dpf embryos per group were infected into their HBV with low (approximately 125 CFU), medium (approximately 250 CFU) and high doses (approximately 1,250 CFU) of *M. marinum* expressing DsRed2. Ten embryos were also injected with Mock solution as a control. Embryos were imaged at 6 hpi and 72 hpi using a fluorescence stereomicroscope. Images were processed using the Icy FPC protocol with yolk sac autofluorescence subtracted from each FPC value presented. Experiment was performed twice, with data from one representative experiment shown. (A-C) Three bacterial fluorescence images are shown for zebrafish larvae 72 hpi infection with low (A), medium (B) and high (C) doses of *M. marinum*. Images were selected which show the range of infection burden for each dose, i.e. one larva with a low burden, one with an average burden and one with a high burden. White polygon shows larva outline, white numbers refer to graph shown in (D), and insets show corresponding brightfield images. Scale bar 100 µm. (D) Graph shows FPC for each image in (A-C), according to the numbers shown in white in (A-C).

During Experiment 2 we also compared the FPC values with those obtained through CFU plating using MycoPrep Kit treatment (Section 3.3.4.2.1), for the same infected zebrafish larvae. A positive overall correlation was observed at $r^2 = 0.6576$ (Figure 3.9D), which also reflects the variability in the individual CFU plating values obtained. It had been anticipated that the loss of bacteria during the lysis and decontamination processes, potentially resulting in the differential killing of bacteria between samples as well as a reduction in the recovery of bacteria overall, would result in large variations in the CFU plating data for the low dose infection group in particular due to the relatively
small numbers of bacteria involved. This in turn was expected to negatively affect the correlation between the data from the two measurements for the low infection dose group, and the data reflect this (Figure 3.9A). Correspondingly, the data demonstrates a greater correlation for the medium and high infection dosage groups (Figure 3.9B-C).

It is therefore proposed that variations in the effects of lysis and decontamination reported by both myself and other groups results in a high degree of variability in the CFU plating values, making a comparison between the FPC and CFU plating values for individual zebrafish larvae difficult. In keeping with this, individual fish data for FPC and CFU plating values reported by Adams et al. would appear to show similar variation to those presented in this thesis, although the data was not shown in a direct comparison. Adams et al. did present in the supplementary information an analysis of the correlation between average Log_{10} FPC and average Log_{10} CFU values for four different treatment groups, which provided a value for Pearson’s Coefficient of Correlation \( r = 0.9341 \)\textsuperscript{5}. This analysis led to our expectation that the data obtained from the two methods would show a high degree of correlation. Indeed, analysis of the correlation between average Log_{10} FPC and average Log_{10} CFU values for Experiment 2 shows an \( r^2 = 0.9746 \) (Figure 3.9E), which is comparable to the previous data.

We concluded that the Icy FPC protocol alone and with post-processing yolk sac subtraction can be used to accurately distinguish between zebrafish showing varying bacterial burdens based upon the results of Experiments 1 and 2. In Experiment 1, the FPC values for individual zebrafish larvae accurately reflected the observed differences in bacterial burden from images. In Experiment 2, we observed FPC data accurately reflecting images, high correlation between the injection dose group averages for FPC and CFU plating values, as well as positive correlation between individual zebrafish FPC and CFU plating values. The Icy FPC protocol with post-processing yolk sac region subtraction was therefore selected for use in future experiments to determine the bacterial burden over whole zebrafish. These experiments could involve the assessment of infecting strain virulence, intended for use with TCSs deletion mutant strains (attempted generation of these strains is described in Chapter 5). The Icy FPC protocol alone was selected for use in future experiments to determine the bacterial burden over the HBV region (Section 3.3.4.4), described below and in Chapter 4, as yolk sac region subtraction is not required for analysis of this region.
Figure 3.9. FPC as an accurate measure of relative *M. marinum* burden in zebrafish. (A-F) Twenty 2 dpf embryos per group were infected into their HBV with low (approximately 125 CFU), medium (approximately 250 CFU) and high doses (approximately 1,250 CFU) of *M. marinum* expressing DsRed2. Ten embryos were also injected with Mock solution as a control. Embryos were imaged at 6 hpi and 72 hpi using a fluorescence stereomicroscope. Following imaging at 72 hpi larvae were lysed and plated for CFU counting. Images were processed using the Icy FPC protocol with yolk sac region subtraction, fish lysed using MycoPrep Kit and colonies counted after 5 days. Experiment was performed twice, with data from one representative experiment shown. (A-D) Graphs show FPC vs CFU plating. Sample size (n): 24 (A), 21 (B), 21 (C) and 66 (D). (A) Low dose infections, $r^2 = 0.2434$. (B) Medium dose infections, $r^2 = 0.4917$. (C) High dose infections, $r^2 = 0.6067$. (D) Pooled data for all infection doses, $r^2 = 0.6578$. (E) Correlation between average FPC per fish and average CFU count per fish data. Sample size (n): 24 (Low), 21 (Medium) and 21 (High), $r^2=0.9746$, p=0.1019. (F) FPC measurements for two fish imaged in dorsal and left lateral orientations 72 hpi with medium dose of *M. marinum* expressing DsRed2.
Effect of zebrafish orientation on FPC values

We found that some zebrafish larvae can be imaged in different orientations without significantly altering the FPC values obtained, such as for zebrafish A in Figure 3.9F, which returned FPC values of 846 pixels in the dorsal orientation and 883 pixels in the lateral orientation. This data is in agreement with that reported by other groups\[5\]. However, zebrafish B returned an FPC value of 412 pixels when imaged in the dorsal orientation but 0 pixels when imaged in the lateral orientation (Figure 3.9F).

We concluded that zebrafish orientation during imaging must be standardised during an experiment similarly to the image acquisition settings to ensure that the data can be compared. All zebrafish were therefore manually positioned in the same orientation for imaging in all subsequent experiments.

Determination of M. marinum dosage for zebrafish infection experiments

We wished to quantify zebrafish bacterial burden and leukocyte recruitment to the HBV region to assess the effects of Bac5 treatment on M. marinum infection progression (detailed in Chapter 4). Limited stocks of Bac5 were available for these experiments, and we therefore chose to assess both bacterial burden and leukocyte recruitment in the HBV region following HBV injections. Quantification of bacterial burden in the HBV at 3 dpi with M. marinum following confocal microscopy has been described previously\[41\], presented as bacterial volume in the HBV and determined using a three-dimensional surface-rendering feature of Imaris rather than FPC. Quantification of the bacterial burden of whole zebrafish at 5 dpi with M. marinum to the HBV by FPC has also been described by Torraca et al.\[328\]. We believe that FPC quantification of zebrafish bacterial burden at early time points following M. marinum infections to the HBV using fluorescence microscopy has not been reported previously. We therefore wished to verify that M. marinum infection of the HBV can be visualised by fluorescence microscopy at the desired early time points.

The analysis of the images taken at 6 hpi in Experiment 2 (Figures 3.7-3.8 for 72 hpi analyses) is described here and was performed to optimise the infection dose. The FPC values for the low infection dose group ranged from 0 to 279 pixels, with the majority of the infected embryos showing little detectable infection (Figure 3.10D). The low dose of infection (approximately 125 CFU) was therefore deemed unsuitable for use in future
infection experiments assessing infection progression parameters as a reduction in the
infection burden at 6 hpi due to the use of a less virulent strain or an effective treatment
could not be detected. FPC values for the medium and high dose infections
(approximately 250 and 1,250 CFU respectively) were in a detectable range for all
infected zebrafish larvae at 6 hpi (Figure 3.10E-F), and both doses deemed suitable for
use future infection experiments assessing infection progression parameters. The
survival of infected zebrafish larvae was monitored throughout Experiment 2. There
were no dose-dependent effects of *M. marinum* infection on zebrafish survival during
the course of the experiment, confirming that both the medium and high infection doses
could be used (Figure 3.10.B).

The medium dose of approximately 250 CFU was selected for future zebrafish infection
experiments. The field typically use injection doses of 50-200 CFU *M. marinum* to the
HBV for leukocyte recruitment studies, thus the selected dose of 250 CFU is in line with
the field standard for infections to the HBV.
Figure 3.10. FPC of relative *M. marinum* burden in zebrafish 6 hpi. (A-F) Twenty 2 dpf embryos per group were infected into the HBV with low (approximately 125 CFU), medium (approximately 250 CFU) and high doses (approximately 1,250 CFU) of *M. marinum* expressing DsRed2. Ten zebrafish embryos were injected with Mock solution as a control. Images were taken at 6 hpi at a fixed z-axis depth for all zebrafish, determined using the infected zebrafish embryos as a guide, using standardised image acquisition and magnification settings. The Icy FPC protocol with yolk sac region subtraction was then performed on the acquired images. The threshold was defined as the highest intensity pixel in the uninfected control images found outside of the yolk sac regions. Data presented for the same fish as in Figures 3.7 and 3.8. Experiment was performed twice, with data from one representative experiment shown. (A) Overlay images showing brightfield and bacterial fluorescence channels cropped to the head region for three representative zebrafish embryos 6 hpi with each infection dose - low, medium and high. Scale bar 100 µm. (B) Survival of zebrafish larvae following infection with low, medium and high doses of *M. marinum*. (C) Graph showing log10 FPC values for each infection dose group at 6 hpi. (D-F) Graphs show FPC values for low (D), medium (E), and high (F) doses at 6 hpi. Sample size (n): 24 for low, 21 for medium, and 21 for high dose injection groups.
3.3.4.4 Development of an image processing method to permit simultaneous analysis of bacterial burden and leukocyte recruitment in the HBV region

We wished to combine the optimised techniques to quantify the bacterial burden of *M. marinum*-infected zebrafish using a fluorescence stereomicroscope with techniques for the quantification of zebrafish leukocyte recruitment. To prevent repeated anaesthetisations of zebrafish and facilitate the simultaneous monitoring of bacterial burden and leukocyte recruitment during infection, we wished to perform live-imaging of transgenic zebrafish using a fluorescence stereomicroscope only. Confocal microscopy is traditionally used during manual quantification of leukocyte recruitment to the zebrafish HBV. To eliminate counting bias associated with manual leukocyte counting during live-imaging we wished to quantify leukocyte recruitment from acquired images using standardised image acquisition and analysis parameters for all injection groups. This would also provide a more medium-throughput method of analysis than manual leukocyte counting.

To enable the medium-throughput quantification of the bacterial burden and leukocyte recruitment from images, it is desirable to provide an image of single plane of focus along the z-axis for analysis rather than a z-stack of images taken throughout the entire HBV region. Therefore, images of zebrafish were taken in the dorsal view as this provides the most complete snapshot of the HBV region in a single z-plane due to the reduced depth of the HBV region in this orientation compared to the lateral orientation. The in-focus z-slice was defined using the depth from the top of the head in which bacterial fluorescence was seen in the majority of infected zebrafish groups; the approximate location is shown in Figure 3.11A. Image slices at this depth were then extracted for all zebrafish in the experiment and analysed using both the Icy FPC protocol to quantify the bacterial burden and the Icy Spot Detector plugin[^230] (developed by Fabrice de Chaumont and Jean-Christophe Olivo-Marin, Figure 3.11.C,D) to quantify leukocyte recruitment.

During image processing, auto-fluorescence from the yolk sac and lymph nodes in the neck was seen to affect bacterial pixel count values determined by the Icy FPC protocol and leukocyte counts determined using the Icy Spot Detector plugin, respectively. In addition, the HBV is not readily defined in the dorsal view. We therefore defined a standardised region for analysis by cropping the selected z-slices at the otic vesicle,
referred to as the HBV region throughout this thesis. Bacterial burden and leukocyte recruitment were determined within the zebrafish head as far as the otic vesicle (HBV region), as shown in Figure 3.11A-B. An advantage of performing analyses in this way is that wider differences in bacterial burden and leukocyte recruitment to the head region following HBV injection could be determined than recruitment to the HBV alone.

Figure 3.11. Quantification of leukocyte recruitment to the HBV region of zebrafish from fluorescence images using Icy Spot Detector plugin. (A) Brightfield images of a 2 dpf zebrafish embryo taken in the lateral view using a fluorescence stereomicroscope. White circle indicates the otic vesicle, white polygon the HBV, red dashed line the image crop line (image region left of the line, covering the head, analysed), green dashed line the approximate location of the z slices selected for analysis. Scale bar 100 µm. (B) Brightfield images of a 2 dpf zebrafish embryo taken in the dorsal view using a fluorescence stereomicroscope. White polygons indicate the otic vesicle. The dorsal view was used for all spot detection analyses in this thesis. Images are shown cropped at the otic vesicle for spot detection analysis. (C) Icy Spot Detector plugin used to quantify fluorescent spots in images – plugin was developed by Fabrice de Chaumont and Jean-Christophe Olivo-Marin[230], and made available for use in the open-source Icy platform. The Icy Spot Detector plugin requires the user to define the channel for detection, whether bright spots or dark spots should be detected, and the scale and sensitivity of the detection parameters for analysis. The scale parameter defines the size in pixels of an object for it to be counted as a spot. The sensitivity parameter defines the intensity of the pixels of an object relative to the maximum intensity for the whole image for the object to be counted as a spot. The plugin provides a summary in excel and an output image displaying the number of spots counted in the image. (D) Panel showing overlay and spot detector output images of two casper Tg(mpx:GFP) zebrafish embryos 4 hpi to the HBV with Mock or M. marinum expressing DsRed2 (Mmar). Images were taken in the dorsal view using a fluorescence stereomicroscope. Overlay images show brightfield, GFP neutrophil fluorescence and M. marinum DsRed2 fluorescence channels. Spot detector output images show the analysed GFP neutrophil fluorescence channels only. Scale bar 100 µm.
3.3.4.5 Quantification of zebrafish leukocyte recruitment to the HBV injection site

3.3.4.5.1 Overview

We wished to monitor the recruitment of leukocytes to the zebrafish HBV following injections over several time points, to enable us to determine the longer-term effects on the zebrafish. We aimed to select three time points which would allow us to determine the early-, mid- and longer-term effects of injections on zebrafish leukocyte recruitment to the injection site and the bacterial burden of infected zebrafish. The leukocyte recruitment quantifications were to be used in this project to inform on the effects of *M. marinum* TCSs deletion or Bac5 treatment on leukocyte recruitment to the infection, as well as Bac5 treatment on leukocyte recruitment to the injection site in the absence of infection.

Macrophages are known to be recruited to the site of *M. marinum* infections only a few hours after injection\(^{[26]}\), and therefore the assessment of the effects of *M. marinum* TCSs deletion or Bac5 treatment on macrophage recruitment to the infection can be performed within the first 24 hours. It is known that neutrophils directly interact with and phagocytose infecting mycobacteria in animal infection models as well as during human TB infection\(^{[188]}\). However, it has been reported that neutrophils show little interest in infecting *M. marinum* bacilli 3-6 hpi to the zebrafish HBV at 30 hpf, to the extent that *M. marinum* bacilli appear to be invisible to neutrophils in terms of chemotaxis and phagocytosis\(^{[363]}\). We therefore did not expect to see significant neutrophil recruitment in response to infection with *M. marinum* until 2-4 dpi of the zebrafish when early granuloma formation begins. The later-stage time point of 72 hpi was therefore selected solely for the assessment of the effects on zebrafish neutrophil recruitment to the infection of *M. marinum* TCSs deletion or Bac5 treatment.

Previous data indicated that Bac5 increases the production of the neutrophil chemoattractant IL-8 and the enzyme iNOS in epithelial cells (Sam Willcocks unpublished data). We therefore hypothesised that Bac5 would increase neutrophil recruitment to the injection site in the presence and absence of *M. marinum* infection immediately following injections. It is also known that neutrophils play a protective role in the early stages of infection in animal models of TB\(^{[188]}\). In addition, neutrophils can
protect the zebrafish host from mycobacterial infections by killing of internalised mycobacteria from infected macrophages through NAPDH oxidase dependent mechanisms\cite{363}. We therefore also hypothesised that the earlier recruitment of neutrophils to the injection site by Bac5 may increase phagocytosis and killing of the infecting mycobacteria, reducing bacterial burden. To enable testing of these hypotheses, we also wished to select earlier time points to quantify zebrafish neutrophil recruitment and bacterial burden in response to injections.

The following sections describe the selection of the earlier time points which will be used in subsequent experiments for the analysis of macrophage recruitment, neutrophil recruitment and bacterial burden.

3.3.4.5.2 Investigation of zebrafish leukocyte recruitment timings in response to HBV infection with M. marinum

To determine which time points might be useful to study effects on leukocyte recruitment following injection to the HBV, images of the HBV region were acquired over the first 24 hours of zebrafish infection with \textit{M. marinum}. The experiment was performed separately for macrophages and neutrophils.

During imaging, it was seen that double transgenic animals expressing both mCherry-labelled macrophages and GFP-labelled neutrophils had been inadvertently included in the experiment to assess macrophage recruitment timings. Zebrafish embryos expressing only mCherry-labelled macrophages were included in the analysis, providing 7 zebrafish injected with a Mock solution and 3 with a medium dose of \textit{M. marinum}. In addition, during the experiment to assess neutrophil recruitment timings the agarose gel mold moved twice, disrupting the imaging timings. The first movement occurred after the 4 hpi time point and was discovered an hour later after the 5 hpi time point. Zebrafish were repositioned and the experiment continued at 7 hpi. Three zebrafish in the Mock injection group were not successfully repositioned, providing 7 zebrafish injected with a Mock solution for comparison with 10 injected with a medium dose of \textit{M. marinum}. The second movement occurred during overnight imaging at 19 hpi and necessitated the termination of the experiment at this time point.
Images were analysed and zebrafish scored as positive or negative for leukocyte recruitment to the HBV region, scoring is represented graphically in Figure 3.12. The scoring revealed that all 7 Mock–injected zebrafish showed macrophage recruitment to the HBV from 2-4 hpi, and then the macrophages left the HBV region until only one zebrafish showed recruitment from 8-24 hpi. Similarly, most Mock–injected zebrafish showed neutrophil recruitment to the HBV from 3-13 hpi, and then the neutrophils left the HBV region until only two zebrafish showed recruitment from 15-19 hpi. By contrast, all of the infected zebrafish demonstrated leukocyte recruitment after 4 hpi. The data can be interpreted as Mock-injected zebrafish embryos demonstrating leukocyte recruitment to the wounding damage up until 8 hpi, whereas infected zebrafish showed sustained leukocyte recruitment as a result of the infection.

It was concluded that suitable early-stage and mid-stage time points would be in the ranges of 2-8 hpi and 8-24 hpi respectively. The early-stage time point of 3 hpi was selected as an accepted early stage time point used by the field which is practicable in larger scale experiments involving longer injection timings prior to the commencement of imaging. The mid-stage time point of 24 hpi was selected to allow zebrafish embryos to recover from repeated anaesthetisations.

Figure 3.12. Leukocyte recruitment timings following HBV infection with M. marinum. 2 dpf Tg(mpeg-1:mCherry) or casper Tg(mpx:GFP) zebrafish embryos were injected into their HBV with Mock or a medium dose of M. marinum (Mmar) expressing GFP or DsRed2, respectively. Zebrafish embryos were immobilised in 2% low-melting point agarose and live-imaged using a fluorescence stereomicroscope. Z-stack images of the HBV region were acquired every hour at 2-10 hpi and every 2 h at 12-24 hpi for macrophage recruitment determinations or every hour at 2-4 hpi and every 2 h at 7-19 hpi for neutrophil recruitment determinations. Images were assessed for leukocyte recruitment to the HBV and zebrafish scored positive or negative for recruitment. (A-B) Graphs displaying the percentage of zebrafish demonstrating macrophage recruitment (A) and neutrophil recruitment (B) over the course of the experiments are shown. Sample size (n): Mock=7, Mmar=3 for macrophage recruitment and Mock=7, Mmar=10 for neutrophil recruitment. Each experiment was performed once.
3.3.4.5.3 Determination of zebrafish macrophage recruitment timings in response to HBV infection with M. marinum

The macrophage recruitment findings at 3 hpi and 24 hpi were verified using a greater number of Tg(mpeg-1:mCherry) zebrafish embryos per group to establish statistical significance. To prevent double transgenic animals being included in the experiment, zebrafish embryos were screened as mCherry positive but GFP-negative prior to use in the experiment. Following injections to the HBV with Mock and M. marinum, significant macrophage recruitment was seen to the infection 3 hpi and a non-significant trend for elevated macrophage recruitment 24 hpi in response to the infection as expected (Figure 3.13). It was established that a scale parameter of 3 (~7 pixels) and sensitivity parameter of 20% were optimal for the detection of macrophages in the HBV region.

Figure 3.13. Verification of macrophage recruitment timings following HBV infection of zebrafish with M. marinum. (A-C) Two dpf Tg(mpeg-1:mCherry) zebrafish embryos were injected into the HBV with Mock or a high dose of M. marinum expressing GFP (Mmar). Zebrafish embryos were live-imaged using a fluorescence stereomicroscope and z-stack images of the HBV region acquired at 3 hpi and 24 hpi. In-focus z-slices were extracted, cropped and processed for macrophage recruitment as described previously (Section 3.3.4.3). (A) Representative images of Mock and Mmar injected embryos are shown for each time point. Scale bar 100 µm. (B-C) Macrophages in the HBV region were quantified from fluorescence images of zebrafish embryos using Icy Spot Detector plugin at 3 hpi (B) and 24 hpi (C). Sample size (n): 24, 34. Experiment was performed once. Error bars represent S.E.M. Unpaired t-test. **p<0.01.
We concluded that 3 hpi and 24 hpi were suitable time points for the analysis of the effects of different bacterial strains or treatment with chemical compounds on zebrafish macrophage recruitment. Therefore, these time points, along with the identified detection parameters, were selected for subsequent experiments, described in Chapter 4.

3.3.4.5.4 Determination of zebrafish neutrophil recruitment timings in response to HBV infection with M. marinum

The neutrophil recruitment findings at 3 hpi and 24 hpi were verified using a greater number of casper Tg(mpx:GFP) zebrafish embryos per group to establish statistical significance. Issues with the fluorescence stereomicroscope were encountered when attempting to image the zebrafish embryos at 3 hpi. These issues were resolved and imaging proceeded at the later time point of 6 hpi, which remains within the initially identified early-stage time point range of 2-8 hpi.

Following injections to the HBV with Mock and M. marinum, significant neutrophil recruitment was seen to the infection after 72 h, but not 6 h or 24 h as expected (Figure 3.14B). It was established that a scale parameter of 4 (~13 pixels) and sensitivity parameter of 20% were optimal for the detection of neutrophils in the HBV region. The GFP fluorescent protein provides higher intensity fluorescence than mCherry, which requires a larger scale parameter to be set than for the macrophage experiment. Bacterial burden in infected zebrafish increased between 24 hpi and 72 hpi, but did not noticeably increase between 6 hpi and 24 hpi (Figure 3.14C).

We concluded that 6 hpi and 24 hpi were suitable time points for the analysis of the effects of different bacterial strains or treatment with chemical compounds on zebrafish neutrophil recruitment. Therefore, these time points, along with the identified and detection parameters, were selected for subsequent experiments, described in Chapter 4.
Figure 3.14. Verification of neutrophil recruitment timings and bacterial burden following HBV infection of zebrafish with *M. marinum*. (A-C) Twenty-four 2 dpf casper Tg(*mpx:GFP*) zebrafish embryos were injected into the HBV with Mock or a medium dose of *M. marinum* expressing DsRed2 (Mmar). Zebrafish embryos were live-imaged using a fluorescence stereomicroscope and z-stack images of the HBV region acquired at 6 hpi, 24 hpi and 72 hpi. In-focus z-slices were then extracted, cropped and processed for FPC analysis and neutrophil recruitment as described previously (Section 3.3.4.3). Experiment was performed once. Error bars represent S.E.M. (A) Representative images of Mock and Mmar injected embryos are shown for each time point. Scale bar 100 µm. (B) Neutrophils in the HBV region were quantified from fluorescence images of zebrafish embryos using Icy Spot Detector plugin. Sample size (n): 24, 24. Unpaired t-test. **p<0.01. (C) Bacterial burden in the HBV region of *M. marinum*–infected zebrafish embryos was quantified from fluorescence images using Icy FPC protocol. Sample size (n): 24.
3.3.4.6 Quantification of bacterial dissemination in infected zebrafish by development of a bacterial distance distribution protocol

To inform on infection progression in zebrafish we also wished to perform quantification of bacterial dissemination from images acquired by fluorescence microscopy. To achieve this, we used the Spot Detector plugin and other features available in Icy to provide a read-out of bacterial clusters in the zebrafish. As a first step, our collaborator Alexandre Dufour developed a script which can be run in Icy on a single image to determine the bacterial distance distribution from the injection site (Appendix 2 Figure A7). Development of the Icy Bacterial Distance Distribution protocol to enable the application of bacterial distance distribution analysis to folders of images was performed with the assistance of Matthew Martin.

The developed protocol utilises the Icy Wavelet Spot Detector protocol block, to identify bacterial clusters and their distances from a user-defined site, e.g. the injection site (Figure 3.15). The protocol block identifies spots (or clusters) in images using the same analysis as the Spot Detector plugin but requires the user to define the desired outputs. The protocol requires the user to define three ROIs on the image for analysis (the whole fish, yolk sac region and a point for the distance determination), the image channel for analysis, and the scale and sensitivity detection parameters. The protocol outputs are: a binary image showing all detections; an output image showing all detections outside of the yolk sac region (utilises Icy Spot Detector plugin code); and an excel file containing the total number of clusters identified as well as the distance in pixels of the identified clusters and the frequency histogram bin position for each cluster (bin size 10 pixels). The binary and detection output images can be used to confirm that the distance distribution calculations have been performed satisfactorily for each image in the batch. The protocol was designed to subtract the yolk sac region data from the whole zebrafish data due to the high levels of autofluorescence seen there (Section 3.3.4.2.2). Removal of the protocol blocks associated with subtraction of the yolk sac region would permit the application of the Bacterial Distance Distribution protocol to different experiments.
Figure 3.15. Icy protocol developed to quantify the number of bacterial clusters and their distance distribution throughout the zebrafish. (A-C) Protocol workflow from L to R in A) through C). Scripts contained within blocks are provided in Appendix 2 Figures A8-11. **Block 0**: Folder loop - performs the operation for all files of a user-defined extension within the selected folder. **Block 8**: File to sequence – opens the next image in the folder. **Block 9**: Extract channel – extracts the channel defined by the user in Block 7 for analysis. **Block 11**: Get ROI from sequence – recovers ROI information from the opened image file. **Block 13**: Restore ROIs – adds ROIs back to the extracted channel for analysis. **Block 14**: Extract Fish/yolk sac/injection ROIs – extracts the areas of the image marked by each ROI for analysis. **Block 17**: Wavelet Spot Detector Block – protocols version of the Spot Detector Plugin, this identifies clusters over user-defined scale and sensitivity parameters which have been defined in Block 16. Block 12 ensures that detection is performed for one ROI at a time. Block 15 adds the fish ROI prior to analysis. The user must define whether bright spots over a dark background or dark spots over a light background are to be detected in Block 17 (Wavelet Spot Detector Block) as for the Spot Detector Plugin. **Block 1**: Create Save Directory – creates a user-defined folder in which to save the outputs. **Block 21**: Save Binary Sequence – saves the binary output image file from Block 17 (Wavelet Spot Detector Block), identifiable as binary sequence by addition of file suffix using Blocks 2 and 3, and directed to save by Block 10 (Path to file). Binary output image was set up to contain data over the entire image analysed, with no subtractions. **Block 25**: Export to excel – saves the data for the identified bacterial clusters in terms of their distances from ROI injection site and their histogram bin position (bin size 10 pixels), identifiable as the excel file output by addition of the suffix using Blocks 4 and 5, and directed to save by Block 6. Blocks 22, 23 and 24 ensure the subtraction of the yolk sac region from the results exported to the excel file. **Block 28**: Export with ROIs and Detection – exports an output image showing all bacterial clusters detected. Output image is generated from the original opened image (Block 8 output) and the excel output data (Block 25 output) with yolk sac region subtraction being entered as a detection token to Block 28 by the use of Block 26. Block 26 repackages the input (Block 8) and wavelet spot detector (Block 17/25) results to a format suitable for further processing by the spot detector plugin components – the format is referred to as a detection token in the spot detector plugin code. The Spot detector plugin is used to add an ROI to the original image marking each detected spot to permit visualisation of the results.

The Bacterial Distance Distribution protocol was used to determine the distance from the injection site of bacterial clusters in infected zebrafish from Experiment 1, which
had previously been processed using the Icy FPC protocol to determine bacterial burden (Section 3.3.4.2.2.2). Only one of the ten zebrafish demonstrated bacterial dissemination out of the head region, Fish 7 (Figure 3.16A,C). A zebrafish which did not show bacterial dissemination out of the head region, Fish 9, was selected for comparison (Figure 3.16B,D). Analysis using the Bacterial Distance Distribution protocol was performed for each of the processed images (which are marked with the required ROIs) and the output images shown in Figure 3.16C-D. Counted bacterial clusters are indicated by the blue circles and disseminated infection can be seen in the tail region of Fish 7 but not Fish 9 (Figure 3.16C-D). The bacterial distance distributions are displayed for each fish in Figure 3.16E-F and reflect the dissemination observations from images.

We concluded that the development of a protocol which determines the amount and degree of bacterial dissemination following *M. marinum* zebrafish infection as a measure of infection progression was successful and it can be used in future experiments.
Figure 3.16. Quantification of *M. marinum*-zebrafish infection progression using the Icy Bacterial Distance Distribution protocol. (A-J) Ten 2 dpf *casper Tg(mpx:GFP) zebrafish embryos were infected with a medium dose of *M. marinum* expressing DsRed2 and imaged in the lateral view using a fluorescence stereomicroscope 6 dpi. The same 10 fish used in Figure 3.7 were analysed. (A-B) Overlay images showing the brightfield and bacterial fluorescence channels are shown for two zebrafish larvae. Images show zebrafish larvae outlined with a green polygon, yolk sac regions outlined with a white polygon and the HBV injection sites marked by a green point. Yolk sac regions are outlined in the Icy software with black polygons which have been re-coloured white in (A-B) for clarity of display in this thesis. Scale bar 100 µm. (C-D) Images from (A-B) are shown overlaid with the spot detector output images. Detected spots are indicated by blue circles. Yolk sac polygons are shown in black as seen in the Icy output in the whole fish images, but have been re-coloured white in the inset images for clarity of display. Scale bar 100 µm. (E) Bacterial distance distribution of fish shown in (A, C). (F) Bacterial distance distribution of fish shown in (B, D).
3.3.5 Quantification of zebrafish immunity gene expression in response to *M. marinum* infection by qRT-PCR

During experiments to assess the effect of Bac5 treatment on the zebrafish and infection progression in the zebrafish (detailed in Chapter 4), we wished to quantify the zebrafish immune response following HBV injections at the selected early and late time points to provide a more complete picture of immunity gene expression following Bac5 treatment. Quantification of the zebrafish immune response to systemic *M. marinum* infection by qRT-PCR has been reported previously at 4 dpi, with *il-1β* and *mmp-9* expression levels showing the greatest induction[338]. Transcriptomic analysis of the zebrafish gene expression profile over the first five days of *M. marinum* infection has also been reported[25]. Expression of most genes was found to follow three phases: an early phase between 2 and 4 hpi with clear induction of expression of many genes, a mid-phase between 4 hpi and 1 dpi with few genes displaying differential expression, and a late phase from 2-5 dpi with many genes showing striking induction after 4 dpi.

We therefore wished to determine whether induction of expression of *il-1β* and *mmp-9* following *M. marinum* infection could be detected by qRT-PCR before 4 dpi, particularly at 24 hpi. As we wished to assess the results of localised injections to the zebrafish HBV, it was hypothesised that increases in cytokine production in response to the infection may be more readily detected in zebrafish head samples than samples of whole fish in which the changes may be diluted by the inclusion of uninfected tissue.

Samples of zebrafish heads were taken following euthanasia by cutting with a scalpel as indicated in Figure 3.17A. It can be seen that detectable cytokine induction following *M. marinum* injection was found in whole zebrafish samples but not in samples of zebrafish heads, contrary to the hypothesis (Figure 3.17B). This finding would suggest that a localised infection with *M. marinum* generates a systemic immune response in the zebrafish. Although cytokine induction of 2.5- to 4-fold was seen for infected whole zebrafish samples, the differences were not statistically significant. The observed trend for the induction of *il-1β* expression is consistent with the transcriptomics study, which demonstrated moderate induction of *il-1β* at 1 dpi of approximately 2-fold which was not statistically significant. There was no induction of *mmp-9* at 1 dpi in the transcriptomics study. That we observed a trend for moderate induction of *mmp-9* may
be due to differences in the experimental methods, quantification methods or zebrafish lines and bacterial strains between the two studies.

We concluded that experiments to analyse the immune response of zebrafish to *M. marinum* infection at early time points will be possible, though large sample sizes of whole fish will be required to achieve statistical significance at these early stages.

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**Figure 3.17. qRT-PCR analysis of *M. marinum*-infected zebrafish 24 hpi.** (A) Schematic showing region taken for zebrafish head samples, separation from the body was performed using a scalpel. (B) Two dpf Tg(mpeg-1:mCherry) zebrafish embryos were injected into their HBV with a medium dose of *M. marinum*. Samples were taken of whole fish and fish heads at 24 hpi. Fold changes of *il-1β* and *mmp-9* mRNA levels were assessed by qRT-PCR, normalised to 18S and expressed relative to one control sample. Each data point represents a pool of 4 fish, n=12. Error bars represent S.E.M. Kruskal-Wallis with Dunn’s post test showed no significant differences. Experiment was performed once. Zebrafish drawing taken from the Dallman Group image repository.

### 3.4 Discussion

The optimisation of methods to quantify *M. marinum* infection progression in THP-1 cells and zebrafish was described in this chapter. Firstly, we optimised *M. marinum* infection of THP-1 cells in a 96-well plate format leading to the selection of a cell seeding density of 5x10⁵ cells per well and an MOI of 1 for future experiments (Sections 3.3.1.2 and 3.3.1.3). In addition, we selected the sampling of duplicate wells of resting THP-1 cells during 96-well plate assays for qRT-PCR assessment of gene expression levels in future experiments (Figure 3.3). We also confirmed increased expression of the
pro-inflammatory genes *IL-1β*, *TNF-α* and *MMP-9* by resting and PMA-activated THP-1 cells following 24 hours of infection with *M. marinum* (Figure 3.3). During zebrafish HBV infection experiments, we determined the timings and analysis parameters for use in quantification of zebrafish leukocyte recruitment and bacterial burden in the HBV region (Figures 3.11-3.13). In addition, we selected sampling of whole zebrafish during localised HBV infection experiments for qRT-PCR assessment of gene expression levels (Figure 3.17). We confirmed trends for increased expression of the pro-inflammatory genes *il-1β* and *mmp-9* in whole zebrafish samples following 24 hours of infection with *M. marinum* (Figure 3.17), as assessment of gene expression levels by qRT-PCR following *M. marinum* infection at such an early time point has not been widely reported. The determination of these experimental conditions permits the monitoring of THP-1 cell and zebrafish infection progression in future experiments.

This chapter also described the development and/or optimisation of techniques for the quantification of *M. marinum*-zebrafish infection progression parameters from fluorescence microscopy images of live embryos and larvae. The aim was to advance the quantification techniques available for use in longitudinal studies using the zebrafish embryo model, which is made possible through the transparency of zebrafish larvae and is one of the main benefits of this animal model over other systems. As a first step, we validated and optimised an Icy FPC protocol and post-processing method to determine the bacterial burden of infected zebrafish from images. This protocol and processing method permit accurate quantification of bacterial burden and eliminate the issues seen with variable results through CFU plating (Figures 3.5-3.8). We validated the Icy FPC protocol for use in the zebrafish to provide the binary image output, which is not provided in other published FPC protocols and can be used to confirm correct application of the FPC procedure. This step is advantageous in that fluorescence originating from other sources, such as a fluorescent artefact in an image or unusually high zebrafish yolk sac autofluorescence (Section 3.3.4.2.2.3), can be readily detected and steps taken to exclude them from the bacterial burden quantifications. We also developed a post-processing protocol to allow the use of a lower background threshold value whilst excluding yolk sac autofluorescence (Section 3.3.4.2.2.3). This prevents the loss of bacterial burden information through overlap with yolk sac autofluorescence intensity values when analysing images of whole zebrafish embryos and larvae, which
occurs when using other FPC tools. During this work, we identified the standardisation of zebrafish orientation during imaging as a critical step for the application of the Icy FPC protocol both to different samples within experiments and to samples within separate experiments (Figure 3.9), similarly to the known requirement for standardised image acquisition settings. This conflicts with data for three zebrafish presented by Adams et al. which indicated that zebrafish orientation did not affect FPC quantification to a significant degree\[5\]. The different conclusions may be explained by the small sample sizes involved. In summary, we optimised a full protocol for accurate determination of zebrafish bacterial burden through quantification of fluorescence in images with a view to combining this technique with others for infection progression quantification during longitudinal studies.

We also optimised the use of the Icy Spot Detector Plugin to quantify zebrafish leukocytes from images taken using a fluorescence stereomicroscope (Figures 3.11-3.13). During the course of this PhD project, Ellett et al. also developed a method for the quantification of zebrafish leukocytes from fluorescence microscopy images, which provides a read-out of “leukocyte units” that is proportional to the number of leukocytes in the image\[89\]. Previously, zebrafish leukocyte recruitment to the HBV in response to injections had been described by manual counting during confocal microscopy of both live and fixed samples\[320, 328\]. Manual counting is time consuming and does not permit the analysis of large numbers of live zebrafish samples, whilst fixing of samples to facilitate larger sample sizes is destructive and prevents longitudinal study. Quantification of leukocytes from fluorescence microscopy images therefore represented a significant advancement in the techniques available for infected zebrafish image analysis at the time that the optimisation of the Icy Spot Detector plugin was conducted. Quantification of leukocytes from fluorescence microscopy images also provides the ability to combine leukocyte enumeration with other quantification techniques.

Finally, we reported the development of a protocol for the quantification of the amount and degree of bacterial dissemination from images of infected zebrafish (Figure 3.15 and Figure 3.16), permitting the first image quantification of the degree of dissemination. Quantification of the amount of bacterial dissemination and average bacterial cluster size, has been described by Torraca et al. during the course of this
Previously, bacterial dissemination was assessed by manual counting of the number of bacterial clusters and manual scoring of cluster size, which is time consuming and did not permit the analysis of large numbers of zebrafish embryos. As the successful dissemination of infection is critical during mycobacterial pathogenesis, quantification of dissemination is a powerful tool for assessing the virulence of an infecting bacterial strain to identify bacterial factors required for pathogenesis and assessing the potential of an anti-mycobacterial therapy to protect against mycobacterial infections. The Icy Bacterial Distance Distribution protocol described here provides the ability to perform rapid, sequential analysis of dissemination. The protocol is therefore an important addition to the available techniques to quantify *M. marinum* infection progression in the zebrafish.

The development of techniques and imaging protocols to enable the high-throughput analysis of *M. marinum* infection of zebrafish from images using a single infection progression parameter has been described by other groups in the literature. One example is the Ellett *et al.* leukocyte quantification discussed above\(^{[89]}\). A second is the method described by Takaki *et al.* to permit the high-throughput quantification (in a 96-well plate format) of infected zebrafish bacterial burden using automated plate reader analysis without anaesthesia or cryo-anaesthetisation of larvae and automated fluorescence microscopy imaging for FPC analysis\(^{[320]}\). The FPC tool utilised by Takaki *et al.*, which was that first described in Adams *et al.*\(^{[5]}\), was used in validation experiments with the Icy FPC protocol during this thesis. It is possible to imagine the use of these techniques with each other and/or the Icy protocols described here (the Icy FPC protocol to quantify bacterial burden, Icy Spot Detector Plugin to quantify leukocyte number and Icy Bacterial Distance Distribution protocol to quantify bacterial dissemination) for the simultaneous quantification of different combinations of infection progression parameters from images of *M. marinum*-infected zebrafish.

To permit the combined analysis of multiple infection progression parameters (bacterial burden and leukocyte recruitment) in the HBV region, we developed an image processing protocol for z-stack images of zebrafish taken in the dorsal view (Figure 3.11). We believe the use of this image processing protocol in Chapter 4 of this thesis represents the first instance of simultaneous quantification of these infection progression parameters from images of *M. marinum*-infected zebrafish. The
quantification of multiple infection progression parameters from the same fluorescence microscopy image during longitudinal studies permits the analysis of a greater number of zebrafish samples and analysis of each parameter at an increased number of time points. This is achieved through the elimination of the requirements for separate imaging for quantification, which is limited by the recovery period between repeated anaesthetisations of zebrafish. Therefore, the techniques presented in this chapter represent a significant advancement in the infected zebrafish image quantification techniques available for longitudinal studies using the *M. marinum*-zebrafish model.
4 Investigating the effect of selected host defence peptides (HDPs) on *M. marinum* infection progression in THP-1 cells and the zebrafish

4.1 Introduction

There is evidence that HDPs achieve host protection against infections through their direct antimicrobial and immunomodulatory functions, with their immunomodulatory functions thought to be the most important (Section 1.4). Several studies have demonstrated the importance of cathelicidins in TB infections (Section 1.4.5.2) and that exogenous HDP treatments during infections can be beneficial to the host (Section 1.4.4.3). We hypothesised that an exogenous peptide would be capable of immunomodulation in the zebrafish and that treatment with exogenous cathelicidins may be beneficial to the human host during TB infections. We selected a bovine cathelicidin family member, bactenecin-5 (Bac5), for studies of the efficacy of treating mycobacterial infections with exogenous HDPs in the *M. marinum*-zebrafish infection model. We also selected two control HDPs for our investigations: the fish epinecidin-1 (EPI), of the piscidin family of HDPs, which possesses immunomodulatory but no direct antimycobacterial activities; and the human cathelicidin LL-37, which possesses both direct antimycobacterial activities and immunomodulatory activities (Table 1.3).

4.2 Aims

The aim of this work was to test the hypothesis that the exogenous peptide Bac5, which has no direct antimycobacterial activity, would be capable of immunomodulation which would benefit the zebrafish host during mycobacterial infections.

The detailed hypotheses were:

1) Bac5 would possess direct bacteriocidal activities against *E. coli* but not *M. marinum*
2) Bac5 would modulate THP-1 cell gene expression in the presence and absence of *M. marinum* infection
3) Bac5 would perform immunomodulation in uninfected zebrafish embryos and larvae and also during the early stages of local infection with *M. marinum*,

137
specifically through increasing macrophage recruitment, increasing neutrophil recruitment and modulation of gene expression

4) Bac5 treatment would benefit the zebrafish host during *M. marinum* infection through reducing bacterial burden and increasing survival

4.3 Results

4.3.1 Selected HDPs demonstrate direct bacteriocidal activity *in vitro*

To determine the direct effects of the selected HDPs on *M. marinum*, 96-well plate assays incubating logarithmic-phase *M. marinum* with varying concentrations of the HDPs for 24 hours were performed with CFU plating after the incubations. The pooled data shows that LL-37 demonstrates the expected direct bacteriocidal activity against *M. marinum*, with 90% killing after 24 hours of incubation with concentrations above 15 ng/µl (Figure 4.1A). No direct bacteriocidal effects against *M. marinum* are observed following 24 hours of incubation with LL-37 concentrations under 15 ng/µl, or with any concentration of Bac5 or EPI (Figure 4.1A). A 96-well plate assay was also performed for varying concentrations of Bac5 with logarithmic-phase *E. coli*. The pooled data demonstrates the expected direct bacteriocidal of Bac5 against *E. coli*, with >90% killing following 24 hours of incubation with all tested concentrations of Bac5 (Figure 4.1B).

![Figure 4.1. Direct bacteriocidal effects of HDPs on *M. marinum* and *E. coli*. (A) *M. marinum* CFU count following 24 hours (h) of incubation with varying concentrations of HDPs LL-37, bactenecin-5 (Bac5) and EPI. (B) *E. coli* CFU count following 24 h of incubation with varying concentrations of Bac5. Data pooled from three independent experiments is shown (n=9). Error bars represent S.E.M. One-way analysis of variance (ANOVA) with Bonferroni's multiple comparison post-test, significance compared to 0 ng/µl treatment is shown. ***p<0.001.](image-url)
These experiments confirm that the obtained sample of Bac5 is active and testing of its activities in THP-1 cells and zebrafish can proceed.

4.3.2 Effect of HDPs on *M. marinum* infection progression in THP-1 cells

4.3.2.1 Effect of HDPs on host immunity gene expression of infected and uninfected THP-1 cells

To determine the effect of HDPs on the expression of immunity genes by infected and uninfected THP-1 cells, resting THP-1 cells were treated with 15 ng/µl HDPs for 24 hours before qRT-PCR analysis of their immunity gene expression. Treatment of both uninfected and *M. marinum*-infected THP-1 cells with LL-37 led to consistent trends for increased expression of *IL-1β* and *MMP-9*, which are not statistically significant (Figure 4.2). Treatment with both exogenous peptides resulted in increased immunity gene expression, reflecting their immunomodulatory activities. Treatment with EPI and Bac5 significantly increased *IL-1β* expression for both *M. marinum*-infected and uninfected THP-1 cells (Figure 4.2). EPI treatment increased *IL-1β* expression of uninfected THP-1 cells by 3.5-fold and that of *M. marinum*-infected cells by 2-fold. Bac5 treatment increased *IL-1β* expression of THP-1 cells by 2-fold independent of *M. marinum* infection. Furthermore, EPI treatment leads to a significant 4-fold increase in the expression of *MMP-9* for *M. marinum*-infected THP-1 cells and a trend for increased expression by uninfected cells. In addition, treatment with Bac5 resulted in trends for increased expression of *MMP-9* and *TNF-α* for *M. marinum*-infected THP-1 cells only. We observed no effects of LL-37 or EPI treatment on *TNF-α* expression by THP-1 cells.
Figure 4.2. Effect of HDPs on THP-1 cell gene expression. Fold change of IL-1β, TNF-α and MMP-9 mRNA levels in uninfected and _M. marinum_ -infected resting THP-1 cells following incubation with 15 ng/µl HDPs for 24h. THP-1 cells were incubated for 48 h without PMA, followed by infection for 3 h with log-phase _M. marinum_ at an MOI of 1, and then incubation with or without 15 ng/µl HDPs, the highest effective concentration of the HDPs (LL-37, Figure 4.1). Relative expression levels were assessed by qRT-PCR at 24 hpi, the previously determined optimum incubation time (Section 3.3.1.4). Samples were normalised to 18S and expressed relative to one control sample. Data pooled from two independent experiments is shown (n=12). Each data point represents two treatment wells. Error bars represent S.E.M. Kruskal-Wallis with Dunn’s post-test performed within _M. marinum_ infection groups for each gene. **p<0.01, *p<0.05.

4.3.3 Effect of Bac5 on _M. marinum_ infection progression in zebrafish following Bac5 and _M. marinum_ co-injection to the HBV
4.3.3.1 Effect of Bac5 on macrophage recruitment and host immunity gene expression following co-injections to the zebrafish HBV

4.3.3.1.1 Overview

To test whether Bac5 is able to chemoattract macrophages and modulate zebrafish immunity gene expression, we locally injected Mock solution or Bac5 into the HBV of zebrafish embryos at 2 days post-fertilisation (dpf). To determine whether Bac5 treatment can affect macrophage recruitment and modulate zebrafish immunity gene expression in response to M. marinum infection, we also injected M. marinum or co-injected M. marinum and Bac5. To determine the effects of Bac5 on macrophage recruitment dynamics following a single injection, and quantify any longer-term effects, we performed the experiment as a longitudinal study wherein individual embryos were tracked to enable comparison of the results between the 3 hpi and 24 hpi time points. A schematic of the experiment is shown in Figure 4.3. The experiment utilised transgenic zebrafish expressing mCherry-tagged macrophages, the Tg(mpeg-1:mCherry) line.

Figure 4.3. Schematic of co-injection experiments to determine the effects of bactenecin-5 (Bac5) on zebrafish macrophage recruitment or host immunity gene expression. Two days post-fertilisation (dpf) Tg(mpeg-1:mCherry) zebrafish embryos were injected into their HBV with Mock, Bac5, Mock/M. marinum (Mmar) or Bac5/Mmar. 10ng Bac5 was injected for both Bac5 treatment groups and a medium dose of Mmar expressing GFP for both infection groups. As sampling for qRT-PCR is destructive, three identical sets of injections were performed, with each set comprising all four injection groups. The first set of zebrafish embryos was live-imaged using a fluorescence stereomicroscope and z-stack images of the HBV region acquired at 3 hours post-injection (hpi) and 24 hpi. Imaging of Set 1 was undertaken as a longitudinal study with individual zebrafish maintained in separate wells of 24-well plates to enable comparison of the results at 3 hpi and 24 hpi. Images were processed and the Icy Spot Detector plugin used to determine the number of macrophages recruited to the HBV region of embryos at 3 hpi and 24 hpi (Section 3.3.4.4 and 3.3.4.5). The cytokine response of the zebrafish embryo sets were analysed by qRT-PCR at 6 hpi, 24 hpi and 96 hpi. Zebrafish drawing taken from the Dallman group image repository.
4.3.3.1.2 Effect of Bac5 on macrophage recruitment to the HBV following co-injections to the zebrafish HBV

To determine whether Bac5 is able to chemoattract macrophages in *M. marinum*-infected and uninfected zebrafish, we locally injected Mock solution, Bac5, *M. marinum* or *M. marinum* and Bac5 to the HBV of zebrafish embryos at 2 dpf. Figure 4.4 and Figure 4.5 show images acquired in one representative experiment at 3 hpi and 24 hpi as fluorescence overlays. Full image panels are shown to illustrate the differences in macrophage recruitment whilst reflecting the variability in the individual cell counts. Quantification of macrophage recruitment is shown in Figure 4.6.
3 hpi

Mock  Bac5  Mock/Mmar  Bac5/Mmar

mpeg-1:mCherry; M. marinum
Figure 4.4. Effect of injection of bactenecin-5 (Bac5) to the HBV on zebrafish macrophage recruitment to the HBV region at 3 hpi. Two dpf Tg(mpeg-1:mCherry) zebrafish embryos were injected into their HBV with Mock, Bac5, Mock/M. marinum (Mmar) or Bac5/Mmar. 10ng Bac5 was injected for both Bac5 treatment groups and approximately 190 CFU Mmar for both infection groups. Zebrafish embryos were live-imaged using a fluorescence stereomicroscope and z-stack images of the HBV region acquired at 3 hpi and 24 hpi. Fluorescence overlay images of the HBV region of zebrafish embryos at 3 hpi are shown for one representative experiment. As the experiment was undertaken as a longitudinal study, zebrafish are presented in numbered rows to permit comparison with 24 hpi data in Figure 4.6. White line represents zebrafish head outline as seen in brightfield images. Scale bar 100 µm. Experiment was performed three times. Sample size (n): 12, 12, 15, 14.
24 hpi

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*mpeg-1:mCherry; M. marinum*
Figure 4.5. Effect of injection of bactenecin-5 (Bac5) to the HBV on zebrafish macrophage recruitment to the HBV region at 24 hpi. Two dpf Tg(mpeg-1:mCherry) zebrafish embryos were injected into their HBV with Mock, Bac5, Mock/M. marinum (Mmar) or Bac5/Mmar. 10ng Bac5 was injected for both Bac5 treatment groups and approximately 190 CFU Mmar for both infection groups. Zebrafish embryos were live-imaged using a fluorescence stereomicroscope and z-stack images of the HBV region acquired at 3 hpi and 24 hpi. Fluorescence overlay images of the HBV region of zebrafish embryos at 24 hpi are shown for one representative experiment. Experiments were performed as a longitudinal study, therefore individual zebrafish are presented in the same rows as in Figure 4.5. White line represents zebrafish head outline as seen in brightfield images. Scale bar 100 µm. Experiment was performed three times. Sample size (n): 12, 12, 15, 14.
We observed significant macrophage recruitment following injection of Bac5 to the HBV at 3 hpi (Figure 4.6A) which continued until 24 hpi (Figure 4.6B). Injection of *M. marinum* to the HBV led to significant macrophage recruitment at 3 hpi (Figure 4.6A). Significant macrophage recruitment was also seen following the co-injection of Bac5 and *M. marinum* at 3 hpi (Figure 4.6A). At 24 hpi we observed a consistent trend for increased average macrophage recruitment for the co-injection group compared to the Mock- and Mock/*M. marinum*-injected groups, although the differences were not statistically significant (Figure 4.6B). Overall, it can be seen that injection of Bac5, with and without *M. marinum*, leads to the recruitment of 6-7 additional macrophages at 3 hpi compared to injection alone, which is an increase in the number of macrophages associated with the HBV region of 50% compared to baseline levels.

![Figure 4.6: Injection of bactenecin-5 (Bac5) to the HBV increases zebrafish macrophage recruitment to the HBV region](image)

**A** 3 hpi  
**B** 24 hpi

**Figure 4.6. Injection of bactenecin-5 (Bac5) to the HBV increases zebrafish macrophage recruitment to the HBV region.** (A-B) Two dpf *Tg(mpeg-1:mCherry)* zebrafish embryos were injected into their HBV with Mock, Bac5, Mock/*M. marinum* (Mmar) or Bac5/Mmar. 10ng Bac5 was injected for both Bac5 treatment groups and approximately 190 CFU Mmar for both infection groups. Zebrafish embryos were maintained individually to enable longitudinal study. Zebrafish embryos were live-imaged using a fluorescence stereomicroscope and z-stack images of the HBV region acquired at 3 hpi and 24 hpi. Macrophages in the HBV region were quantified from fluorescence images of zebrafish using Icy Spot Detector plugin at 3 hpi (A) and 24 hpi (B). Data pooled from three independent experiments is shown. Sample size (n): 41, 37, 61, 53. Error bars represent S.E.M. One-way ANOVA with Bonferroni’s post-test. *p<0.05, ***p<0.001.

The data from the longitudinal aspect of the study show that Bac5 does not affect macrophage recruitment dynamics beyond an initial increase in recruitment, seen by comparing macrophage recruitment between 3 hpi and 24 hpi for the different injection groups (Figure 4.7). Full data from one representative experiment is shown in Figure...
4.7A-D, where the change in HBV region macrophage count of individual zebrafish over the course of the experiment is shown. Comparing Figure 4.7A and Figure 4.7B, it can be seen that Bac5 treatment alone does not affect macrophage recruitment dynamics. The data also show that Bac5 treatment does not affect macrophage recruitment dynamics to *M. marinum* infection, seen by comparing Figure 4.7C and Figure 4.7D. Pooled data from three independent experiments demonstrates that long-term macrophage recruitment dynamics are not affected by Bac5 treatment (Figure 4.7E).
Figure 4.7. Injection of bactenecin-5 (Bac5) to the HBV does not affect zebrafish macrophage recruitment to the HBV region over time. (A-E) Two dpf Tg(mpeg-1:mCherry) zebrafish embryos were injected into their HBV with Mock, Bac5, Mock/M. marinum (Mmar) or Bac5/Mmar. 10ng Bac5 was injected for both Bac5 treatment groups and approximately 190 CFU Mmar for both infection groups. Zebrafish embryos were maintained individually to enable longitudinal study. Zebrafish embryos were live-imaged using a fluorescence stereomicroscope and z-stack images of the HBV region acquired at 3 hpi and 24 hpi. Macrophages in the HBV region were quantified from fluorescence images of zebrafish using Icy Spot Detector plugin at 3 hpi and 24 hpi. (A-D) Data from one representative experiment is shown. Macrophages were quantified for individual zebrafish at the indicated time points for all injection groups: Mock (A), Bac5 (B), Mock/Mmar (C) and Bac5/Mmar (D). Sample size (n): 12, 12, 15, 14. (E) Data pooled from three independent experiments is shown. Sample size (n): 41, 37, 61, 53. Error bars represent S.E.M. Data shown is from the same experiments as Figure 4.6.
4.3.3.1.3 Effect of Bac5 on host immunity gene expression following co-injections to the zebrafish HBV

In addition to assessing its effects on macrophage recruitment, we also determined whether the exogenous HDP Bac5 is able to modulate gene expression in uninfected and *M. marinum*-infected zebrafish during the same experiments. qRT-PCR was performed to assess the expression levels of several genes that function in immunity which have previously been reported to be elevated in *M. marinum* infection of the zebrafish (*il-1β*, *mmp-9* and *tnfa*) and/or following Bac5 treatment of cell culture cells (*il-1β*, *mmp-9* and *cxcl-c1c*). Expression of the antiviral cytokine *ifnphi1* was also quantified as a negative control because we do not expect Bac5 to activate the viral immune response.

Analysis by qRT-PCR revealed the expected undetectable expression levels of *ifnphi1* for all injection groups throughout the experiment. Expression of *tnfa* was also found to be at undetectable levels for all samples at 6 hpi and 24 hpi. At 6 hpi and 24 hpi consistent trends were seen for expression of the other tested immunity genes, although they are not statistically significant. At 6 hpi both injection with *M. marinum* and Bac5 led to trends for increases of 1.5- to 2-fold in the expression of *il-1β*, *mmp-9* and *cxcl-c1c* compared to Mock-injected controls (Figure 4.8A). The trend for a 2-fold increase in the average expression levels for these three genes following HBV infection with *M. marinum* continues at 24 hpi (Figure 4.8B). However, at 24 hpi Bac5-injected zebrafish samples displayed the reverse trend to that at 3 hpi, with decreases in the average expression of all three genes.

At 96 hpi, infection with *M. marinum* led to significant increases in the average expression levels of all four immunity genes tested independent of Bac5 treatment, with *il-1β* showing 45-fold increases, *mmp-9* 30- to 35-fold increases and *cxcl-c1c* 35- to 45-fold increases in expression compared to Mock-injected controls (Figure 4.8C). In addition, *tnfa* expression was only detectable in our experiments with *M. marinum* infection at 96 hpi. Early treatment with Bac5 does not affect the average expression level of *il-1β* or *mmp-9* at 96 hpi independent of *M. marinum* infection. Trends for increased expression of *cxcl-c1c* and *tnfa* following Bac5 treatment were observed in the presence of *M. marinum* infection only, although the differences are not statistically significant (Figure 4.8C,D).
Figure 4.8. Injection of bactenecin-5 (Bac5) to the HBV does not affect cytokine gene expression levels of uninfected and M. marinum-infected zebrafish. (A-D) Two dpf Tg(mpeg-1:mCherry) zebrafish embryos were injected into their HBV with Mock, Bac5, Mock/M. marinum (Mmar) or Bac5/Mmar. 10ng Bac5 was injected for both Bac5 treatment groups and approximately 190 CFU Mmar for both infection groups. Fold changes of il-1β, mmp-9, cxcl-c1c and tifa mRNA levels were assessed by qRT-PCR, normalised to 18S and expressed relative to one control un.injected (uninj.) sample. Samples were taken at 6 hpi (A), 24 hpi (B) and 96 hpi (C, D). tifa mRNA levels were undetectable in samples taken at 6 hpi and 24 hpi, and in uninfected samples taken at 96 hpi. tifa mRNA levels are shown expressed relative to Mock/Mmar. Data pooled from three different independent experiments to Figure 4.6 is shown. Zebrafish used for image analysis at 3 hpi and 24 hpi were sampled for qRT-PCR immediately after imaging at 24 hpi. Error bars represent S.E.M. Kruskal-Wallis with Dunn’s post-test; statistical significance between M. marinum-infected and uninfected groups is displayed relative to the Mock control group only for clarity. **p<0.01, *p<0.05. Each data point in (A-B) represents a pool of three fish (n=36), each data point in (C) represents an individual fish (n=15).
4.3.3.2 Effect of Bac5 on neutrophil recruitment and bacterial burden following co-injections to the zebrafish HBV

4.3.3.2.1 Overview

Having determined the effects of Bac5 on macrophage recruitment and host immunity gene expression, we wished to determine the effects of Bac5 on other infection progression parameters. To test whether Bac5 is able to chemoattract neutrophils, we locally injected Mock solution or Bac5 into the HBV of zebrafish embryos at 2 dpf. To determine whether Bac5 treatment can affect neutrophil recruitment to M. marinum infection and bacterial burden, we also injected M. marinum or co-injected M. marinum and Bac5. A schematic of the longitudinal experiment is shown in Figure 4.9. The experiment utilised transgenic zebrafish expressing GFP-tagged neutrophils with mutations leading to their transparency to aid in longer-term monitoring of the zebrafish by microscopy, the casper Tg(mpox:GFP) line. The rationale for assessing the effects of Bac5 on neutrophil recruitment in the presence and absence of M. marinum infection is detailed in Section 3.3.4.5.1 and on bacterial burden following HBV injections in Section 3.3.4.4.

**Figure 4.9. Schematic of co-injection experiments to determine the effects of bactenecin-5 (Bac5) on zebrafish neutrophil recruitment or M. marinum bacterial burden.** Two dpf casper Tg(mpox:GFP) zebrafish embryos were injected into their HBV with Mock, Bac5, Mock/M. marinum (Mmar) or Bac5/Mmar. 10ng Bac5 was injected for both Bac5 treatment groups and approximately 250 CFU Mmar expressing DsRed2 for both infection groups. The experiment was undertaken as a longitudinal study with individual zebrafish maintained in separate wells of 24-well plates to enable comparison of the results over time. All zebrafish embryo injection groups were live-imaged using a fluorescence stereomicroscope and z-stack images of the HBV region acquired at 6 hpi, 24 hpi and 72 hpi. Images were processed and the Icy Spot Detector plugin used to determine the number of neutrophils recruited to the HBV region of embryos at 6 hpi, 24 hpi and 72 hpi (Section 3.3.4.4 and 3.3.4.5). The bacterial burden in the HBV region of infected embryos and larvae was quantified using the Icy FPC protocol as described in Section 3.3.4.2. Zebrafish drawing taken from the Dallman Group image repository.
4.3.3.2.2  Effect of Bac5 on neutrophil recruitment to the HBV following co-injections to the zebrafish HBV

To determine whether Bac5 treatment affects neutrophil recruitment in uninfected and *M. marinum*-infected zebrafish, we locally injected Mock solution, Bac5, *M. marinum* or *M. marinum* and Bac5 to the HBV of zebrafish embryos at 2 dpf. Figure 4.10, Figure 4.11 and Figure 4.12 show images acquired in one representative experiment at 6 hpi, 24 hpi and 72 hpi, as fluorescence overlays. Full image panels are shown to illustrate the trends for neutrophil recruitment whilst reflecting the variability in the cell counts expected with the zebrafish model. Quantification of neutrophil recruitment is shown in Figure 4.13.

Images for each time point were acquired using standardised settings which had been optimised for the early time points. Upon processing images acquired at 72 hpi using these standardised settings it was noted that neutrophil fluorescence was overexposed in these images, making counting using the Spot Detector plugin inaccurate at this time point as individual neutrophil cells could not always be resolved from clusters. All images were therefore also processed using the Icy FPC protocol to determine the number of fluorescent pixels due to neutrophils in each image over the background threshold, shown in Figure 4.13. The background threshold was determined using z-slices of the top of the head (dorsal side) of Mock-injected zebrafish containing out-of-focus neutrophils only, resulting in only in-focus neutrophils being included in quantifications.
### 6 hpi

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*mpx:GFP; M. marinum*
Figure 4.10. Effect of injection of bactenecin-5 (Bac5) to the HBV on zebrafish neutrophil recruitment to the HBV region at 6 hpi. Two dpf casper Tg(mpx:GFP) zebrafish embryos were injected into their HBV with Mock, Bac5, Mock/M. marinum (Mmar) or Bac5/Mmar. 10ng Bac5 was injected for both Bac5 treatment groups and approximately 250 CFU Mmar expressing DsRed2 for both infection groups. Zebrafish embryos were live-imaged using a fluorescence stereomicroscope and z-stack images of the HBV region acquired at 6 hpi, 24 hpi and 72 hpi. Fluorescence overlay images of the HBV region of zebrafish embryos at 6 hpi are shown for one representative experiment. As the experiment was undertaken as a longitudinal study, zebrafish are presented in numbered rows to permit comparison with 24 hpi and 72 hpi data in Figures 4.12-13. White line represents zebrafish head outline as seen in brightfield images. Scale bar 100 µm. Experiment was performed three times. Sample size (n): 12, 12, 13, 12.
24 hpi

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mpx::GFP, *M. marinum*
Figure 4.11. Effect of injection of bactenecin-5 (Bac5) to the HBV on zebrafish neutrophil recruitment to the HBV region at 24 hpi. Two dpf casper Tg(mpox:GFP) zebrafish embryos were injected into their HBV with Mock, Bac5, Mock/M. marinum (Mmar) or Bac5/Mmar. 10ng Bac5 was injected for both Bac5 treatment groups and approximately 250 CFU Mmar expressing DsRed2 for both infection groups. Zebrafish embryos were live-imaged using a fluorescence stereomicroscope and z-stack images of the HBV region acquired at 6 hpi, 24 hpi and 72 hpi. Fluorescence overlay images of the HBV region of zebrafish embryos at 24 hpi are shown for the same representative experiment as in Figure 4.11. Experiments were performed as a longitudinal study, therefore zebrafish are presented in the same rows as in Figure 4.11. White line represents zebrafish head outline as seen in brightfield images. Scale bar 100 µm. Experiment was performed three times. Sample size (n): 12, 12, 13, 12.
72 hpi

Mock  Bac5  Mock/Mmar  Bac5/Mmar

mpx:GFP; M. marinum
Figure 4.12. Effect of injection of bactenecin-5 (Bac5) to the HBV on zebrafish neutrophil recruitment to the HBV region at 72 hpi. Two dpf casper Tg(mpx:GFP) zebrafish embryos were injected into their HBV with Mock, Bac5, Mock/\emph{M. marinum} (Mmar) or Bac5/Mmar. 10ng Bac5 was injected for both Bac5 treatment groups and approximately 250 CFU Mmar expressing DsRed2 for both infection groups. Zebrafish embryos were live-imaged using a fluorescence stereomicroscope and z-stack images of the HBV region acquired at 6 hpi, 24 hpi and 72 hpi. Fluorescence overlay images of the HBV region of zebrafish embryos at 72 hpi are shown for the same representative experiment as in Figure 4.11. Experiments were performed as a longitudinal study, therefore zebrafish are presented in the same rows as in Figure 4.11-12. White line represents zebrafish head outline as seen in brightfield images. Scale bar 100 µm. Experiment was performed three times. Sample size (n): 12, 12, 13, 12.

At 6 hpi, significant increases in the average neutrophil counts are seen with the combined effect of Bac5/Mmar co-injections compared to Mock-injected embryos, with trends seen for increases following individual Bac5 and \emph{M. marinum} injections, although the differences were not statistically significant. The average neutrophil pixel count data
shows a significant increase following Bac5 injection compared to Mock-injected embryos. The pixel count data also shows trends for increases with both *M. marinum* injection groups and for Bac5 and *M. marinum* co-injections compared to injections with *M. marinum* only, although these differences were not statistically significant. The lack of significant neutrophil recruitment in response to *M. marinum* infection alone was expected at this early time point (Section 3.3.4.5). Interestingly, treatment with Bac5 leads to a greater increase in the average neutrophil count compared to Mock-injected embryos than infection with *M. marinum*. Overall, it can be seen that injection of Bac5, with and without *M. marinum*, leads to the recruitment of 4-6 additional neutrophils compared to injection alone, which is an increase in the number of neutrophils in the HBV region of 20-30% at 6 hpi compared to baseline levels.

By 24 hpi, recruitment of neutrophils in response to both Bac5 and *M. marinum* has decreased such that neutrophil levels have returned to near baseline levels (Mock-injected embryo levels), and no significant differences are seen in the counts data. For the neutrophil pixel count data, significant differences in the average number of pixels are seen only with the combined effect of Bac5/Mmar co-injections at 800 FPC compared to Mock injections at 400 FPC.

Significant additional neutrophil recruitment of approximately 15 cells was observed at 72 hpi with *M. marinum* as expected, seen by comparing *M. marinum*-injected zebrafish groups with Mock-injected zebrafish groups for both neutrophil count and neutrophil pixel count data. Neutrophil recruitment to the infection was not affected by Bac5 treatment, as seen by comparing groups injected with *M. marinum* only with Bac5/MMar at the 72 hpi time point. In addition, injection with Bac5 does not affect neutrophil recruitment to the HBV region at 72 hpi, seen by comparing Bac5-injected with Mock-injected zebrafish larvae.
Figure 4.13. Injection of bactenecin-5 (Bac5) to the HBV does not affect zebrafish neutrophil recruitment to the HBV region. (A-C) Two dpf casper Tg(mpx:GFP) zebrafish embryos were injected into their HBV with Mock, Bac5, Mock/M. marinum (Mmar) or Bac5/Mmar. 10ng Bac5 was injected for both Bac5 treatment groups and approximately 250 CFU Mmar expressing DsRed2 for both infection groups. Zebrafish embryos were live-imaged using a fluorescence stereomicroscope and z-stack images of the HBV region acquired at 6 hpi, 24 hpi and 72 hpi. Neutrophils in the HBV region were quantified from fluorescence images of zebrafish embryos using Icy Spot Detector plugin (A, C, E) and Icy FPC protocol (B, D, F) at 6 hpi (A, B), 24 hpi (C, D) and 72 hpi (E, F). Sample size (n): 39, 37, 38, 43. Data pooled from three independent experiments is shown. Error bars represent S.E.M. One-way ANOVA with Bonferroni's post-test. *p<0.05, **p<0.01, ***p<0.001.

The results of the longitudinal aspect of the study are shown in Figure 4.14. Full data from one representative experiment is shown in Figure 4.14A-D, where the change in HBV region neutrophil count of individual zebrafish over the course of the experiment is
shown. The data show that Bac5 does not affect the dynamics of neutrophil recruitment after 6 hpi, which can be seen by comparing the profiles in Figure 4.14A and Figure 4.14B. Bac5 does not appear to affect the dynamics of neutrophil recruitment in the presence of *M. marinum* infection, as shown by comparing Figure 4.14C and Figure 4.14D. The rates of neutrophil recruitment in the three independent experiments are summarised in Figure 4.14E, and also show no effect of early Bac5 treatment on long-term neutrophil recruitment dynamics.
Figure 4.14. Injection of bactenecin-5 (Bac5) to the HBV does not affect zebrafish neutrophil recruitment to the HBV region over time. (A-E) Two dpf casper Tg(mpx:GFP) zebrafish embryos were injected into their HBV with Mock, Bac5, Mock/M. marinum (Mmar) or Bac5/Mmar. 10ng Bac5 was injected for both Bac5 treatment groups and approximately 250 CFU Mmar expressing DsRed2 for both infection groups. Zebrafish embryos were kept as individuals in 24-well plates. Zebrafish embryos were live-imaged using a fluorescence stereomicroscope and z-stack images of the HBV region acquired at 6 hpi, 24 hpi and 72 hpi. Neutrophils in the HBV region were quantified from fluorescence images of zebrafish embryos using Icy Spot Detector plugin. (A-D) Data from one representative experiment is shown. Neutrophils were quantified for individual zebrafish at the indicated time points for all injection groups: Mock (A), Bac5 (B), Mock/Mmar (C) and Bac5/Mmar (D). (E) Data pooled from three independent experiments is shown. Sample size (n): 39, 37, 38, 43. Error bars represent S.E.M. Data shown is from the same experiments as in Figure 4.13.
4.3.3.2.3 Effect of Bac5 on bacterial burden of zebrafish following localised infection with M. marinum

In addition to assessing neutrophil recruitment, we also determined whether Bac5 treatment affects bacterial burden of M. marinum-infected zebrafish in the same experiments. Images are shown above in Figure 4.10, Figure 4.11, and Figure 4.12. Quantification of bacterial burden in infected zebrafish is shown in Figure 4.15.

The data show the expected small increase, of approximately double, in bacterial burden between 6-24 hpi and a large increase of approximately 6-fold at 24 hpi to 72 hpi during early granuloma formation. The results revealed that treatment with Bac5 does not significantly affect the bacterial burden of infected zebrafish at any of the time points analysed in this study (Figure 4.15).

Figure 4.15. Injection of bactenecin-5 (Bac5) to the HBV does not affect bacterial burden of localised M. marinum infection. (A-C) Two dpf casper Tg(mpx:GFP) zebrafish embryos were injected into their HBV with Mock/M. marinum (Mmar) or Bac5/Mmar. 10ng Bac5 was injected for Bac5 treatment group and approximately 250 CFU Mmar expressing DsRed2 for both groups. Zebrafish embryos were live-imaged using a fluorescence stereomicroscope and z-stack images of the HBV region acquired at 6 hpi, 24 hpi and 72 hpi. Bacterial burden in the HBV region of zebrafish embryos was quantified from fluorescence images by fluorescent pixel count (FPC) using Icy FPC protocol, at 6 hpi (A), 24 hpi (B) and 72 hpi (C). Sample size (n): 38, 43. Data pooled from the same three independent experiments as Figure 4.13 is shown. Error bars represent S.E.M. Unpaired t-test showed no significant differences.
As previously mentioned, these experiments were performed as a longitudinal study. Full data from one representative experiment is shown in Figure 4.16A-B, where the change in bacterial burden of individual zebrafish over the course of the experiment is shown. It can be seen by comparing the trend in the data for the *M. marinum*-injected group (Figure 4.16A) with the *M. marinum* and Bac5 co-injected group (Figure 4.16B) that treatment with Bac5 does not lead to large differences in the rate of bacterial burden increase during infection. When comparing data for the pooled experiments (Figure 4.16C-D) it can be seen that overall, co-injection of *M. marinum* with Bac5 leads to a small reduction in the rate of bacterial burden increase in infected zebrafish between 24 hpi and 72 hpi compared to zebrafish injected with Mock/*M. marinum*, although the reduction in growth rate is not significant.
Figure 4.16. Injection of bactenecin-5 (Bac5) to the HBV does not affect bacterial burden of localised *M. marinum* infection over time. (A-D) Two dpf *casper Tg(mpx:GFP)* zebrafish embryos were injected into their HBV with Mock/*M. marinum* (Mmar) or Bac5/Mmar. 10ng Bac5 was injected for Bac5 treatment group and approximately 250 CFU Mmar expressing DsRed2 for both groups. Zebrafish embryos were maintained individually in 24-well plates. Zebrafish embryos were live-imaged using a fluorescence stereomicroscope and z-stack images of the HBV region acquired at 6 hpi, 24 hpi and 72 hpi. Bacterial burden in the HBV region of zebrafish embryos was quantified from fluorescence images by fluorescent pixel count (FPC) using Icy FPC protocol, at 6 hpi, 24 hpi and 72 hpi. (A-B) Data from one representative experiment is shown for *M. marinum*-injected (A) and *M. marinum* and Bac5 co-injected (B) zebrafish. Sample size (n): 13, 12. Data from one of the same three independent experiments as Figures 4.14-4.16 is shown. (C-D) Data pooled from the same three independent experiments as Figure 4.14-4.16 is shown on a log_{10} scale (C) and linear scale (D). Sample size (n): 38, 43. Error bars represent S.E.M. Unpaired t-test showed no significant differences.

### 4.3.4 Effect of Bac5 on *M. marinum* infection progression in zebrafish following sequential injections of *M. marinum* and Bac5 to the HBV

#### 4.3.4.1 Effect of Bac5 on neutrophil recruitment and bacterial burden following sequential injections to the zebrafish HBV
4.3.4.1.1 Overview

Co-injections of *M. marinum* and Bac5 did not lead to any lasting effects on zebrafish leukocyte recruitment, bacterial burden or host immunity gene expression. This could be due to degradation of Bac5 over time after the initial injection, providing diminishing effects over time. In addition, Bac5 treatment was performed at very early stages of infection, prior to granuloma formation, when neutrophils are not expected to play a role in the control of the infection. One of our hypotheses was that Bac5 would lead to increased recruitment and antimicrobial activities of neutrophils resulting in a reduction in infectious burden (Section 3.3.4.5.1). We therefore chose not to repeat co-injection experiments which had demonstrated trends for changes in infection progression parameters to establish significance, but decided to focus on further experiments to investigate the effects of Bac5 on *M. marinum* infection progression in the zebrafish due to time constraints. We decided to perform repeated treatments of zebrafish with Bac5 and also later treatments with Bac5 at the early granuloma formation stage of infection (2-4 dpi) when neutrophils play a role in the control of the infection. This was indicated in the co-injection experiment data by the significant increases in neutrophil recruitment at 72 hpi and in immunity gene expression at 96 hpi in response to *M. marinum* infection.

To determine whether single and repeated doses of Bac5 can affect zebrafish neutrophil recruitment, survival and bacterial burden at the early granuloma formation stage of *M. marinum* infection, we performed sequential injections of *M. marinum* and Bac5 to the HBV. We locally infected zebrafish embryos with *M. marinum* to the HBV at 2 dpf as previously, and then locally injected Mock solution or Bac5 into the HBV of zebrafish embryos at 48 hpi or both 48 hpi and 96 hpi. A schematic of the experiment is shown in Figure 4.17. Initially, we wished to test the effects of repeated doses of Bac5 every 48 h for the first 10 days of infection. Preliminary trials indicated that injection of Bac5 to the zebrafish HBV after 6 dpf would not be possible due to developmental changes in larval physiology (difficulties of needle penetration into larvae owing to increased epithelium rigidity combined with shrinking of the accessible HBV region) and that infected zebrafish would not be expected to survive until 14 dpi. We therefore focussed on the effects of single or double treatments with Bac5 during the first 5 days of infection when infected zebrafish could be expected to survive in accordance with our Project License.
under the Animals (Scientific Procedures) Act 1986. The experiment utilised transgenic zebrafish expressing GFP-tagged neutrophils with mutations leading to their transparency to aid in longer-term monitoring of the zebrafish by microscopy, the casper Tg(lysC:GFP) line. The transgenic line used in the sequential injections experiments differs from the line used for the co-injection experiments by necessity as the original line used, casper Tg(mpx:GFP), ceased regular breeding in between the experiments. Both transgenic lines lead to labelling of neutrophil cells with GFP.

**Figure 4.17. Schematic of sequential injection experiments to determine the effects of bactenecin-5 (Bac5) on zebrafish neutrophil recruitment or M. marinum bacterial burden.** Two dpf casper Tg(lysC:GFP) zebrafish embryos were injected into their HBV with approximately 220 CFU Mmar expressing DsRed2. All zebrafish embryos were live-imaged using a fluorescence stereomicroscope and images of the HBV region acquired at 24 hpi for the determination of bacterial burden using the Icy FPC protocol (Section 3.3.4.2). Embryos were then separated into four groups of equal infection distribution and burden (FPC of approximately 650), with any surplus infected zebrafish discarded at this point. HBV injections of Mock or 10ng Bac5 were performed at 48 hpi for all four groups and again at 96 hpi for two groups (Group 3 received Mock injections and Group 4 received 10ng Bac5 injections at both time points). All zebrafish embryo injection groups were live-imaged using a fluorescence stereomicroscope and z-stack images of the HBV region acquired at 72 hpi and 120 hpi. Z-slices which showed the HBV region in focus were extracted from the z-stacks. These slices were then processed using Icy Bioimage Analysis software to determine the number of neutrophils recruited to the HBV region (Icy Spot Detector plugin) and the bacterial burden (Icy FPC protocol) in the HBV region for all embryos (Section 3.3.4.2, 3.3.4.4, 3.3.4.5). Zebrafish survival was recorded daily from 72 hpi (5 dpf). Zebrafish drawing taken from the Dallman Group image repository.

### 4.3.4.1.2 Effect of Bac5 on zebrafish neutrophil recruitment in M. marinum-infected zebrafish following sequential injections to the HBV

To determine whether Bac5 treatment affects neutrophil recruitment to M. marinum infection during the early granuloma formation stage, we locally infected zebrafish with M. marinum at 2 dpf and later treated with Bac5 at 48 hpi and 96 hpi.
Figure 4.18. Injection of bactenecin-5 (Bac5) to the HBV after 48 hours of M. marinum infection does not affect zebrafish neutrophil recruitment. (A-D) Two dpf casper Tg(LysC:GFP) zebrafish embryos were injected into their HBV with approximately 220 CFU M. marinum. Zebrafish embryos were live-imaged using a fluorescence stereomicroscope at 24 hpi, individual bacterial burdens calculated using the Icy FPC protocol, and embryos were separated into four groups of equal infection burden. Two groups were treated with Mock or 10ng Bac5 at 48 hpi and two groups were treated with repeated doses of Mock or 10ng Bac5 at 48 hpi and 96 hpi. Zebrafish embryos were live-imaged using a fluorescence stereomicroscope and z-stack images of the HBV region acquired at 72 hpi and 120 hpi. (A-B) Representative fluorescence overlay images acquired at 72 hpi (A) and 120 hpi (B) during one experiment are shown. All images acquired during the same representative experiment are shown as fluorescence overlays in Appendix 3 Figures A12-13. (C-D) Neutrophils in the HBV region were quantified from fluorescence images of zebrafish embryos using Icy Spot Detector plugin at 72 hpi (C) and 120 hpi (D). The scale and sensitivity parameters used for these analyses differed to those previously described for earlier time points – scale 3 and sensitivity 20% were used. These changes were necessary as the exposure times used in image acquisition were reduced from the co-injection experiment settings to eliminate the issue of image overexposure. Sample size (n): 111, 109 in (C) and 47, 43, 45, 41 in (D). Data pooled from three independent experiments is shown. Error bars represent S.E.M. Unpaired t-test (C) and one-way ANOVA with Bonferroni’s post-test (D) showed no significant differences.

Quantification of neutrophil recruitment to M. marinum infection at the HBV at 72 hpi with M. marinum and following 24 hours of single injection treatment with Bac5 (Bac5 48 hpi) revealed that Bac5 does not affect neutrophil recruitment to M. marinum
infection (Figure 4.18A). At 120 hpi with *M. marinum*, trends for changes in neutrophil recruitment following Bac5 treatments are seen (Figure 4.18B). Single treatment with Bac5 led to reduced average neutrophil recruitment and double treatment led to increased average neutrophil recruitment to the HBV region, although the differences were not statistically significant.

### 4.3.4.1.3 Effect of Bac5 on bacterial burden of infected zebrafish following sequential injections to the HBV

In addition to assessing neutrophil recruitment to the HBV region, we also determined whether Bac5 treatment affects bacterial burden of *M. marinum*-infected zebrafish in the same experiment. Images are shown above in Figure 4.18 and in Appendix 3 Figures A12-13. Quantification of bacterial burden in infected zebrafish is shown in Figure 4.19. The data revealed that single or repeated treatment with Bac5 does not significantly affect the bacterial burden of infected zebrafish at any of the time points analysed in this study (Figure 4.19).

**Figure 4.19.** Injection of bactenecin-5 (Bac5) to the zebrafish HBV after 48 hours of *M. marinum* infection does not affect bacterial burden. (A-B) Two dpf casper Tg(LysC:GFP) zebrafish embryos were injected into their HBV with approximately 220 CFU *M. marinum*. Zebrafish embryos were live-imaged using a fluorescence stereomicroscope 24 hpi, individual bacterial burdens calculated using the Icy FPC protocol, and embryos were separated into four groups of equal infection burden. Two groups were treated with Mock or 10ng Bac5 at 48 hpi and two groups were treated with repeated doses of Mock or 10ng Bac5 at 48 hpi and 96 hpi. Zebrafish embryos were live-imaged using a fluorescence stereomicroscope and z-stack images of the HBV region acquired at 72 hpi and 120 hpi. Bacterial burden of *M. marinum*-infected zebrafish embryos was quantified from fluorescence images by fluorescent pixel count (FPC) using Icy FPC protocol at 72 hpi (A) and 120 hpi (B). Sample size (n): 111, 109 in (A) and 47, 43, 45, 41 in (B). Data pooled from the same three independent experiments as Figure 4.18 is shown. Error bars represent S.E.M. Unpaired t-test (A) and one-way ANOVA with Bonferroni’s post-test (B) showed no significant differences.
4.3.4.1.4 Effect of Bac5 on zebrafish health and survival

In addition to quantifying the effects of Bac5 treatment on zebrafish neutrophil recruitment and bacterial burden, we also monitored zebrafish survival after 72 hours of *M. marinum* infection. Zebrafish survival was scored at 72 hpi prior to imaging, 96 hpi and at 115 hpi prior to imaging at 120 hpi.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Signs of distress</th>
<th>Epithelium weakening</th>
<th>Dead</th>
<th>Healthy</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock 48 hpi</td>
<td>8</td>
<td>0</td>
<td>1</td>
<td>47</td>
<td>56</td>
</tr>
<tr>
<td>Bac5 48 hpi</td>
<td>10</td>
<td>0</td>
<td>3</td>
<td>43</td>
<td>56</td>
</tr>
<tr>
<td>Mock 48 hpi &amp; 96 hpi</td>
<td>9</td>
<td>0</td>
<td>2</td>
<td>45</td>
<td>56</td>
</tr>
<tr>
<td>Bac5 48 hpi &amp; 96 hpi</td>
<td>8</td>
<td>3</td>
<td>5</td>
<td>41</td>
<td>57</td>
</tr>
</tbody>
</table>

Data from three independent experiments is shown. Some zebrafish larvae from all treatment groups displayed signs of distress in each independent experiment, zebrafish larvae died following injection with a double dose of Bac5 in each independent experiment, zebrafish larvae died following injection with a double dose of Mock solution and a single dose of Bac5 during two independent experiments, and zebrafish larvae displayed epithelium weakening in two independent experiments.

When accumulating the survival data, it was noted that zebrafish health may be reduced for those groups treated with Bac5 compared to Mock solution. At 5 dpf zebrafish enter protection under the Animals (Scientific Procedures) Act 1986 as they begin free feeding. These changes permit the monitoring of larval health and distress according to their ability to control buoyancy, ability to control swimming direction and their interest in feeding. Some larvae demonstrated signs of distress such as floating and lack of feeding, whilst others demonstrated epithelium weakening near the injection site during manipulation for imaging, seen as the bursting of the HBV region (affected zebrafish were euthanized). Table 4.1 shows the total number of fish displaying signs of distress or epithelium weakening for each treatment group over the course of three independent experiments. No zebrafish larvae displayed signs of distress until 115 hpi, with all those in distress euthanized immediately at 115 hpi according to the Animals (Scientific Procedures) Act 1986 and scored as dead in the survival analysis. Therefore the observations in Table 4.1 reflect the results from the survival data: that the majority
of zebrafish larvae displaying signs of distress were from the Bac5 treatment groups and only zebrafish from the Bac5 treatment group displayed weakened epithelia.

The results of the survival assessments revealed that single injections of either Mock or Bac5 solutions to the zebrafish HBV lead to a 4.5% higher survival rate than double treatment of the paired solutions (Figure 4.20), though the differences were not statistically significant. The results also demonstrated that single or double injection with Bac5 reduced survival compared to Mock injections by approximately 8% (Figure 4.20), although the differences were not statistically significant.

![Figure 4.20. Effect of injection of bactenecin-5 (Bac5) to the HBV after 48 hours of M. marinum infection on zebrafish survival. Two dpf casper Tg(Lyz:GFP) zebrafish embryos were injected into their HBV with approximately 220 CFU M. marinum. Zebrafish embryos were live-imaged using a fluorescence stereomicroscope 24 hpi and individual bacterial burdens calculated using the Icy FPC protocol. Embryos were separated into four groups of equal infection burden at 24 hpi. Two groups were treated with Mock or 10ng Bac5 at 48 hpi and two groups were treated with repeated doses of Mock or 10ng Bac5 at 48 hpi and 96 hpi. Data for survival of all zebrafish treatment groups pooled from the same three independent experiments as Figures 4.18-20 is shown. Sample size (n): 47, 43, 45, 41. Survival analysis showed no significant differences.](image)

### 4.3.4.2 Effect of Bac5 on host immunity gene expression following sequential injections to the zebrafish HBV

#### 4.3.4.2.1 Overview

Having determined the effects of Bac5 treatment on neutrophil recruitment, bacterial burden and survival at the early granuloma formation stage of *M. marinum*-zebrafish infection, we determined the effects of Bac5 treatment on host immunity gene expression at the same stage. To determine whether single and repeated doses of Bac5 can alter the zebrafish immunity gene expression response to *M. marinum* infection at the early granuloma formation stage, we performed sequential injections of *M. marinum*
and Bac5 to the HBV. We locally infected zebrafish embryos with *M. marinum* to the HBV at 2 dpf as previously, and then locally injected Mock solution or Bac5 into the HBV of zebrafish embryos at 72 hpi or 90 hpi. The expression of selected genes was quantified in samples of whole zebrafish larvae at 96 hpi by qRT-PCR. A schematic of the experiment is shown in Figure 4.21. The experiment utilised transgenic zebrafish expressing mCherry-tagged macrophages, *Tg(mpeg-1:mCherry)*, to maintain a consistent zebrafish line background with previous host immunity gene expression data acquired during co-injection experiments (Figure 4.8).

![Figure 4.21. Schematic of sequential injection experiments to determine the effects of bactenecin-5 (Bac5) on zebrafish immunity gene expression.](image)

Two dpf *Tg(mpeg-1:mCherry)* zebrafish embryos were injected into their HBV with approximately 180 CFU *M. marinum* expressing GFP. All zebrafish embryos were live-imaged using a fluorescence stereomicroscope and images of the HBV region acquired at 24 hpi for the determination of bacterial burden using the Icy FPC protocol (Section 3.3.4.2). Embryos were then separated into four groups of equal infection distribution and burden, with any surplus infected zebrafish discarded at this point. HBV injections of Mock or 10ng Bac5 were performed at 72 hpi for groups 1 and 2 and 90 hpi for groups 3 and 4. The cytokine response of the zebrafish embryos was analysed by qRT-PCR at 96 hpi for all groups. Zebrafish drawing taken from the Dallman Group image repository.

### 4.3.4.2.2 Effect of Bac5 on immunity gene expression by infected zebrafish following sequential injections to the HBV

We assessed the expression levels of the same genes as for the co-injection experiments, genes which function in immunity and have previously been reported to be elevated in *M. marinum* infection of the zebrafish and/or following Bac5 treatment of cell culture cells (*il-1β, mmp-9, cxcl-c1c* and *tnfa*). As a negative control, expression of the antiviral cytokine *ifnphi1* was also quantified.

The qRT-PCR data showed that *ifnphi1* expression was undetectable for all groups throughout the experiment as expected. We observed no effect of Bac5 treatment at 72
hpi on expression levels of il-1β, mmp-9 and tnfa, seen by comparing the results from 72 hpi treatment with Mock- to Bac5-treated groups (Figure 4.22). Expression of cxcl-c1c increased 2-fold following treatment with Bac5 at 72 hpi, though the difference was not statistically significant. Injections at 90 hpi led to trends for increases in the expression levels of the tested immunity genes, although the differences were not statistically significant. Treatment with Bac5 at 90 hpi showed trends for reduced average expression levels for all of the immunity genes tested compared to Mock-injected groups. In addition, injection alone at 90 hpi led to trends for increased average expression of il-1β and cxcl-c1c and significantly increased expression of mmp-9 compared to injection alone at 72 hpi.

**Figure 4.22.** Injection of bactenecin-5 (Bac5) to the HBV of *M. marinum*-infected zebrafish 72 hpi or 90 hpi does not affect zebrafish immunity gene expression. Two dpf Tg(mpeg-1:mCherry) zebrafish embryos were injected into their HBV with 180 CFU *M. marinum* expressing GFP. Zebrafish embryos were live-imaged using a fluorescence stereomicroscope at 24 hpi, individual bacterial burdens calculated using the Icy FPC protocol, and embryos were separated into four groups of equal infection burden. Each group was injected into their HBV with Mock or 10ng Bac5 at 72 hpi or 90 hpi. Fold changes of il-1β, mmp-9, cxcl-c1c and tnfa mRNA were assessed at 96 hpi by qRT-PCR, normalised to 18S and expressed relative to one control. Data pooled from two independent experiments is shown (n=20). Each data point represents an individual fish. Error bars represent S.E.M. Kruskal-Wallis with Dunn’s post-test. **p<0.01, *p<0.05.
4.4 Discussion

4.4.1 Bac5 lacks direct antimycobacterial activities
Experiments to determine the direct effects of the HDPs on *M. marinum* revealed significant direct bacteriocidal effects for human LL-37, resulting in 90% killing of *M. marinum* over 24 hours of co-incubation, but no direct killing activities for bovine Bac5 or fish EPI (Figure 4.1). These results agreed with previous studies which demonstrated that LL-37 possesses direct antimycobacterial activities\(^{199, 307}\). Previous work undertaken by our collaborator demonstrated that Bac5 possesses direct bacteriocidal effects against *E. coli* (Sam Willcocks, unpublished data). The sample of Bac5 used in this study was proven to be active with significant killing activity seen when Bac5 and *E. coli* were co-incubated for 24 hours (Figure 4.1). It has been shown that EPI demonstrates bacteriostatic activities against several bacteria, including *P. aeruginosa*\(^{235, 236}\), and it would be useful to perform experiments to confirm the bacteriostatic abilities against this pathogen of the sample of EPI used in this study. Although such experiments were not performed, later experiments testing the effect of the HDPs against THP-1 cells did demonstrate effects with the sample of EPI used and it is therefore considered to be active.

4.4.2 Bovine Bac5 is capable of immunomodulation of human THP-1 cells independent of *M. marinum* infections
The effects of treatment with EPI and Bac5 on *M. marinum* infection in THP-1 cells were not previously known. Infected THP-1 cell treatment for 24 hours with Bac5 resulted in significantly increased *IL-1β* expression and treatment for 24 hours with EPI resulted in significantly increased *IL-1β* and *MMP-9* expression (Figure 4.2). Neither peptide increased expression of *TNF-α* by THP-1 cells, consistent with previous data for the treatment of THP-1 cells with the derivative of a bactenecin family member (IDR-1002) resulting in no detectable induction of *TNF-α*\(^{221}\). During infection, *M. marinum* bacilli would be expected to come into contact with resting macrophages in the initial stages of the infection and with activated macrophages once the bacilli have infected them. Therefore, the response of resting THP-1 cells during our experiments indicates that the exogenous peptides Bac5 and EPI, which may play roles in the control of natural
mycobacterial infections, could potentially have a beneficial effect for the human host if used to treat tuberculous disease.

### 4.4.3 Bovine Bac5 increases initial macrophage recruitment during the early stages of *M. marinum* infection of zebrafish embryos

Experiments to test the effects of the exogenous peptide Bac5 on the progression of *M. marinum* infection in zebrafish embryos were performed initially by assessing the effect of injections of *M. marinum*, Bac5 and co-injections of *M. marinum* and Bac5. We assessed the ability of Bac5 to protect the zebrafish from mycobacterial infection through quantification of bacterial burden. We also assessed the ability of Bac5 to perform immunomodulation in zebrafish by quantification of leukocyte recruitment and host gene expression in the presence and absence of *M. marinum* infection. We quantified recruitment of macrophages in response to Bac5 injections as they are considered the key cell type in mycobacterial infections. We also quantified neutrophil recruitment based upon our preliminary data from epithelial cell line work indicating that Bac5 may lead to increased neutrophil recruitment and bacterial killing capabilities and reports that neutrophils may play a protective role in early mycobacterial infections (Section 3.3.4.5.1).

Treatment of 2 dpf zebrafish with Bac5 increased macrophage recruitment to the HBV region by 50% and neutrophil recruitment to the HBV region by approximately 25% at 3-6 hpi (Figure 4.6 and Figure 4.13). Significant recruitment of macrophages at 3 hpi (Figure 4.6) but not neutrophils at 6 hpi (Figure 4.13) was seen following injection of *M. marinum* to the HBV, in agreement with previous reports[^53] ,[^328] ,[^363] . Additionally, co-injection with Bac5 resulted in a consistent trend for increased leukocyte recruitment compared to injection with *M. marinum* only at both 3-6 hpi and 24 hpi. However, the observed small initial increases in leukocyte recruitment may not be of biological significance as these do not translate into a reduction in bacterial burden, discussed below. In addition, monitoring of leukocyte recruitment over the course of these longitudinal experiments revealed that there were no lasting effects of Bac5 treatment on leukocyte recruitment (Figure 4.7 and Figure 4.14).
Our data is consistent with recent studies demonstrating increased recruitment of leukocytes in invasive murine infection models of *S. aureus* and *E. coli* in response to treatment with IDR peptides derived from a bactenecin family member, IDR-1 and IDR-1002\[221, 293\]. In addition, IDR-1002 was shown to lack direct monocyte chemoattractive activity, which may indicate that the increased zebrafish leukocyte recruitment to the infection site in response to Bac5 injection is due to immunomodulatory activities of Bac5 rather than direct effects. It would therefore be useful to determine which chemokines are induced upon Bac5 treatment of the zebrafish and lead to the observed increased leukocyte recruitment.

The assessment of other infection progression parameters during zebrafish co-injection experiments revealed trends for changes in response to Bac5 injections which should be further investigated. For example, there was a consistent trend for reduced average bacterial burden of *M. marinum*-infected zebrafish co-injected with Bac5 compared to those co-injected with Mock solution (Figure 4.15). In addition, comparing bacterial burden increases between 24 hpi and 72 hpi revealed a small reduction for Bac5 treated zebrafish (Figure 4.16). These trends may be attributed to inaccuracies in the initial dose arising from the reduced preparation volumes for the Bac5 co-injection solutions, necessitated by limited peptide stocks. Further experiments using equal volumes of Mock and Bac5 solutions for the resuspension of the injection bacteria would determine whether Bac5 treatment affects the bacterial burden of *M. marinum*-infected zebrafish.

Trends for immunomodulation following treatment of zebrafish with Bac5 could also be further investigated. We observed trends for increased expression of pro-inflammatory cytokines *il-1β* and *mmp-9* as well as the chemokine *cxcl-c1c* at 6 hpi, decreased expression of the same genes by 24 hpi (interestingly more striking in the presence of *M. marinum*), and increased average expression levels at 96 hpi of *cxcl-c1c* and *tnfa* (Figure 4.8). These gene expression trends are consistent with the observed significant initial increases in leukocyte recruitment at 3-6 hpi and return towards baseline leukocyte recruitment levels at 24 hpi. That significant lasting differences in gene expression following Bac5 treatment are not seen indicates that the leukocytes which are recruited initially are not activated, supporting the data demonstrating no effect on neutrophil recruitment and bacterial burden at 72 hpi. Further or more extensive experiments to assess the expression of other pro-inflammatory genes by qRT-PCR or
fluorescence in situ hybridisation (FISH), or a larger transcriptomics study, were not undertaken as no significant differences in expression were seen with the initial group of 4 genes which we thought likely to show differential expression upon Bac5 treatment (Section 4.3.3.1.3).

In the studies of invasive infection with S. aureus, mice treated with IDR-1002 and IDR-1 were shown to produce increased levels of the chemokine MCP-1 and anti-inflammatory cytokine IL-10, whilst the expression levels of pro-inflammatory cytokines TNF-α and IL-6 were shown to remain unchanged or decrease\cite{221,293}. This led to the conclusion by Nijnik et al. that protection of mice against S. aureus infection by IDR-1002 is not associated with an overall inflammatory response\cite{221}. These conclusions are consistent with the trend for the initial increase in chemokine expression, no significant increase in pro-inflammatory cytokine expression and possible anti-inflammatory effects following treatment of zebrafish with Bac5 presented here in co-injection experiments. The gene expression data at 24 hpi could indicate that treatment with Bac5 has resulted in immunomodulation to control excess inflammation, a feature of HDP action\cite{50,195}. Alternatively, Bac5 could enhance healing of the injection wound, reducing inflammatory gene expression by 24 hpi, as HDPs and IDR peptides have previously been shown to enhance wound repair in murine and porcine models\cite{147,194}. Therefore, it would be useful to further investigate the potential reduction in inflammation at 24 hpi with Bac5 and determine the cause.

Our data on the zebrafish gene expression response to M. marinum is consistent for the most part with a recent transcriptomics study describing RNA-Seq analysis of uninfected and M. marinum-infected zebrafish embryo gene expression over the first 5 days of systemic M. marinum infection\cite{25}. We observed trends for increases in the average expression levels of il-1β, mmp-9 and cxcl-c1c at 6 and 24 hpi with M. marinum, and significant increases in expression of all three genes at 4 dpi (Figure 4.8). In addition, we were able to detect tnfa expression in M. marinum-infected samples only at 4 dpi, indicating an increase in expression in response to M. marinum infection. The Benard et al. transcriptomics data revealed three phases of the zebrafish gene expression response to M. marinum infection\cite{25}. The early phase demonstrated significant increases in expression of many pro-inflammatory cytokines and mmp-9 at 2-4 hpi, which decline thereafter in the mid-phase of 6-24 hpi, before peaking at 4-5 dpi.
in the late phase (which runs from 2-5 dpi). Details of the expression profiles of *il-1β*, *mmp-9*, *tnfa* and *tnfb* (the second zebrafish orthologue of human *TNF-α*) over the five day infection time course are provided in the study. However, the expression profile of *cxcl-c1c* is not described, with profiles shown instead for the chemokines *cxcl-8a* and *cxcl-18b*. The study undertaken by Benard *et al.* demonstrated a non-significant increase in *il-1β* expression and significant increases in the expression of *cxcl-18b* and *mmp-9* at 6 hpi, which is broadly consistent with our data. The data presented by Benard *et al.* are also consistent with our results at 24 hpi, in that no significant increases in the expression of *il-1β*, *mmp-9*, *cxcl-8a* and *cxcl-18b* were observed at this mid-phase time point. However, the data in the transcriptomics study demonstrates no induction of gene expression at 24 hpi, whilst our data shows a trend for moderate increases in expression of the tested genes. Finally, the transcriptomics study demonstrated significant induction of all of the genes of interest - *il-1β*, *mmp-9*, *cxcl-8a*, *cxcl-18b*, *tnfa* and *tnfb* – to markedly higher levels than at the early time points, which is again reflected in our gene expression data (Figure 4.8). Therefore, whilst we did not assess the zebrafish gene expression response to *M. marinum* infection in the early phase, our data were consistent with the mid- and late-phase responses identified in the Benard *et al.* transcriptomics study. The observed small differences in gene expression between the two studies can be explained by differences in the experimental methods, quantification methods or zebrafish lines and bacterial strains used. That our gene expression data reflected those obtained in an independent study lends weight to our conclusions on the effect of Bac5 on zebrafish gene expression in the presence and absence of *M. marinum* infection.

From the co-injection experiments we concluded that the bovine cathelicidin Bac5 is capable of immunomodulation in the zebrafish independently of *M. marinum* infection, as evidenced by the significant macrophage recruitment at 3 hpi following treatment with Bac5. By performing the co-injection experiments as a longitudinal study, we were able to conclude that there was no lasting immunomodulation of zebrafish after 24 hpi with Bac5 and no benefit to the host during *M. marinum* infection of Bac5 treatment.
4.4.4 Bovine Bac5 does not affect *M. marinum* infection progression of zebrafish embryos at the early granuloma formation stage

There are several reasons why treatment with Bac5 of zebrafish embryos at very early infection stages may not be expected to lead to lasting differences in *M. marinum* infection progression. One explanation is that Bac5 would be expected to degrade over time after the initial injection through the action of proteases and therefore its effects will diminish over time. In addition, Bac5 treatment was performed at very early stages of infection, prior to granuloma formation, when neutrophils are not expected to play a role in the control of the infection. Previous data indicated that Bac5 increases the production of the neutrophil chemoattractant IL-8 and the enzyme iNOS in epithelial cells (Sam Willcocks unpublished data), Therefore, we expected Bac5 to have an effect on neutrophil recruitment to the site of infection and hypothesised that Bac5 would lead to increased antimicrobial activities of neutrophils resulting in a reduction in infectious burden. As we treated with Bac5 before neutrophils are expected to play a role in the host defence against mycobacterial infections, we may have missed lasting effects of Bac5 on *M. marinum* infection progression.

We therefore assessed the effect of sequential injections of Bac5 at a later stage of infection with *M. marinum* on infection progression in zebrafish, that of early granuloma formation when neutrophils begin to respond to the infection. We observed no effect on zebrafish neutrophil recruitment or bacterial burden at 72 hpi following a single Bac5 treatment at the early granuloma stage of infection (Figure 4.18 and Figure 4.19). It was expected from the 6 hpi co-injection experiments result that injection with Bac5 at 48 hpi with *M. marinum* would lead to increased neutrophil recruitment. However, the co-injection data did indicate that neutrophil recruitment at 6 hpi in response to Bac5 injection had begun to return to baseline levels by 24 hpi, which could explain why no additional neutrophil recruitment was seen in the sequential injection experiments. Another possibility is that neutrophil recruitment in response to infected macrophages at 72 hpi provides more potent chemoattraction than Bac5 injection, and therefore the *M. marinum* infection is masking any effects of Bac5. Thus, these experiments are consistent with the co-injection data which indicated no long-term effects of early Bac5 treatments at the granuloma formation stage.
The sequential injection experiments assessing zebrafish neutrophil recruitment and bacterial burden involved the robust statistical analysis of very large sample sizes at 72 hpi, followed by the separation of the samples into further treatment groups at 96 hpi. Therefore, trends for changes in the tested parameters were observed at 120 hpi and it would be useful to increase the sample sizes to investigate these trends. For example, a single injection of Bac5 to zebrafish at 48 hpi with *M. marinum* led to a small reduction in the average neutrophil recruitment to the infection site whilst sequential injections of Bac5 to zebrafish at 48 hpi and 96 hpi with *M. marinum* led to a small increase in the recruitment of neutrophils to the infection site at 120 hpi. Moreover, single or double treatment with Bac5 lead to trends for reduced zebrafish health and survival compared to Mock-injected groups (Figure 4.20). Future experiments with larger sample sizes could include quantification of zebrafish health by scoring according to the parameters identified in the sequential injection experiments (Table 4.1), to determine the effects of Bac5 treatment on zebrafish health at early developmental stages and/or early infection stages.

We also assessed the effects of sequential injections of Bac5 at the early granuloma formation stage of zebrafish infection with *M. marinum* on host immunity gene expression. A single Bac5 injection to zebrafish at 72 hpi with *M. marinum* had no effect on host immunity gene expression at 96 hpi (Figure 4.22). A single Bac5 injection to zebrafish at 90 hpi with *M. marinum* demonstrated trends at 96 hpi for reduced average expression levels of the four immunity genes tested – *il-1β, mmp-9, cxcl-c1c* and *tnfa*. As the initial panel of 4 genes did not show significant differences in expression following Bac5 treatment, further experiments to investigate the effects of Bac5 treatment on the expression of other genes by qRT-PCR, FISH or a transcriptomics approach were not undertaken. From the existing qRT-PCR data we concluded that treatment of *M. marinum*-infected zebrafish with Bac5 at the early granuloma formation stage does not affect host immunity gene expression to a biologically significant degree which might affect the outcome of infection in zebrafish.

Overall, the sequential injection experiments data is consistent with that obtained in the *M. marinum* and Bac5 co-injections in that Bac5 treatment did not lead to any lasting effects on neutrophil recruitment, bacterial burden or host immunity gene expression. From the sequential injections experiments data we concluded that later or repeated
Bac5 treatment during *M. marinum* infection of zebrafish did not improve its immunomodulatory activity or the infection outcome.

**4.4.5 Implications for Bac5 as a novel TB therapy**

We believe that the co-injection and sequential injection experiments presented here represent the first investigations of the immunomodulatory activities of Bac5 in zebrafish and of the immunomodulatory activities of an exogenous HDP in the *M. marinum*-zebrafish model. These experiments to assess the effects of Bac5 treatment on the zebrafish and *M. marinum* infection progression were conducted using localised treatment with Bac5 and localised infection of zebrafish in the HBV. Experiments were conducted in this way for several interrelated reasons: Bac5 stocks available for these experiments were limited; systemic treatment of zebrafish embryos and larvae with Bac5 would require additional experiments to investigate the cytotoxicity of Bac5 and appropriate dosage for treatments at these developmental stages; and time constraints.

The field standard is to quantify leukocyte recruitment in response to a treatment or infection following injections to the zebrafish HBV cavity. A limitation of assessing other effects of Bac5 injections using localised Bac5 treatment to the HBV is that only a small proportion of host cells, those which are present in and around the HBV, would be exposed to the treatment and respond. This in turn could potentially limit the observed effects of Bac5 on host gene expression and bacterial burden of infected zebrafish which would not be the case for experiments involving systemic treatments and systemic infections. There exists a precedent for determining zebrafish bacterial burden in the HBV following HBV infections with *M. marinum* [41]. Cambier *et al.* successfully determined the importance of bacterial and zebrafish host factors in mycobacterial infections through quantification of HBV bacterial burden following infection of WT and MyD88 morpholino zebrafish with WT and deletion mutant strains of *M. marinum* [41]. It is also important to note that our data on the expression of a small group of immunity genes obtained from localised *M. marinum* infection of zebrafish did broadly reflect the data from a larger transcriptomics study into the response to a systemic *M. marinum* infection as discussed above (Section 4.4.3). We proceeded with our experiments in order to provide valuable, if limited, information on the effects of Bac5 treatment on the zebrafish as this had not been previously reported.
Ideally, experiments in zebrafish embryos and larvae would have been undertaken using systemic treatment of Bac5, by bathing or caudal vein injection, and systemic infections with *M. marinum* through caudal vein injection. As a first step, potential as a TB treatment would be confirmed through assessment of the toxicity of Bac5 in humans and zebrafish followed by determination of the required dosage for systemic treatment of zebrafish embryos and larvae. Investigations into the effect of systemic Bac5 treatment on zebrafish gene expression in the presence and absence of *M. marinum* infection and infected zebrafish bacterial burden would then proceed. Experiments investigating the effects of systemic Bac5 treatment could be performed in future to confirm that Bac5 does not significantly affect zebrafish gene expression or bacterial burden, although this may not be of interest in light of the results from the local treatment experiments presented here. Future experiments of particular interest would be those investigating the effect of systemic Bac5 treatment on bacterial dissemination following systemic infections of zebrafish embryos, which could be determined using the Icy Bacterial Distance Distribution protocol described in Chapter 3 (Section 3.3.4.6).

We hypothesised that Bac5 treatment would lead to reduced bacterial dissemination through its immunomodulatory effects increasing bacterial killing by neutrophils and other cells, but were unable to test this hypothesis during the local treatment experiments. Experiments to determine the effects of Bac5 treatment on *M. marinum* infection progression in adult zebrafish would also be of particular interest, which is discussed further below.

Taking the co-injections and sequential injections experiments as a whole, we were able to form several conclusions on the effects of localised treatment with Bac5 on the zebrafish and on the progression of localised *M. marinum* infection in the zebrafish. These experiments demonstrated that the bovine cathelicidin Bac5 is capable of moderate initial immunomodulation in the zebrafish independently of *M. marinum* infection, as evidenced by the significant macrophage recruitment at 3 hpi following treatment with Bac5. However, localised treatment with Bac5 did not lead to lasting immunomodulation of the zebrafish, either through continued leukocyte recruitment or modulation of host gene expression. In addition, localised treatment with Bac5 did not benefit the host and lead to a significant reduction in the bacterial burden of infected
zebrafish during our investigations, which may be attributed to the use of localised treatment as discussed above or to the lack of lasting immunomodulation.

That we see no lasting effects following injection of Bac5 which benefit the zebrafish host during *M. marinum* infection is contrary to our original hypothesis, which was based upon a previous study in the murine model by Rivas-Santiago *et al.*[276]. Their study used a peptide derived from a bactenecin family member, IDR-1018, which demonstrated modest direct antimicrobial activity versus *M. tuberculosis*. Treatment with IDR-1018 reduced bacterial burden and host inflammation in the murine model of TB using both the virulent, drug-susceptible H37Rv strain and an MDR strain of *M. tuberculosis*. We provide two further explanations for the lack of lasting immunomodulation and of a reduction in *M. marinum*-infected zebrafish bacterial burden following Bac5 treatment in our experiments.

Firstly, we suggest that the additional macrophages recruited upon injection of Bac5 may include resting macrophages which would benefit the infecting bacilli and act to oppose any other beneficial effects of Bac5 treatment of the zebrafish. Cambier *et al.* described the evasion of microbicidal macrophages (expressing iNOS) and recruitment of permissive, resting macrophages (which lack iNOS expression) by mycobacteria for their benefit during infections of mice and zebrafish embryos leading to increased bacterial burden[41]. Furthermore, Volkman *et al.* reported that bacteria-induced production of MMP-9 increased macrophage recruitment at 5 dpi (once granuloma formation has occurred) and contributed to granuloma maturation and increased bacterial burden in infected zebrafish[345]. These studies suggest that continued treatment with Bac5, or treatment with Bac5 at later infection stages once granuloma formation has occurred, may benefit the infecting bacilli through recruitment of resting macrophages to the injection sites. Therefore, it is important to determine the state of the macrophages recruited by Bac5 through antibody staining against iNOS and to further investigate the modulation we observed by Bac5 of *mmp-9* expression in the zebrafish.

Secondly, we propose that any additional resting leukocytes recruited by Bac5 are not activated upon arrival at the infection site, and thus there is no increase in bacterial killing, due to a lack of activator and effector molecules of the immune system in our
zebrafish embryo model of early infection. Cambier et al. reported that knockdown of zebrafish MyD88 led to increased bacterial burden at later infection stages, suggesting that recruitment of macrophages with microbicidal abilities by IL-1 is important in the control of infection\[41\]. However, IL-1 expression by zebrafish embryos could not be detected at 3 hpi with *M. marinum*, leading to the lack of microbicidal macrophage recruitment during early infection\[41\]. Furthermore, Rivas-Santiago et al. successfully used IDR-1018 to reduce the bacterial burden and pneumonia in the lungs of infected mice at a late, progressive stage of TB infection\[276\]. Hernandez-Pando et al. demonstrated that this second stage of pulmonary TB in mice possesses the highest levels of IFNγ\[135\]. This suggests that any additional recruited macrophages during the study undertaken by Rivas-Santiago et al. would be activated by their interaction with IFNγ and IL-1, leading to the observed increased bacterial killing. Collectively, these studies suggest that the stage of infection and actions of the adaptive immune system are important in determining the effect of any additional recruited macrophages to the injection site. Thus the lack of IL-1 and IFNγ production, part of the Th1 immune response, in the zebrafish embryo infection model may prevent protective effects of Bac5 treatment.

Therefore, the work presented here serves to highlight the benefits of using the zebrafish embryo infection model to study the innate immune system only, which has indicated the importance of the adaptive immune system in mycobacterial infection control and treatment. Combining the experiments presented here with experiments in adult zebrafish to investigate the effects of Bac5 on *M. marinum* infection would confirm whether the adaptive immune system is required for the beneficial effects on bacterial burden of treatment with Bac5, and, by extension, other HDPs and their derivatives. These studies could be crucial in determining the suitability of HDPs and their derivatives as potential novel immunotherapies for the treatment of human TB infections and of the stages of infection at which treatment with HDPs may be efficacious.

Whilst our results do not promote the use of Bac5 alone as a treatment for TB disease at this stage, it may be desirable to use Bac5 as an adjuvant in treatments to protect against mycobacterial infections. Recent studies have demonstrated that HDPs and IDR peptides possess adjuvant activities\[3, 39, 43, 100, 150, 167, 294, 336\]. These reports have led to
the suggestion that such peptides may be best administered with other adjuvant components\[195, 217\]. The identification of suitable combinations of Bac5 with other adjuvant components to provide lasting protective immune responses against mycobacterial infections is therefore of interest. The zebrafish embryo model of TB infection is limited to the study of the initial stages of granuloma formation in a setting where the adaptive immune system is not yet functional. The reported adjuvant activities of HDPs and IDR peptides involve either interactions with or the actions of the adaptive immune system. Therefore, it would be necessary to investigate the potential of Bac5 as an adjuvant in mycobacterial infection therapies in the adult zebrafish infection model.

That our experiments were performed on zebrafish embryos lacking an adaptive immune system and at the early acute phase of the infection renders them at the equivalent stage as a naïve human or mouse host in the first 2-3 days of the infection. This being the case, our experiments can be considered to be of relevance to studies on the effects of Bac5, as well as other HDPs and their derivatives, as prophylaxis. Prophylactic treatment of at-risk individuals, such as those in intensive care units or HIV-infected individuals, may represent a major step forwards in the control of many infections. In particular, the use of HDPs and their derivatives as prophylaxis for household members who are constantly exposed to mycobacteria was suggested by Rivas-Santiago et al. as a major step forwards in the treatment and prevention of tuberculous disease\[276\]. Studies by other groups have indicated that such peptides do act prophylactically when administered prior to exposure to bacteria\[144, 181, 221, 237-239, 252, 291\]. However, the data presented here indicate that the use of Bac5 in a prophylactic strategy against TB infection may not be successful.

Concerns on the toxicities of HDPs\[50, 128, 195\], as well as our observations on zebrafish health following Bac5 treatment, render the investigation of the cytotoxicity of Bac5 using both zebrafish embryo and adult models important. If Bac5 does not prove to be toxic to zebrafish embryos, it would be possible to generate transgenic zebrafish for use in studying the effects of Bac5 as prophylaxis against \textit{M. marinum}, and by extension mycobacterial, infections. Transgenic zebrafish constitutively expressing Bac5 would permit the assessment of the effects of Bac5 and protection from infections throughout
zebrafish development and serve as a model for gene therapy treatment of household members or HIV-infected individuals.
5 Generation of TCSs deletion mutants in *M. marinum*

5.1 Introduction

TCSs are ubiquitous in bacteria but absent in higher eukaryotes making them attractive and safe targets for new therapies or live vaccine strains\(^{[36]}\). Bacterial TCSs regulate gene expression in response to external environmental changes, permitting the adaptation of the bacteria to their new environment or stress placed upon them by the actions of the host immune system\(^{[36, 170]}\). Through these actions TCSs control many physiological processes, including virulence and antibiotic resistance\(^{[36, 170]}\). There are 12 complete TCSs present in the *M. tuberculosis* genome, which have been implicated in virulence by many studies of TCSs mutant strains (summarised in Table 1.5). However, conflicting results have been obtained through the use of multiple different TB infection models, none of which fully reflect human TB. The natural infection pairing of *M. marinum*-zebrafish, which reflects the early stages of human TB infection in the embryo model\(^{[53]}\) and later stages in the adult model\(^{[203, 244, 256]}\), could be used to provide greater clarity on the role of mycobacterial TCSs in virulence. *M. marinum* possesses homologues to 11 of the complete *M. tuberculosis* TCSs and these formed the basis of this study.

5.2 Aims

The aim of this work was to test the hypothesis that targeting of some of the selected mycobacterial TCSs would benefit the zebrafish host during infections due to their role in virulence. To test this aim, we intended to screen *M. marinum* TCSs deletion mutants for attenuation in the zebrafish embryo infection model, identifying potential novel TB drug targets for future studies.

Our initial hypotheses were:
1) TCSs between *M. tuberculosis* and *M. marinum* would show conserved protein sequences and similar genomic contexts
2) Mycobacteria-adapted recombineering or sucrose counter-selection could be used to generate *M. marinum* TCSs deletion mutants
3) *M. marinum* TCSs deletion mutants could be screened for attenuation in the zebrafish embryo model through quantification of infection progression parameters (bacterial burden, bacterial dissemination, leukocyte recruitment, and host immunity gene expression)

5.3 Results

5.3.1 Investigation of TCSs relatedness between *M. tuberculosis* and *M. marinum*

5.3.1.1 Overview

To study the relatedness of the 11 selected TCSs and orphaned RRs and SKs of *M. tuberculosis* with their *M. marinum* homologues, protein sequence and genomic context alignments were performed for each TCSs or orphan in both species. The sequence alignments provide a direct measure of the relatedness of the proteins in both organisms, whilst the genomic context can be used to infer functional conservation. A high degree of protein sequence similarity and the gene of interest being in a similar genomic environment in both organisms are suggestive of a conserved function.

5.3.1.2 Investigation of TCSs relatedness between *M. tuberculosis* and *M. marinum*

The four orphaned RRs in the *M. tuberculosis* genome are Rv0260, Rv0818, Rv2884 and Rv3143. The orphaned SK in the *M. tuberculosis* genome is DosT, part of the DosR/S/T TCSs. As there is no DosT homologue in the *M. marinum* genome, this protein is described below with *M. tuberculosis* DosS. Analysis of the protein sequence identities and genomic contexts of the orphaned RRs revealed a coding sequence identity range of 66-90% and similar genomic environments between these genes in *M. marinum* and *M. tuberculosis* (data not shown).

One of the 12 complete TCSs of *M. tuberculosis* (Rv0600c/Rv0601c/TcrA) has no homologue in *M. marinum* and therefore does not form part of these investigations. Sequence identities for the remaining 11 TCSs are summarised in Table 5.1, whilst example alignments and genomic context views for the DosR/S/T system are shown in
Figure 5.1 (the remaining protein alignments and genomic context views are provided in Appendix 4 Figures A14-A23). These investigations revealed a coding sequence identity range of 74-99% between *M. marinum* and *M. tuberculosis* for all TCSs proteins in this study. Fifteen of the twenty-two proteins showed higher than the average 85% maximum coding sequence amino acid identity between orthologous regions of the genomes.\[326\].

The genomic context studies are summarised in Table 5.2, with scoring according to similarity of the genomic environment for 6 flanking genes in the upstream and downstream locations for the TCSs in each organism. The analysis revealed that PdtAR is in the same genomic context between the two organisms, eight TCSs have few to moderate differences in the genomic context, and three TCSs are in largely different genomic contexts.
Table 5.1 Coding sequence amino acid identities of selected TCSs proteins between *M. tuberculosis* and *M. marinum*.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Length in <em>M. tuberculosis</em></th>
<th>Length in <em>M. marinum</em></th>
<th>Coding sequence amino acid identity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DosR</td>
<td>217</td>
<td>216</td>
<td>90</td>
</tr>
<tr>
<td>DosS</td>
<td>578</td>
<td>585</td>
<td>79</td>
</tr>
<tr>
<td>KdpD</td>
<td>860</td>
<td>839</td>
<td>82</td>
</tr>
<tr>
<td>KdpE</td>
<td>226</td>
<td>226</td>
<td>85</td>
</tr>
<tr>
<td>MprA</td>
<td>228</td>
<td>232</td>
<td>95</td>
</tr>
<tr>
<td>MprB</td>
<td>504</td>
<td>511</td>
<td>86</td>
</tr>
<tr>
<td>MtrA</td>
<td>228</td>
<td>225</td>
<td>99</td>
</tr>
<tr>
<td>MtrB</td>
<td>567</td>
<td>559</td>
<td>92</td>
</tr>
<tr>
<td>NarL</td>
<td>216</td>
<td>216</td>
<td>94</td>
</tr>
<tr>
<td>Rv0845</td>
<td>422</td>
<td>402</td>
<td>76</td>
</tr>
<tr>
<td>PdtaR</td>
<td>205</td>
<td>205</td>
<td>93</td>
</tr>
<tr>
<td>PdtaS</td>
<td>501</td>
<td>499</td>
<td>85</td>
</tr>
<tr>
<td>PhoP</td>
<td>247</td>
<td>240</td>
<td>96</td>
</tr>
<tr>
<td>PhoR</td>
<td>485</td>
<td>481</td>
<td>74</td>
</tr>
<tr>
<td>PrrA</td>
<td>236</td>
<td>236</td>
<td>97</td>
</tr>
<tr>
<td>PrrB</td>
<td>446</td>
<td>449</td>
<td>95</td>
</tr>
<tr>
<td>RegX3</td>
<td>227</td>
<td>227</td>
<td>99</td>
</tr>
<tr>
<td>SenX3</td>
<td>410</td>
<td>408</td>
<td>84</td>
</tr>
<tr>
<td>TcrX</td>
<td>234</td>
<td>235</td>
<td>94</td>
</tr>
<tr>
<td>TcrY</td>
<td>475</td>
<td>504</td>
<td>81</td>
</tr>
<tr>
<td>TrcR</td>
<td>257</td>
<td>254</td>
<td>91</td>
</tr>
<tr>
<td>TrcS</td>
<td>509</td>
<td>514</td>
<td>79</td>
</tr>
</tbody>
</table>

Protein sequence information was sourced from TubercuList and MarinoList, with amino acid identities calculated from NCBI BLAST searches.
Figure 5.1. Coding sequence amino acid alignments and genomic context views of DosR/S/T TCSs proteins in *M. tuberculosis* and *M. marinum*. (A-C) Coding sequence alignments of TCSs proteins to be studied, created using ClustalX2. Mtb = *M. tuberculosis* sequence, and Mmar = *M. marinum* sequence. * = fully conserved residue, : = residue conserved within a group of residues highly similar in their properties, . = residue conserved within a group of residues moderately similar in their properties. (A-B) Coding sequence alignments of DosR (A) and DosS (B) from both species. The second DosR homologue in *M. marinum*, MMAR_3480, is also shown in (A). (C) Coding sequence alignment of DosS and DosT of *M. tuberculosis*. (D) Genomic context view of DosR/S operon, created in Geneious. The *M. tuberculosis* sequence is shown first. Yellow = TCSs genes, blue = homologous genes between the two organisms, red = different genes between the two organisms. For clarity, the gene name prefix “MMAR_” has been omitted from the *M. marinum* genomic views. The red bar indicates the position in the *M. tuberculosis* sequence of the additional gene in the *M. marinum* sequence (MMAR_1520). The green bar indicates the position in the *M. marinum* sequence of the additional gene in the *M. tuberculosis* sequence (Rv3128c).

Table 5.2. Summary of TCSs genomic context comparisons between *M. tuberculosis* and *M. marinum*.

<table>
<thead>
<tr>
<th>TCSs</th>
<th>Genomic context similarity score</th>
<th>Upstream genomic context</th>
<th>Downstream genomic context</th>
</tr>
</thead>
<tbody>
<tr>
<td>PdtaR</td>
<td>+++</td>
<td>Identical</td>
<td>Identical</td>
</tr>
<tr>
<td>MtrA/B</td>
<td>+++</td>
<td>2 gene insertion in Mtb</td>
<td>Identical</td>
</tr>
<tr>
<td>PdatS</td>
<td>+++</td>
<td>1 gene insertion Mtb</td>
<td>Identical</td>
</tr>
<tr>
<td>PrrA/B</td>
<td>+++</td>
<td>6 gene insertion Mmar</td>
<td>Identical</td>
</tr>
<tr>
<td>RegX3/SenX3</td>
<td>+++</td>
<td>1 gene insertion Mtb</td>
<td>Identical</td>
</tr>
<tr>
<td>DosR/S</td>
<td>++</td>
<td>1 gene difference</td>
<td>Single gene insertion in both genomes</td>
</tr>
<tr>
<td>MprA/B</td>
<td>++</td>
<td>3 gene and 1 gene insertion in Mtb</td>
<td>4 gene insertion in Mtb</td>
</tr>
<tr>
<td>NarL/Rv0845</td>
<td>++</td>
<td>1 gene difference, 1 gene insertion in Mmar</td>
<td>10 gene insertion in Mtb</td>
</tr>
<tr>
<td>PhoP/R</td>
<td>++</td>
<td>Flanking genes differ after first 2 genes</td>
<td>2 gene and 1 gene insertion in Mmar</td>
</tr>
<tr>
<td>KdpD/E</td>
<td>+</td>
<td>Kdp operon in different orientations, flanking genes differ after operon</td>
<td>Flanking genes differ</td>
</tr>
<tr>
<td>TcrX/Y</td>
<td>+</td>
<td>Flanking genes differ</td>
<td>Flanking genes differ after first 2 genes</td>
</tr>
<tr>
<td>TrcR/S</td>
<td>+</td>
<td>Flanking genes differ after first 3 genes</td>
<td>Flanking genes differ</td>
</tr>
</tbody>
</table>

The genomic environment of TCSs were compared between *M. tuberculosis* and *M. marinum* for the first six upstream and downstream flanking genes using Tuberculist and MarinoList. Scoring for similarity of the genomic environment was: ++++ = identical genomic environments; +++ = highly similar genomic environments (one flank identical); ++ = moderately similar genomic environments (some changes to both flanks); + = little similarity between the genomic environments (major differences to flanking regions).
5.3.2 Attempted generation of *M. marinum* ΔTCSs operon mutants by mycobacteria-adapted recombineering

5.3.2.1 Overview

We attempted to knock-out entire TCSs systems in *M. marinum* by mycobacteria-adapted recombineering\[^{296, 339, 340}\], with the intention of deleting both the response regulator and sensor kinase genes to provide a clean removal of all components of the regulatory system. This would allow the investigation of the role of the TCSs in mycobacterial survival *in vitro* and *in vivo* using the zebrafish embryo model. There are no reports of mycobacteria-adapted recombineering being used previously to construct mutants in *M. marinum*, although it has been successfully used in related species (*M. smegmatis* and *M. tuberculosis*)\[^{339}\]. We wished to use recombineering as it is much faster and easier than methods previously used for mutant construction in *M. marinum*, such as sucrose counter-selection, and it also allows the generation of defined in-frame deletions. If recombineering was successful in *M. marinum*, it would make genetic manipulation of this organism much easier, as occurred with *M. tuberculosis*.

Recombineering relies on the expression of mycobacteriophage-encoded proteins that increase the rate of homologous recombination\[^{339}\]. Introducing the helper plasmid pJV53 by transformation, followed by induction of the expression of the phage proteins by addition of acetamide, and then introducing an allelic exchange substrate (AES) by a second transformation event leads to the loss of the desired genomic region through targeted recombination events (Figure 5.2). The AES consists of a hygromycin resistance cassette flanked by \(~1\) Kb upstream and \(~1\) Kb downstream of the region to be knocked out of the *M. marinum* genome.
Figure 5.2. Schematic showing the recombineering method. (1) Upstream and downstream homologous regions, each with a 3 codon-overlap to the targeted gene/operon, are amplified from the target genome locus using primers 1-2 and 3-4. (2) These homologous regions (“mutator” fragments) are inserted to a cloning vector, pCR-Blunt of the Zero Blunt Cloning Kit, for further amplification and sequence-verification. (3) Sequenced fragments are then sub-cloned into the destination vector pYUB854 in the desired orientations either side of the hygromycin resistance cassette. This generates the allelic exchange substrate (AES), which consists of the two flanking homology regions and the resistance cassette. (4) M. marinum is transformed with the helper plasmid pJV53/pJV53ApR. (5) Following induction of the phage recombination-promoting proteins gp60 and gp61 by 0.2% acetamide for one doubling time (5h for M. marinum), competent cells containing the expressed phage proteins are prepared. (6) The linearised AES is generated by restriction enzyme digest of pYUB854::AES. (7) The competent cells from step (5) are transformed with 0.1 µg of linearised AES. Following recovery, the M. marinum chromosome will contain the hygromycin resistance cassette in place of the targeted region, in this case a TCSs operon, due to targeted homologous recombination events. Grey dashed lines guide visualisation of the cloning strategy to generate the linearised AES, blue dashed lines denote homologous recombination events, and black dashed lines mark the external boundaries of the AES flanking regions on the M. marinum chromosome.

5.3.2.2 Mycobacteria-adapted recombineering

The selected TCSs are arranged in operons and co-transcribed, with the exception of the PdtaR/S system which is separated in the M. marinum genome. The DosR operon also encodes a universal stress protein (USP), whose function we would like to take the opportunity to investigate. Therefore, primers (Section 2.1.2, Table 2.4) were designed
to amplify the upstream and downstream flanking regions required for 13 AES (PdtAR, PdtAS, DosR/S, DosR/S + USP, and the 9 other TCSs operons). Twenty-five of the 26 flanking sequences were successfully cloned. The remaining region, the upstream flank for TrcR/S, could not be successfully amplified by standard or asymmetric PCR. We therefore continued with experiments to target the remaining 12 TCSs sequences.

Recombineering was initially trialled during my MRes rotation with the DosR/S + USP AES only (Round 1), with all subsequent rounds being performed during the PhD project. Table 5.3 summarises the conditions used for each of the rounds of recombineering undertaken during this PhD project. Following the failure of Round 5 of recombineering we continued attempts to generate the desired ΔTCSs strains in *M. marinum* by sucrose counter-selection (Section 5.3.3). During the course of this work, valuable input on the conditions required for successful transformation of *M. marinum* and deletion mutant selection was provided by Dr Galina Mukamolova. We therefore returned to mycobacteria-adapted recombineering for Rounds 6-7.
Table 5.3. Summary of recombineering rounds undertaken during this project.

<table>
<thead>
<tr>
<th>Round</th>
<th>Strain background</th>
<th>Concentration acetamide (%)</th>
<th>Induction time (hours)</th>
<th>Competent cells</th>
<th>AES amount (µg)</th>
<th>AES</th>
<th>Growth and recovery temperature (°C)</th>
<th>Recovery time</th>
<th>Agar plate</th>
<th>Number of colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>London WT</td>
<td>0.2</td>
<td>5</td>
<td>Fresh</td>
<td>0.1</td>
<td>DosR/S + USP</td>
<td>28.5</td>
<td>5</td>
<td>7H11</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>London GFP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>London DsRed</td>
<td></td>
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<td></td>
<td></td>
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<td>3</td>
</tr>
<tr>
<td>2</td>
<td>London WT</td>
<td>0.2</td>
<td>5</td>
<td>Frozen</td>
<td>0.1</td>
<td>DosR/S + USP</td>
<td>28.5</td>
<td>5</td>
<td>7H11</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>London GFP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>DosR/S + USP</td>
<td>28.5</td>
<td>5</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>London DsRed</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>DosR/S + USP</td>
<td>28.5</td>
<td>5</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>London WT</td>
<td>0.2</td>
<td>5</td>
<td>Frozen</td>
<td>0.1</td>
<td>DosR/S + USP</td>
<td>28.5</td>
<td>5</td>
<td>7H11</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>London GFP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>RegX3/SenX3/TcrX/Y</td>
<td>28.5</td>
<td>5</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>London DsRed</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>RegX3/SenX3/TcrX/Y</td>
<td>28.5</td>
<td>5</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>London WT</td>
<td>0.20, 0.15, 0.10, 0.05 and 0.00</td>
<td>1, 3 and 5</td>
<td>Fresh</td>
<td>0.05, 0.10, 0.25, 0.50 and 1.00</td>
<td>DosR/S + USP</td>
<td>28.5</td>
<td>5</td>
<td>7H11</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>London GFP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>RegX3/SenX3/TcrX/Y</td>
<td>28.5</td>
<td>5</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>London DsRed</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>RegX3/SenX3/TcrX/Y</td>
<td>28.5</td>
<td>5</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>London WT</td>
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<td>5</td>
<td>Fresh</td>
<td>0.1</td>
<td>All 12 AES</td>
<td>28.5</td>
<td>5</td>
<td>7H11</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
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<tr>
<td></td>
<td>London DsRed</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>6</td>
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<td>0.2</td>
<td>5</td>
<td>Fresh, concentrated</td>
<td>0.1</td>
<td>DosR/S + USP</td>
<td>28.5</td>
<td>5</td>
<td>7H11</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Leicester WT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PhoP/R RegX3/SenX3</td>
<td>28.5</td>
<td>5</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Round</td>
<td>Description</td>
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</tr>
<tr>
<td>1</td>
<td>All three <em>M. marinum</em> parent strains (WT, GFP and DsRed) and the DosR/S + USP AES constructed by Faye Rodgers were utilised.</td>
<td>0.2</td>
<td>7H10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-3</td>
<td>Rounds 2 and 3 utilised competent cells from a single batch prepared prior to Round 2. <strong>Round 4:</strong> To identify the optimal protocol for recombineering in <em>M. marinum</em>, the concentration of acetamide for pJV53 protein expression induction and the amount of AES during transformation were varied. Reduced acetamide concentrations and induction times were trialled as reduced strain growth occurred in both prior attempts to prepare induced pJV53-containing competent cells following acetamide addition. An acetamide concentration of 0% was included in the event that leaky expression of the recombineering proteins from pJV53 occurs in <em>M. marinum</em>. The AES concentrations were varied in transformations of cells which had been induced with 0.2% acetamide and not induced. For other acetamide concentrations, the published quantity of AES (0.1 µg) was used. As no colonies were obtained for any of the trialled conditions, the published acetamide concentration and AES amount were used in all further experiments. <strong>Round 5:</strong> To confirm that essentiality of the TCSs targeted thus far had not resulted in the failure of recombineering, all 12 AES which had been successfully constructed, each targeting a different locus, were used. <strong>Round 6:</strong> To eliminate strain differences as the cause of our difficulties, the strain of <em>M. marinum</em> used by Dr Mukamolova’s group at Leicester University, denoted “Leicester”, was trialled alongside our own <em>M. marinum M</em> strain, denoted “London”. <strong>Round 7:</strong> Recommendations provided by Dr Mukamolova for deletion mutant generation were applied to recombineering: longer protein induction times to compensate for reduced strain growth in practice compared to published doubling times, freshly prepared competent cells for each round which were 10x concentrated compared to previous rounds, increased AES quantities in transformation reactions, low and high temperatures for cell growth and recovery throughout the recombineering process, longer cell recovery times following transformations, and selection on 7H10 plates following transformations for up to one month (previously two weeks on 7H11 plates which contain increased malachite green had been used). No colonies were obtained using the lower AES quantity.</td>
<td>5 and 12</td>
<td>Fresh, concentrated</td>
<td>0.1 and 1.0</td>
<td>DosR/S + USP DosR/S MprA/B PhoP/R RegX3/SenX3 TcrX/Y</td>
<td>32</td>
<td>16</td>
<td>16x DosR/S + USP 22x DosR/S 18x MprA/B 18x PhoP/R 0x RegX3/SenX3 17x TcrX/Y 24x DosR/S + USP 25x DosR/S 24x MprA/B 18x PhoP/R 22x RegX3/SenX3 14x TcrX/Y</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
5.3.2.3 Characterisation of putative TCSs deletion mutant strains: Overview

Putative ΔTCSs operon mutants were characterised by PCR to confirm the deletions. Four sets of PCR primers were designed for each TCSs targeted to probe the region of interest, including the genomic context (Figure 5.3). An additional set were designed for the DosR/S system. The PCRs to investigate the presence of hygromycin in the appropriate genomic context would be sufficient to identify a mutant (primers 3-4 and 5-6). However, as there can be no positive control for these reactions, additional PCR primers were designed to amplify the entire genomic locus in the event that the first PCRs yielded no product. In most instances, the entire region PCRs would provide products for a mutant and WT strain of different size (Section 2.1.2, Tables 2.7 and 2.8).

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**Figure 5.3. Schematic of putative TCSs deletion mutant characterisation PCRs.** HygR = hygromycin resistance cassette, UR = upstream homology region, and DR = downstream homology region. Numbered arrows represent primers used in PCR characterisation with the expected product length displayed with double arrows. Primers displayed above the *M. marinum* chromosome diagram are used for all putative TCSs deletion mutants, those below (7 and 8) were designed and used for characterisations of putative DosR/S + USP mutants only. Primers designed within the hygromycin resistance cassette (1, 2, 4 and 5) are shared for all TCSs, whereas the remaining primers in the respective genomic environments are unique for each TCSs (3, 6, 7, and 8). Primers 1 and 2 are the internal hygromycin cassette characterisation primers, which provide a 521 bp product if the cassette has integrated into the *M. marinum* genome. Primers 3 and 4, and 5 and 6, are the upstream and downstream hygromycin cassette-genome characterisation primers, which provide an ~1500 bp product if the cassette has integrated into the desired location in the *M. marinum* genome (product sizes provided in Table 2.7). When used together, primers 3 and 6 are the full locus characterisation primers, and provide products of different sizes over the whole locus for the respective TCSs deletion mutant or WT strain (product sizes provided in Table 2.7). Primers 7 and 8 are located within the flanking regions of the DosR/S + USP AES, which provide a product of 2019 bp for the AES and 3297 bp for the WT TCSs operon, and therefore indicate whether this AES has integrated into the *M. marinum* genome when gDNA is used as the template for the PCR. The primer numbers used in this figure are shown in Table 2.8 to identify the respective primers for each TCSs targeted.
In addition to the above primers, others were also designed to amplify 540 bp of a gene which is specific to *M. marinum* to confirm that the colonies obtained were the strain of interest (gene MMAR_2865, a putative oxygenase of unknown function). The need for these *M. marinum* confirmation primers (Table 2.4) was indicated by a previous attempt by the Williams group to generate a ΔDosR/S + USP strain that yielded colonies which were similar in appearance to *M. marinum* but were later found to be a contaminant. PCRs using these primers were found to be very reliable and were used throughout this PhD to confirm that *M. marinum* gDNA samples were of sufficient quality for use as templates in PCR reactions.

5.3.2.4 Characterisation of putative TCSs deletion mutant strains obtained through initial rounds of recombineering

Three putative ΔDosR/S + USP colonies in the *M. marinum* DsRed background were obtained during Round 1 of recombineering. Of these, two colonies were successfully grown on and three purified colonies stored for each for later characterisation during the PhD project (6 colonies in total). All 6 colonies obtained in the *M. marinum* DsRed background for both RegX3/SenX3 and TcrX/Y AES in Round 3 were successfully grown on, with one purified colony for each stored for further testing (12 colonies in total). These 18 putative ΔTCSs operon mutants were characterised by PCR using the primers detailed above.

Initial attempts to characterise the putative ΔTCSs operon mutants by colony PCR using the *M. marinum* confirmation and internal hygromycin cassette primers were unsuccessful, with no products obtained. As *M. marinum* is similar to *M. tuberculosis*, in which it is difficult to obtain a product from colony PCRs, gDNA was prepared for each putative ΔTCSs operon mutant. The PCR attempts using gDNA and both the *M. marinum* confirmation and the internal hygromycin cassette primers (primers 1-2, Figure 5.3) were successful. With the internal hygromycin cassette primers, high intensity product bands were seen for the putative ΔDosR/S + USP strains but putative mutants for the other strains displayed products of a similar intensity to the negative control PCRs using WT gDNA and water (Figure 5.4A,B). Elimination of these nonspecific products was attempted by use of different PCR buffers and stocks but was unsuccessful. In light of
these characterisation PCR results, experiments with the putative ΔRegX3/SenX3 and ΔTcrX/Y strains were discontinued.

Further characterisation PCRs were performed for the putative ΔDosR/S + USP mutant strains using the extracted gDNA samples as the template. PCRs with the hygromycin-genome primers were inconclusive in that low intensity products were obtained in some PCR reactions but these data were not replicable (data not shown). PCRs were therefore performed for all of the putative mutant strains with the genomic orientation primers (primers 3 and 6, Figure 5.3). Despite repeated attempts and gradient PCRs, no products were obtained for the genomic orientation PCRs with gDNA samples for the putative ΔDosR/S + USP strains or WT positive control (data not shown).

To characterise the putative ΔDosR/S + USP strains, additional primers were designed within the flanking regions of the AES (primers 7-8, Figure 5.3), which had been successfully amplified by PCR previously. PCRs using these primers and the gDNA samples as templates displayed an unexpected result – product bands for both the mutant and WT strains were obtained for each of the 6 isolates (data not shown). Attempts to purify the mutant strain from the WT background were made by needle homogenisation of overnight liquid cultures to provide a single cell suspension and dilution plating of the cell suspensions. Repeat PCRs using extracted gDNA samples following two rounds of purification demonstrated the same mixed products result (Figure 5.4C).

After the third round of purification a separation occurred, with each of the six original colonies providing red colonies and cream coloured colonies, which are assumed to be due to continued carriage and loss of the DsRed plasmid, respectively. Two colonies of both colours were grown on for each original colony, and then gDNA prepared for two single colonies from each from the onwards growth for testing (from 6 colonies to 24 colonies each for DsRed and WT background strains). To confirm whether separation of a ΔDosR/S + USP strain from a WT background had been achieved, the 48 gDNA samples for colonies with differential DsRed plasmid carriage were tested for the presence of the hygromycin cassette using the internal hygromycin primers (primers 1-2, Figure 5.3). The PCRs revealed that all strains carried the hygromycin cassette, Figure 5.5 shows the results for the first twelve colonies for each strain background.
Representative purified strains and parent strains of putative ΔDosR/S + USP mutants were further characterised by Southern Blotting (Section 5.3.4).

**Figure 5.4. Characterisation PCRs for putative TCSs deletion mutants.** (A-B) PCRs using internal hygromycin cassette primers, expected product if cassette present in genome = 521 bp. Positive control = pYUB854 AES parent vector (pYUB), negative controls = WT gDNA and water. All PCRs were performed using the same mastermix, the control reactions were halved and run on both gels for ease of reference. Characterisation PCRs for the putative ΔDosR/S + USP and ΔRegX3/SenX3 strains are shown in (A), with PCRs for the putative ΔTcrX/Y strains shown in (B). (C) PCRs using flanking region genome locus primers for ΔDosR/S + USP characterisations, expected product sizes are 2019 bp for ΔDosR/S + USP and 3297 bp for WT. Positive control = WT gDNA, negative control = water. The test sample for ΔDosR/S + USP 3 did not grow in liquid culture and was therefore not used. PCRs were performed using gDNA samples from individual colonies for the independent putative mutant strains from each TCSs AES transformation in (A-B). PCRs in (C) were performed using gDNA samples for putative ΔDosR/S + USP strains following two rounds of purification by needle homogenisation and dilution plating.

The colonies obtained in recombineering Round 7 from transformation of the London and Leicester *M. marinum* strains were successfully grown on and stored for analysis. gDNA samples were prepared for the putative ΔDosR/S and ΔDosR/S + USP strains only for characterisation by PCR. PCRs using the *M. marinum* confirmation primers were successful and the gDNA prepared confirmed as a suitable template for PCRs. PCRs were also attempted with the internal hygromycin resistance cassette primers, with the
positive control reaction of the parent vector providing the expected product, but no products seen with the samples. Example results are provided in Figure 5.6 for DosR/S AES colonies 14-22 in the London strain background. No further characterisations were undertaken and recombineering experiments were discontinued once more.

Figure 5.5. Characterisation PCRs for putative *M. marinum* ΔDosR/S + USP mutants. (A-B) PCRs using internal hygromycin cassette primers, expected product if cassette present in genome = 521 bp. Positive controls = pYUB854 AES parent vector (pYUB) and sucrose counter-selection round 5 DosR/S single crossover 5 (SCS Rnd 5, see Section 5.4). Negative control = WT gDNA. Test PCRs were performed using gDNA samples for putative ΔDosR/S + USP strains obtained during Round 1 of mycobacteria-adapted recombineering following three rounds of purification by needle homogenisation and dilution plating, and single colony purification through restreaking. Gel in (A) displays results for colonies obtained which carry the DsRed plasmid, and gel in (B) displays results for colonies obtained which had lost the DsRed plasmid and returned to the WT *M. marinum* cream colouring.

Figure 5.6. Characterisation PCRs for putative *M. marinum* ΔDosR/S mutants. PCRs using internal hygromycin cassette primers, expected product if cassette present in genome = 521 bp. Positive control = pYUB854 AES parent vector (pYUB), negative controls = WT gDNA and water. Test PCRs performed using gDNA samples for putative ΔDosR/S colonies (numbers 14-22) in the London *M. marinum* M strain background obtained during Round 7 of mycobacteria-adapted recombineering are shown.
5.3.2.5 Investigation of the efficacy of recombineering in *M. marinum*

The failure to generate ΔTCSs mutants in *M. marinum* was investigated by comparing the effects of pJV53 introduction and induction of protein expression on cell growth and transformation efficiency. To inform on the efficacy of recombineering in *M. marinum*, these investigations were also performed with the related species *M. smegmatis*, in which recombineering to generate defined deletion mutants has previously been successful in our laboratory (unpublished data).

5.3.2.5.1 Investigation of the effect of pJV53 carriage and induction of protein expression on *M. marinum* growth

The observed slow growth of strains upon induction with acetamide was further investigated. To test the effect of acetamide induction of protein expression on the growth and survival of *M. marinum*, the growth of *M. smegmatis* and *M. marinum* WT and recombineering strains was monitored in the presence and absence of acetamide induction (Figure 5.7).

Average growth of both *M. smegmatis* and *M. marinum* WT strains was observed to be affected in the presence of pJV53 and 0.2% acetamide throughout the experiments, although statistical significance was not always reached. *M. smegmatis* WT growth was significantly reduced from 32 to 44 hours by the presence of 0.2% acetamide in the growth medium and also from 24 to 48 hours by the presence of pJV53 when incubated both with and without 0.2% acetamide (Figure 5.7A). Growth of *M. marinum* WT was significantly reduced at 96 and 144 hours by the presence of pJV53, whilst the addition of acetamide significantly increased growth of *M. marinum* WT from 96-120 hours and growth of *M. marinum* WT pJV53 from 120-144 hours (Figure 5.7B).

Whilst average growth of *M. marinum* WT was not strongly affected by carriage of pJV53ApR, growth of GFP and DsRed strains were observed to be affected in the presence of pJV53ApR throughout the experiments, although statistical significance was not always reached. Growth of *M. marinum* WT was not significantly affected by the presence of pJV53ApR at any time point (Figure 5.7C). The effects of the introduction of a second plasmid to the *M. marinum* GFP and DsRed strains were more striking than the
effects of carriage of pJV53 on *M. marinum* WT. In both cases, the presence of pJV53ApR significantly reduced growth of the strains from 96 to 144 hours (Figure 5.7D,E). It is worthy of note that the growth of the *M. marinum* GFP and DsRed strains carrying pJV53ApR was extremely adversely affected for a number of the six biological replicates: three cultures each for *M. marinum* GFP pJV53ApR and *M. marinum* DsRed pJV53ApR displayed cell death and lysis during the course of the experiment, returning values for OD$_{600}$ by 144 hours of zero. In addition, three cultures of *M. marinum* DsRed pJV53ApR grown in the presence of acetamide also died during the experiment.

In agreement with the data for WT *M. marinum* and WT *M. marinum* carrying pJV53, the presence of acetamide in the growth medium increased the average growth of *M. marinum* WT pJV53ApR, although the effect was not statistically significant (Figure 5.7C). In contrast to the results with the WT *M. marinum* strains, incubation with acetamide had no effect on growth of the GFP and DsRed parent strains at any time point (Figure 5.7D,E). Incubation of *M. marinum* GFP pJV53ApR with 0.2% acetamide resulted in significantly increased growth between 124-144 hours, which reflects the trends seen for the WT *M. marinum* strains. Incubation of *M. marinum* DsRed pJV53ApR with 0.2% acetamide did not significantly affect growth during the experiment.
Figure 5.7. Growth of *M. smegmatis* (Msm) and *M. marinum* (Mmar) WT and recombineering strains during incubation with acetamide (ac). (A-B) Growth of *M. smegmatis* (A) and *M. marinum* (B) WT and pJV53-containing strains with and without incubation with acetamide. (C-E) Growth of *M. marinum* WT (C), GFP (D) and DsRed (E) background strains with or without pJV53ApR presence and with and without acetamide incubation. Data for the WT strain in the absence of pJV53ApR is repeated from graph (B). *M. marinum* strains containing pJV53ApR were used during recombineering throughout this PhD. Acetamide was added once cultures had reached mid-log phase, which corresponds to the first time point on the graphs – growth up to this point occurred in the absence of acetamide for all groups. For the Msm strains, experiments were performed in triplicate using three biological replicates, and each data point represents the pooled 9 replicates. For the Mmar strains, experiments with one biological replicate for all of the strains were performed six times, and each data point represents the pooled 6 replicates. Repeated measures two-way ANOVA analysis with Bonferroni’s post-test. *p<0.05, **p<0.01, ***p<0.001. Black asterisks indicate significance in relation to parent strain background (without plasmid and acetamide addition), whilst blue asterisks indicate significance between plasmid-containing strains upon acetamide addition.
5.3.2.5.2 Investigation of the effect of pJV53 carriage on transformation efficiency of M. marinum

A low transformation efficiency of M. marinum cells after the induction of protein expression from pJV53 would reduce the success of recombineering. The transformation efficiency of the recombineering strains was therefore investigated using pSMT3, an expression vector which is known to transform well into mycobacterial cells. The competent cells used were WT M. smegmatis and M. marinum strains with and without both pJV53 carriage and induction of protein expression by 0.2% acetamide.

All bacterial strains used had comparable transformation efficiencies for WT cells (Figure 5.8F). Effects on the average transformation efficiency of cells upon addition of pJV53 or pJV53ApR to cell or acetamide to the growth medium were seen for all mycobacterial strains tested, although the large variations in the efficiencies for individual cultures led to a lack of statistical significance for these changes throughout. The average transformation efficiency of the M. smegmatis cells increased 23- to 33-fold upon carriage of pJV53 or incubation with 0.2% acetamide for one doubling time (3 hours, Figure 5.8B). The average transformation efficiency of the M. marinum WT cells increased slightly by 1.25-fold when carrying pJV53. In contrast to the M. smegmatis strain, the average transformation efficiencies of M. marinum WT cells fell slightly upon incubation with acetamide for one doubling time (5 hours) and this fall was more pronounced, at 5-fold, for the strain carrying pJV53 (Figure 5.8B). In contrast to the effects of addition of pJV53 on WT M. marinum cells, addition of pJV53ApR resulted in a 10-fold decrease in the average transformation efficiency which was increased a further 2-fold by incubation with acetamide (Figure 5.8C). Addition of pJV53ApR to both M. marinum GFP and DsRed strains also led to a reduction in transformation efficiency of 3- to 5-fold, which was not significantly affected by incubation with 0.2% acetamide (Figure 5.8D,E).
Figure 5.8. Transformation efficiencies of *M. smegmatis* (Msm) and *M. marinum* (Mmar) strains. 

(A-B) Normalised transformation efficiencies of *M. smegmatis* (A) and *M. marinum* (B) WT and pJV53-containing stains with and without incubation with acetamide (ac). (C-E) Normalised transformation efficiencies of *M. marinum* WT (C), GFP (D) and DsRed (E) background strains with or without pJV53ApR presence and with and without acetamide (ac) incubation. Data for the WT strain in the absence of pJV53ApR is repeated from graph (B). (F) Transformation efficiencies for the parent strains used in these experiments. Acetamide was added once cultures had reached mid-log phase and competent cells were prepared following incubation for one doubling time (3 hours for Msm and 5 hours for Mmar). All strains were transformed with 0.1 µg of pSMT3. Transformation efficiencies were calculated as the number of colonies obtained per transformation reaction divided by the quantity of DNA used in µg, and then normalised to the background strain without addition of acetamide for each graph in (A-E). For the Msm strains, transformation reactions were performed in duplicate for three biological replicates per condition, with experiments performed in triplicate. For the Mmar strains, transformation reactions were performed in duplicate for one sample per condition, with experiments performed eight times. Each bar represents the pooled 18 replicates for Msm and 16 replicates for Mmar strains. Statistical analysis by one-way ANOVA with Bonferroni’s post-test revealed no significant differences between the raw transformation efficiencies when compared to the untreated respective strain background values.
5.3.2.5.3 Investigation of the rate of loss of pJV53 from cells

To investigate the possibility that the carriage of pJV53 itself bestows a fitness cost to cells, the rate of loss of pJV53 on passage in liquid media containing no antibiotic selection was compared between *M. marinum* and *M. smegmatis*. Cell pellet samples were taken from cultures at each passage stage, with the presence of pJV53 verified by colony PCR. The colony PCRs confirmed that pJV53 was lost from *M. marinum* strains during passage 3 but had been maintained in *M. smegmatis* strains until at least passage 6 (Figure 5.9).

![Figure 5.9. Characterisation colony PCRs to compare rate of loss of pJV53 through passaging from *M. smegmatis* and *M. marinum*. (A-B) PCRs using primers to amplify gp60, which is carried on pJV53, expected product if plasmid present = 883 bp. Positive control = pJV53, negative control = *M. marinum* WT colony. Passages (P) without antibiotic selection were undertaken for three biological replicates per strain, with a new passage once cultures reached stationary phase. At each passage step, a sample cell pellet was taken and stored frozen for later analysis. Each replicate is shown in the same well position from L to R for each passage. Experiment was discontinued at the seventh passage due to contamination of 2 *M. marinum* and 1 *M. smegmatis* cultures. All cell pellets for passages 1-6 for *M. marinum* (A) and *M. smegmatis* (B) were tested by colony PCR using the same master mix – control reactions apply to PCRs undertaken for both strains.](image-url)
5.3.3 Attempted generation of *M. marinum* ΔTCSs operon mutants by sucrose counter-selection

5.3.3.1 Overview

Sucrose counter-selection is an alternative method for the construction of targeted deletion mutants in mycobacteria and has previously been used by several groups to construct mutants in *M. marinum* by homologous recombination\[^{[59, 60, 87, 111, 269, 304]}\]. Each group used a different vector for the delivery of the sucrose-sensitive *sacB* gene, with the suicide vector pSMT100 selected for use in this study as it has been used for the construction of mutants in mycobacteria previously by our group and others\[^{[28, 40, 84, 213, 227, 312]}\].

Sucrose counter-selection relies on loss of the desired genomic region through targeted recombination events caused by introduction of a mutagenic construct containing a sucrose-sensitive marker, the *sacB* gene (Figure 5.10). The AESs which had been constructed for mycobacteria-adapted recombineering were used to generate the mutagenic construct by transfer of the AES from the pYUB854::AES constructs to pSMT100. The transfer of all 12 AES was attempted in a single experiment. Transfer to pSMT100 was successful for 7 AES: DosR/S, DosR/S + USP, MprA/B, MtrA/B, PhoP/R, RegX3/SenX3, and TcrX/Y. As the successful cloning of these 7 AES would allow the targeting of 6 separate loci in the *M. marinum* genome, we proceeded to sucrose counter-selection attempts as all 6 TCSs would not be expected to be essential. In the event that sucrose counter-selection was successful for these 7 AES, the remaining AES could be transferred to pSMT100.
5.3.3.2 Sucrose counter-selection

All rounds of sucrose counter-selection performed during this PhD are summarised in Table 5.4. The first attempts, Rounds 1-3, were performed using all three *M. marinum* strain backgrounds and all were unsuccessful. The continued failure to generate the desired deletion mutants led us to seek the advice of Dr Galina Mukomolova at Leicester
University, following her successes with the p2NIL/pGOAL19 system\textsuperscript{[247]} for sucrose counter-selection in \textit{M. marinum} (Dr Galina Mukamolova, personal communication). Rounds 4-8 were then performed incorporating her advice for increasing the transformation efficiencies of \textit{M. marinum} cells to maximise the chances of generating the desired deletion mutants. Dr Mukamolova also kindly provided a sample of the \textit{M. marinum} M strain used by her group, denoted “Leicester”, which was also used in several rounds in this PhD to eliminate strain differences as the cause of our difficulties. Several of these rounds generated colonies of putative single crossover strains for further characterisation in both the London and Leicester WT strain backgrounds, which is described in the next section.

Attempts to generate the putative ΔTCSs mutants in \textit{M. marinum} by sucrose counter-selection were discontinued following Round 8 as there was insufficient time remaining during the PhD to study any mutant strains generated.

The failure to generate ΔTCSs mutants in the London \textit{M. marinum} M laboratory strain was investigated by comparing cell growth and transformation efficiency with the Leicester laboratory in parallel to sucrose counter-selection Rounds 5-8. As \textit{M. marinum} is maintained at 32\textdegree C by the Leicester group and at 28.5 \textdegree C by ourselves in London, the growth and transformation efficiencies of both \textit{M. marinum} M strains were investigated at these temperatures. We observed no effect of \textit{M. marinum} laboratory strain or growth temperature on the average growth and transformation efficiencies (Appendix 5) and therefore on the efficacy of sucrose counter-selection.
Table 5.4. Summary of sucrose counter-selection rounds undertaken during this project.

<table>
<thead>
<tr>
<th>Round</th>
<th>Strain background</th>
<th>Competent cells</th>
<th>Plasmid DNA amount (µg)</th>
<th>pSMT100::AES used</th>
<th>Growth and recovery temperature (°C)</th>
<th>Recovery time (hours)</th>
<th>Agar plate</th>
<th>Colonies obtained</th>
</tr>
</thead>
<tbody>
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<td>1</td>
<td>London WT</td>
<td>Fresh</td>
<td>0.5</td>
<td>DosR/S PhoP/R RegX3/SenX3</td>
<td>28.5</td>
<td>4</td>
<td>7H11, Hyg only</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>London GFP</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>London DsRed</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>2</td>
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<td>0.5</td>
<td>DosR/S PhoP/R RegX3/SenX3</td>
<td>28.5</td>
<td>4</td>
<td>7H11, Hyg only</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>London GFP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
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<td>Frozen</td>
<td>1</td>
<td>DosR/S MprA/B TcrX/Y</td>
<td>28.5</td>
<td>4</td>
<td>7H11, Hyg only</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>London GFP</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>0</td>
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<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
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<td>DosR/S PhoP/R RegX3/SenX3</td>
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<td>16</td>
<td>7H11, Hyg only</td>
<td>0</td>
</tr>
<tr>
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<td>16</td>
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<td></td>
<td></td>
<td></td>
<td>2x DosR/S 1x TcrX/Y</td>
</tr>
<tr>
<td>6a</td>
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<td>DosR/S MprA/B TcrX/Y</td>
<td>28.5</td>
<td>16</td>
<td>7H10, Hyg only</td>
<td>1x DosR/S 4x MprA/B 1x TcrX/Y</td>
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<tr>
<td>6b</td>
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<td>10</td>
<td>DosR/S MprA/B TcrX/Y</td>
<td>32</td>
<td>16</td>
<td>7H10, Hyg only</td>
<td>1x DosR/S 0x MprA/B 19x TcrX/Y</td>
</tr>
<tr>
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<td>Leicester WT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1x DosR/S</td>
</tr>
<tr>
<td>Round</td>
<td>City</td>
<td>WT Strain</td>
<td>Preparation</td>
<td>AES DNA</td>
<td>Transformation Efficiency</td>
<td>Selection Medium</td>
<td>Notes</td>
<td></td>
</tr>
<tr>
<td>-------</td>
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<td>--------------------------</td>
<td>-----------------</td>
<td>-------</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>London WT</td>
<td>Fresh, concentrated</td>
<td>10</td>
<td>DosR/S DosR/S + USP MprA/B PhoP/R RegX3/SenX3 TcrX/Y</td>
<td>32</td>
<td>16</td>
<td>7H10, Hyg only</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Leicester WT</td>
<td>Fresh, concentrated</td>
<td>10</td>
<td>DosR/S DosR/S + USP MprA/B PhoP/R RegX3/SenX3 TcrX/Y</td>
<td>32</td>
<td>16</td>
<td>7H10, Hyg only &amp; SucHyg</td>
<td></td>
</tr>
</tbody>
</table>

**Rounds 1-2:** All three *M. marinum* parent strains (WT, GFP and DsRed) were used. To prevent essentiality of the targeted TCSs from affecting these investigations, three different AES, each targeting a different locus, were used. **Round 3:** The amount of AES DNA used per transformation reaction was raised in an attempt to obtain single crossover strains. **Rounds 4-7:** Following conversations on improving mutant generation efficacy in *M. marinum* with Dr Mukomolova, we decided to use: WT strain backgrounds only; freshly prepared competent cells for each round which were 10x concentrated compared to previous rounds, increased plasmid DNA quantities in transformation reactions, increased temperatures for cell growth and recovery throughout the sucrose counter-selection process, longer cell recovery times following transformations, and selection on 7H10 plates following transformations for up to one month (previously two weeks on 7H11 plates which contain increased malachite green had been used). **Round 8:** Selection for double crossovers was attempted immediately using 10% sucrose-hygromycin (SucHyg) double selection plates. Selection for single crossovers on agar plates was performed using hygromycin (Hyg) only in previous rounds for later selection of double crossovers.
5.3.3.3 **Characterisation of putative TCSs single crossover strains obtained through sucrose counter-selection**

gDNA samples were taken for one single colony purified by re-streaking from all putative TCSs single crossover strains to enable PCR characterisations. PCR reactions were performed for all tested samples using the *M. marinum* confirmation primers, which revealed that all of the tested strains were *M. marinum* and all gDNA samples were suitable for use in PCR reactions as DNA templates.

Characterisations were first undertaken for the colonies obtained during Round 5. PCRs using the internal hygromycin cassette primers to verify carriage of the AES by the tested strains provided the expected product for the positive control reaction and one high intensity product band for a single putative DosR/S AES single crossover strain, colony 5 (Figure 5.11). Lower intensity product bands were also seen for some other putative DosR/S AES single crossover strains as well as some MprA/B single crossover strains (data not shown). Very low intensity product bands were obtained for the TcrX/Y strains, which were of similar intensity or lower than the negative control reactions. Therefore, the 3 strains with the highest intensity product bands each were chosen for the DosR/S AES (colonies 5, 7 and 16) and MprA/B AES (colonies 4, 5 and 24) samples for further characterisations. Each of these strains underwent another round of purification by re-streaking to provide 3 further single colonies for analysis (from 3 strains for each AES to 9).

PCRs using the hygromycin cassette-genome primers for the upstream and downstream regions were performed to confirm the integration of the hygromycin cassette into the genome. For a single crossover strain, a product would only be expected in one direction. The PCRs provided products for the putative DosR/S AES single crossover colony 5 samples for the downstream region (data not shown, see Figure 5.12 for putative double crossover data). No products were obtained for any other putative DosR/S AES single crossover samples or the putative MprA/B AES single crossovers samples in either direction (data not shown). As there are no positive controls available for the MprA/B AES sample reactions in either direction or for the DosR/S AES sample reactions in the upstream direction, all samples were also tested in PCRs using the full locus primers to determine whether these strains possessed the WT locus. These
reactions provided no products for any of the samples (data not shown), which could be attributable to failure of the PCR or of the far increased product size due to presence of a single crossover. Attempts were made to generate double crossovers for all 18 of the strains selected in parallel with these characterisations, as described in Section 5.3.3.4.

Characterisations were also undertaken for the putative single crossover samples obtained from sucrose counter-selection Rounds 6 and 7. All strains obtained during Round 6 were tested, with characterisations of the strains obtained from Round 7 focusing on the DosR/S AES and DosR/S + USP samples. PCRs were performed using the internal hygromycin cassette primers to verify carriage of the AES by the tested strains. The expected product was obtained for the positive control reaction (pSMT100::DosR/S_AES) but no products were obtained for any of the test samples from either round. Experiments using the putative single crossover strains obtained from sucrose counter-selection Rounds 6 and 7 were discontinued.

Figure 5.11. Characterisation PCRs for putative M. marinum DosR/S AES single crossover strains from sucrose counter-selection Round 5. PCRs using internal hygromycin cassette primers, expected product if cassette present in strain = 521 bp. Positive control = pSMT100 AES parent vector (pSMT), negative controls = WT gDNA and water. Test PCRs were performed using gDNA samples for putative single crossover strains following single colony purification. All PCR reactions were performed using the same master mix.

5.3.3.4 Characterisation of putative TCSs double crossover strains obtained through sucrose counter-selection

Alongside the characterisation PCRs, the 18 putative single crossover strains selected from sucrose counter-selection Round 5 (9 DosR/S AES and 9 putative MprA/B AES) were subjected to selection for double crossovers by growth in 5 ml liquid cultures to stationary phase followed by selection on agar plates containing 10% sucrose. In all cases, a lawn of growth was seen following selection on sucrose. Three putative double crossover samples were taken, each from a separate plate, for each strain (27 strains for each AES).
Owing to the lawn of growth obtained, double crossover selection was attempted under different conditions. Three attempts to provide individual colonies through plating on 20% sucrose were made, with each also providing lawns of growth which were not tested. Selection of double crossovers through growth of the strains in liquid medium containing sucrose was also attempted. All putative DosR/S AES single crossover strains were grown in liquid medium containing 10% and 20% sucrose for three passages. Each culture was then plated on both 10% and 20% sucrose, which again provided lawns of growth for all strains. Therefore, the original putative double crossover samples were characterised by PCR.

Characterisation PCRs using the internal hygromycin resistance cassette primers provided the expected product for the positive control reactions and all samples originating from the DosR/S AES colony 5 single crossover strain (Figure 5.11A). No products were seen for reactions with any other DosR/S AES samples (Figure 5.11A) or any of the MprA/B AES samples (data not shown). Characterisation of the MprA/B samples was therefore discontinued. PCRs using the hygromycin cassette-genome primers for the downstream region to verify the integration of the hygromycin cassette into the DosR/S genome locus provided the expected products for all samples originating from the DosR/S AES colony 5 single crossover strain (Figure 5.11B). No products were obtained for any other strains using these primers (Figure 5.11B). PCRs using the hygromycin cassette-genome primers for the upstream region provided no products for any of the samples (data not shown). Optimisation of PCR reactions with these primers was performed by gradient PCRs with gDNA for a putative ΔDosR/S + USP strain obtained through recombineering (as the integrated DosR/S + USP AES contains both primer sites). Following these optimisations, PCRs were repeated with the putative ΔDosR/S + USP strain gDNA as a positive control and all gDNA samples for putative double crossovers originating from the DosR/S AES colony 5 single crossover strain. The expected product was obtained for the positive control but none of the tested putative double crossovers (data not shown). Characterisation of the putative DosR/S AES double crossover strains was therefore verified by Southern Blotting (Section 5.3.4).
5.3.4 Characterisation of putative *M. marinum ΔDosR/S + USP* strains and putative *M. marinum DosR/S AES* double crossover strains by Southern Blotting

5.3.4.1 **Overview**

Characterisation of the putative *M. marinum ΔTCSs* mutant strains generated through mycobacteria-adapted recombineering and sucrose counter-selection was performed by Southern Blotting using a probe to the upstream homologous region of the DosR/S +
USP AES (present in both putative deletions). We used Southern Blotting to test whether the hygromycin cassette had integrated into the target region or an off-target region for the strains generated by recombineering and whether single or double crossovers had been generated by sucrose counter-selection.

### 5.3.4.2 Southern Blotting

The probe was generated from pYUB854::DosR/S+USP_AES by restriction enzyme digest with HindIII, SpeI and SmaI to yield 370 bp in the upstream homologous region of the AES. The gDNA samples to be tested were: *M. marinum* WT gDNA; single colony samples for each of the two independent putative ΔDosR/S + USP mutant strains in the *M. marinum* DsRed background obtained through mycobacteria-adapted recombineering; single colony samples for the corresponding cream coloured strain (WT background) following three rounds of purification from these parent recombineering strains; and two independent putative DosR/S AES double crossovers obtained through sucrose counter-selection (these originated from the same single crossover strain). The positive control used was pYUB854::DosR/S+USP_AES DNA.

Southern Blotting was first attempted using all kits according to manufacturer’s recommendations and gDNA digests of 5 µg using the AcuI restriction enzyme. This yielded Blot 1, which displayed faint labelling of the pYUB854::DosR/S+USP_AES positive control only and high background labelling (Figure 5.13A). Optimisations were therefore performed with positive and negative control gDNA samples until clear labelling of the positive control samples was achieved. The optimisations are summarised in Table 5.5, with key blots shown in Figure 5.13.
Table 5.5. Summary of Southern Blotting optimisations.

<table>
<thead>
<tr>
<th>Blot</th>
<th>Gel thickness (cm)</th>
<th>gDNA (µg)</th>
<th>Plasmid DNA (µg)</th>
<th>Prehybridisation</th>
<th>Probe concentration (ng/ml)</th>
<th>Washes temp. (°C)</th>
<th>Additional wash</th>
<th>Probe detection time (hours)</th>
<th>Results description</th>
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<tr>
<td>1</td>
<td>1.0</td>
<td>5</td>
<td>2</td>
<td>42</td>
<td>1.2</td>
<td>42</td>
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<td>14</td>
<td>Moderate background staining, some labelling positive control</td>
</tr>
<tr>
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<td>1.0</td>
<td>5</td>
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<td>55</td>
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<td>10</td>
<td>0.25</td>
<td>55</td>
<td>1.2</td>
<td>60</td>
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<td>14</td>
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<tr>
<td>5.1</td>
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<td>0.25</td>
<td>55</td>
<td>1.2</td>
<td>60</td>
<td>No</td>
<td>0.25</td>
<td>No background staining, clear labelling positive control</td>
</tr>
<tr>
<td>5.2</td>
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<td>55</td>
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<td>60</td>
<td>No</td>
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<td>As Blot 5.1</td>
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<tr>
<td>5.3</td>
<td>0.6</td>
<td>10</td>
<td>0.25</td>
<td>55</td>
<td>1.2</td>
<td>60</td>
<td>No</td>
<td>2</td>
<td>As Blot 5.1, positive control overexposed</td>
</tr>
</tbody>
</table>

Blot 1: All kits and reagents were used according to manufacturer’s recommendations. Blot 2: Prehybridisation, hybridisation and wash temperatures were raised to 55°C to reduce background staining. Blot 3: Blot 3 was run with three copies of the control samples, separated into three following DNA transfer, and then each portion treated as described. The amount of gDNA was raised and plasmid control DNA lowered to reduce probe sequestration by the positive control and aid visualisation of the results. A positive control of matched copy number to that expected in 10 µg of the *M. marinum* genome was also included (corresponds to 8.9 ng plasmid DNA). Treatment of the gel to increase DNA transfer to the membrane (see Section 2.3.10.3) was performed prior to electroblotting for this and all subsequent blots. Prehybridisation times were increased to 60 minutes and wash temperatures raised to 60°C to reduce background staining. The recommended additional high salt wash to reduce ionic interactions, and thereby background signal, with the UltraHyb buffer was trialled in Blot 3.2. A lower probe concentration to reduce background staining was trialled in Blot 3.3. Blot 4: Use of an uncharged nitrocellulose membrane (kindly donated by Ioly Kotta-Loiziou; manufactured by Invitrogen) to reduce background was trialled. All other blots were performed with the Bright Star Plus positively charged nylon membrane (Thermo Scientific). Blot 5: Blot 5 was again run with three copies of the control samples, separated into three following DNA transfer, and then each portion treated as described. Gel thickness was reduced to aid DNA transfer to the membrane, allowing reduction of the probe detection time to decrease the background staining. *a* = Temperature applies to prehybridisation and hybridisation steps.
Figure 5.13. Optimisation of Southern Blotting. (A-C) Southern Blotting performed using a probe complementary to 370 bp of the upstream homologous region for the DosR/S + USP AES. (A) The two control lanes only of Blot 1 are shown for clarity. 5µg gDNA or 2 µg plasmid DNA (pYUB854 parent vector, pYUB) loaded per well, DNA transfer to Bright Star Plus positively charged nylon membrane, prehybridisation for 30 minutes at 42°C, hybridisation with 1.2 ng/ml probe for 24 hours at 42°C, recommended washes at 42°C and probe detection for 14 hours. Relevant sizes of the GeneRuler 1Kb ladder are marked in the far right lane. (B) Blot 3 was separated into three portions following DNA transfer, with each portion treated differently. Shown is Blot 3.1: 10 µg gDNA loaded per well, prehybridisation for 60 minutes at 55°C, hybridisation and washes at 55°C, and all other conditions as for Blot 1. Lanes: 1 = 250 ng pYUB854::DosR/S+USP_AES, 2 = 250 ng pYUB854::TcrX/Y_AES, 3 = 8.9 ng pYUB854::DosR/S+USP_AES, 4 = 10 µg WT gDNA. Relevant sizes of the GeneRuler 1Kb ladder are marked on the right hand side of the blot – the ladder itself was run on the far left of the gel, which is the far right lane of Blot 3.3 (not shown). (C) Blot 5 was also separated into three portions following DNA transfer, with each portion undergoing probe detection for different lengths of time. Shown are Blots 5.1 and 5.3 which were performed as for Blot 3.1, except probe detection for Blot 5.1 was for 15 minutes and for Blot 5.3 was 2 hours. Lanes: 5 = 250 ng pYUB854::DosR/S+USP_AES, 6 = 8.9 ng pYUB854::DosR/S+USP_AES, 7 = 250 ng pYUB854::TcrX/Y_AES, 8 = 10 µg WT gDNA Relevant sizes of the GeneRuler 1 Kb ladder are marked in the far right lane and apply to both portions of the blot. gDNA samples were digested with AcuI and plasmid samples digested with SpeI and HindIII for all blots.
The optimised blotting conditions were used to test all samples following restriction enzyme digest with NcoI or AcuI. Two restriction enzyme digests were used to provide a full confirmation of the results. For both digests one sucrose counter-selection sample and one recombineering sample following purification did not provide a result (Figure 5.14, lanes 6 and 13, and lanes 4 and 11, respectively).

The putative DosR/S AES double crossover obtained through sucrose counter-selection did not provide labelled bands of the expected size for a double crossover strain for either restriction enzyme digest. A labelled band of a greater size than expected for a single crossover following AcuI digestion was seen (lane 14, expected size 6554 bp), which can be confirmed by comparison to the WT product of 6571 bp in lane 15. The larger size product could be explained by a lack of cleavage by AcuI in the integrated sacB gene and cleavage at the expected location in the pSMT100 plasmid backbone, which would provide a product of 7735 bp. A labelled band of a greater size than expected for a single crossover was also seen following Ncol digestion (Figure 5.14, lane 7, expected size 6037 bp), confirmed by comparison to the similarly-sized WT product at 8358 bp in lane 8. This could again be explained by a lack of cleavage in the sacB gene by Ncol to provide the observed larger product.

The putative ΔDosR/S + USP strains obtained through recombineering provided a labelled band of the expected size for a WT DosR/S locus following NcoI digestion (Figure 5.14, lanes 2, 3, and 5, size 8375 bp), confirmed by comparison to the WT product in lane 8. A second band of lower product size was also obtained for the first strain samples (lanes 5 and 3, lower band, size ~4 Kb). The putative ΔDosR/S + USP strains also provided a labelled band of the expected size for a WT DosR/S locus following AcuI digestion (lanes 9, 10 and 11, size 6571 bp), confirmed by comparison to the WT product in lane 15. A second band of increased product size was also obtained for the first strain samples (lanes 12 and 10, upper band, size >10 Kb). No products of expected size for the deletion mutant were obtained for any of the putative ΔDosR/S + USP strains obtained through recombineering following either restriction enzyme digest.
Figure 5.14. Characterisation by Southern Blotting of putative ΔDosR/S + USP and putative ΔDosR/S mutant strains. Southern Blotting performed using a probe complementary to 370 bp of the upstream homologous region for the DosR/S + USP AES. Positive control = 8.9 ng pYUB854::DosR/S+USP_AES (lane 1). Samples tested were: WT *M. marinum* gDNA, two independent putative ΔDosR/S + USP mutant strains obtained through mycobacteria-adapted recombineering Round 1 (lanes 4-5 and 11-12); two cream coloured colonies (WT background) following three rounds of purification from these parent recombineering strains (lanes 2-3 and 9-10); and two putative ΔDosR/S double crossovers from sucrose counter-selection Round 5 (lanes 6-7 and 13-14). Sample in lanes 2 and 9 corresponds to the parent strain sample in lanes 4 and 11, with the sample in lanes 3 and 10 corresponding to the parent strain sample in lanes 5 and 12. gDNA samples were digested in 10 µg reactions with NcoI (lanes 2-7) or AcuI (lanes 8-15). Plasmid samples were digested with SpeI and XbaI. The GeneRuler 1 Kb ladder is marked in the far right lane. Expected sample sizes for NcoI digest: WT DosR/S locus = 8358 bp, ΔDosR/S + USP = 5641 bp, ΔDosR/S = 5641 bp, and single DosR/S AES crossover = 6037 bp. Expected sample sizes for AcuI digest: WT DosR/S locus = 6571 bp, ΔDosR/S + USP = 5293 bp, ΔDosR/S = 6108 bp, and single DosR/S AES crossover = 6554 bp.

5.4 Discussion

Protein sequence alignments for the orphaned and complete TCSs systems of *M. tuberculosis* and *M. marinum* demonstrated that these systems are highly conserved between the two organisms with greater than 74% sequence identity for the systems selected for study (Table 5.1). Genomic context alignments for the orphaned and complete TCSs systems of *M. tuberculosis* and *M. marinum* demonstrated that 9 of the 12 TCSs loci are found in a similar genomic environment in both organisms (Table 5.2). The high TCSs protein sequence identities between *M. marinum* and *M. tuberculosis*, and the high degree of co-occurrence of genes and conservation of gene order in the TCSs gene flanking regions, confirms that both the TCSs and their gene clusters are highly conserved and would suggest that these gene clusters share function between the two bacterial species. These analyses recommend *M. marinum* as a suitable model for the study of *M. tuberculosis* TCSs.

The generation of the desired *M. marinum* TCSs deletion mutants was attempted in 7 rounds of mycobacteria-adapted recombineering (Table 5.3), with putative deletion
mutant strains obtained during 3 rounds. To counter the difficulties we experienced with recombineering, we varied the TCSs targeted, the *M. marinum* laboratory strain used and the protocol conditions. Through modifications to the published protocol in Round 4, we concluded that the concentration of acetamide used to induce expression of the recombineering helper proteins and the amount of AES used during transformations are not limiting factors in the success of recombineering in *M. marinum*. We concluded that essentiality of the TCSs targeted was not the cause of our difficulties through the targeting of multiple different TCSs loci over the course of our attempts. Through the use of a second laboratory strain in which a related mutagenesis protocol had been successful, we also concluded that recalcitrance to mutagenesis of the *M. marinum* laboratory strain used was not the cause of our difficulties (Rounds 6-7).

Putative TCSs deletion mutants obtained during three separate rounds of recombineering were characterised by PCR investigations of the integration of the hygromycin resistance cassette and TCSs loci. The putative ΔRegX3/SenX3 and ΔTcrX/Y mutants obtained during Round 3 of recombineering (Figure 5.4) and putative ΔDosR/S and ΔDosR/S + USP mutants obtained during Round 7 of recombineering (Figure 5.6) were shown to lack a copy of the hygromycin resistance cassette in the genome. We therefore concluded that these strains were not TCSs deletion mutants but had arisen through spontaneous hygromycin resistance events. Both putative ΔDosR/S + USP mutants obtained during Round 1 of recombineering were verified as containing the hygromycin resistance cassette within the genome (Figure 5.4). Attempts to verify that the hygromycin resistance cassette had integrated into the targeted TCSs locus by PCR characterisations using hygromycin cassette-genome primers were inconclusive (Figure 5.5), necessitating characterisations by Southern Blotting (Figure 5.14). The results demonstrated a lack of products associated with the desired deletion mutants, coupled with products expected for the WT DosR/S locus and an additional product. We concluded that the AES had integrated into an off-target location of the *M. marinum* genome, providing the unknown additional product in the Southern Blotting characterisations, and that the DosR/S locus in these strains remained intact.

The difficulties experienced in generating TCSs deletion mutants in *M. marinum* through mycobacteria-adapted recombineering were unexpected due to the previous successful application of this system to the related mycobacterial species *M. tuberculosis* and *M.*
*smegmatis* by our group (unpublished data). Investigations of the efficacy of recombineering between *M. marinum* and *M. smegmatis* revealed that *M. marinum* is particularly adversely affected by the presence of the recombineering helper plasmid pJV53. Growth of *M. marinum* cells was observed to be significantly reduced by carriage of pJV53 for all three strain backgrounds used during this study (Figure 5.7). In addition, trends for reduced transformation efficiencies for all three *M. marinum* strain backgrounds upon carriage of pJV53 were seen (Figure 5.8). Furthermore, pJV53 was lost from *M. marinum* strains more quickly than *M. smegmatis* following passage (round 2 vs round 6+), indicating a greater fitness cost associated with carriage of pJV53 to *M. marinum* than *M. smegmatis* cells (Figure 5.9). These investigations would indicate that the presence of the helper plasmid pJV53 may reduce the efficacy of recombineering in *M. marinum* compared to other mycobacterial strains through reducing the efficiency of transfer of the AES to cells and cell recovery following transformations. It is also possible that the reduced growth temperature of *M. marinum* leads to decreased activities of the recombineering helper proteins Gp60 and Gp61 compared to other mycobacteria and *E. coli* (in which the method was first described) in which recombineering has been successful. Reduced activities of these key proteins would be a significant barrier to the use of recombineering in *M. marinum*. However, investigation of the activities of these proteins at the two growth temperatures would be difficult to perform and was therefore not undertaken in this study.

Generation of the desired *M. marinum* TCSs deletion mutants was also attempted in 8 rounds of sucrose counter-selection (Table 5.4). To address the difficulties we experienced during sucrose counter-selection, we varied the *M. marinum* laboratory strain used, the TCSs targeted, and the protocol conditions. The improved protocol yielded putative single crossover strains from three independent rounds. We were therefore able to conclude that essentiality of the TCSs targeted was not the cause of our previous difficulties. We also concluded that the *M. marinum* laboratory strain used was not resistant to mutagenesis through comparison with a second laboratory strain in which a different sucrose counter-selection protocol had been successful (Rounds 6-8).

The putative single crossovers obtained during three independent rounds of sucrose counter-selection were characterised by PCR investigations of the carriage of the
hygromycin resistance cassette and TCSs loci. PCR characterisations using the internal hygromycin cassette primers to verify carriage of the AES were performed for all putative TCSs AES single crossovers obtained during Rounds 5 and 6, as well as the DosR/S and DosR/S + USP AES single crossovers obtained during Round 7. All of the strains were found to lack a copy of the hygromycin resistance cassette (Section 5.3.3.3), with the exception of one putative DosR/S AES single crossover obtained during Round 5 (Figure 5.11). We therefore concluded that the remaining strains had arisen through spontaneous hygromycin resistance events and were not the desired single crossovers. Further characterisations of the single crossover using the hygromycin cassette-genome primers revealed that the recombination event occurred at the downstream homology region (Section 5.3.3.3).

Selection for double crossovers from the tested single crossover strains obtained during Round 5 was performed alongside the characterisation PCRs (Section 5.3.3.4). Double crossover selection was therefore performed for the verified single crossover as well as some additional strains. The selection provided the unexpected result of a lawn of growth for all strains, despite attempts using increased concentrations of sucrose during plating and sucrose selection in liquid medium. Growth of the spontaneous hygromycin resistance mutants on the sucrose selection plates would be expected as they did not contain the sucrose-sensitive marker sacB. However, the full growth of the DosR/S AES single crossover strain was unexpected. It is therefore possible that the sacB marker used was non-functional, which is also indicated by the failure of both restriction enzymes used in Southern Blotting to cleave at the expected sites within this gene (Figure 5.14). The functionality of the sacB gene used should therefore be further investigated by PCR amplification from the pSMT100::DosR/S_AES followed by sequence-verification.

PCR characterisations were again performed to investigate the carriage of the hygromycin resistance cassette and TCSs loci in single colonies purified from the lawn of growth obtained from sucrose selection with the verified DosR/S AES single crossover. The putative double crossovers provided the expected results of hygromycin resistance cassette presence following recombination at the downstream homology region using the internal hygromycin cassette primers and hygromycin cassette-genome primers (Figure 5.12). Attempts to verify by PCR that the second recombination
event had occurred at the upstream homology region using the second set of hygromycin cassette-genome primers failed to confirm the event (Figure 5.12), necessitating characterisations by Southern Blotting (Figure 5.14). The results demonstrated products expected for the single crossover strain and a lack of products associated with the desired double crossover. From the Southern Blotting and PCR characterisation results we concluded that the AES and pSMT100 plasmid backbone had successfully integrated into the *M. marinum* genome at the target DosR/S locus, but had failed to complete the second crossover event. Deletion of the DosR/S locus in these strains had therefore been unsuccessful.

The difficulties experienced in generating TCSs deletion mutants in *M. marinum* through sucrose counter-selection were unexpected due to the previous successful application of this method by other groups, including that of Dr Mukamolova at Leicester University (unpublished data). Comparisons of the growth and transformation efficiencies of the *M. marinum* laboratory strains used by our group and the Leicester group confirmed that there are no significant differences between the two strains (Appendix 5). We therefore concluded that recalcitrance to mutagenesis of the *M. marinum* laboratory strain used was not the cause of our initial difficulties generating the desired TCSs deletion mutants by sucrose counter-selection. From the similar colony counts obtained for sucrose counter-selection at both growth temperatures for both laboratory strains (Table 5.4, Rounds 6-7), we concluded that the lower growth temperature used during initial rounds of sucrose counter-selection were not the cause of our initial difficulties generating the desired TCSs deletion mutants.

Attempts to generate TCSs deletion mutants in *M. marinum* for future study of the effects of these systems on virulence in the zebrafish infection model could proceed using the pNIL/pGOAL sucrose counter-selection system\textsuperscript{[247]}. This system is a desirable method given the difficulties experienced in this project due to the blue/white screening step for double crossover colonies enabled by the inclusion of the marker gene *lacZ* in the flexible cassette system. Therefore, a significant reduction in the number of putative mutant strains to be screened is achieved.
6 General Discussion

6.1 Key findings and significance of this study

6.1.1 Chapter 3 – Optimisation of techniques for the assessment of *M. marinum* infection progression in THP-1 cells and the zebrafish infection model

Chapter 3 described the optimisation of infection protocols and techniques for monitoring *M. marinum* infection progression in THP-1 cells and the zebrafish embryo model for the assessment of infecting strain virulence or drug treatment efficacy. For THP-1 cells, 96-well plate assays to quantify infection progression by CFU plating to determine the intracellular bacterial burden and qRT-PCR to determine the host immunity gene expression pattern were described. Following zebrafish HBV infection protocol optimisation, an Icy FPC protocol for the quantification of bacterial burden of infected zebrafish from images was validated in the zebrafish and utilised to eliminate the issues associated with CFU plating and provide binary output images to confirm correct application of the FPC procedure. In addition, a protocol for the quantification of the amount and degree of bacterial dissemination from images was developed to permit the first image quantification of these measurements. Techniques to quantify zebrafish leukocyte recruitment from images and qRT-PCR methods to quantify host immunity gene expression in response to *M. marinum* infection were also optimised.

The described techniques to assess infection progression represent a significant advancement in the image quantifications available for longitudinal studies using the *M. marinum*-zebrafish embryo model. Through these techniques we achieved simultaneous quantification of multiple infection progression parameters following live-imaging of zebrafish embryos and larvae infected with *M. marinum*, permitting medium-throughput longitudinal studies assessing the progression of *M. marinum*-zebrafish infection for the first time. These imaging techniques further recommend the use of the *M. marinum*-zebrafish embryo model to study TB disease compared to other models, as it becomes possible to conduct inexpensive and medium-throughput studies with a focus on host-pathogen interactions over large sample sizes using a natural host-
pathogen pairing. The techniques are particularly beneficial for the study of drug treatment efficacy or the mycobacterial determinants of virulence, infection progression and host-pathogen interactions as they can be applied to screening of drugs and genes of interest, such as TCSs.

6.1.2 Chapter 4 – Investigating the effect of selected HDPs on *M. marinum* infection progression in THP-1 cells and the zebrafish

The application of the techniques for monitoring *M. marinum* infection progression in THP-1 cells and zebrafish embryos to studies of drug treatment efficacy using HDPs was described in Chapter 4, and revealed several key findings. Treatment of resting THP-1 cells with the exogenous HDPs Bac5 and EPI, which lack direct bacteriocidal activity against *M. marinum*, significantly increased expression of immunity genes *IL-1β* and *MMP-9*. Following injection at 2 dpf to the zebrafish embryo HBV, the exogenous peptide Bac5 demonstrated significant leukocyte recruitment and trends for modulation of the zebrafish host immune response independently of *M. marinum* co-injection initially, but no lasting effects on zebrafish leukocyte recruitment, gene expression or *M. marinum* bacterial burden. Single or double Bac5 treatment at a later infection stage, during early granuloma formation, did not improve the long-term effects of Bac5 treatment and demonstrated trends for reduced zebrafish health and survival.

We believe ours is the first study of the immunomodulatory effects of Bac5 in the zebrafish and of an exogenous peptide in the *M. marinum*-zebrafish model. Overall, our findings in THP-1 cells and zebrafish embryos and larvae demonstrate that Bac5 capable of some moderate immunomodulation in the human and zebrafish hosts, in agreement with work presented by other groups. Our data on the effects of Bac5 treatment at early infection stages and/or early developmental stages of the zebrafish showed no improvement of infection outcome and may indicate that Bac5 is not suitable for use as prophylaxis to protect at-risk groups. These findings demonstrate the value of investigating drug treatments in the zebrafish embryo infection model to determine the effects on the innate immune system in isolation. Further investigations into the long-term effects of Bac5, its cytotoxic activities and its effects on the adaptive immune system are required to determine whether Bac5 can be used effectively as a treatment
or as prophylaxis for TB disease. Such studies could utilise the adult zebrafish model and will shed more light on the potential of HDPs as new therapies for TB disease.

6.1.3 Chapter 5 – Generation of TCSs deletion mutants in *M. marinum*

Chapter 5 described attempts to generate targeted TCSs deletion mutants in *M. marinum* by different methods. Mycobacteria-adapted recombineering, which had successfully been used to generate deletion mutants in the related bacteria *M. smegmatis* and *M. tuberculosis* within the group, was found unsuitable for deletion mutant construction in *M. marinum*. This was attributable to strains carrying the recombineering helper plasmid pJV53 displaying a combination of reduced fitness and transformation efficiencies during the critical mutagenesis step. That mycobacteria-adapted recombineering is not applicable to deletion mutant construction in *M. marinum* is significant as the remaining techniques available take markedly longer for mutant construction and thus limit the number of deletion mutant strains which can practicably be generated for study. During attempts to construct TCSs deletion mutant strains using sucrose counter-selection, single crossover strains carrying the mutagenic construct were generated but the generation of double crossovers was not successful. We attributed the difficulties to a possible defective copy of the *sacB* selection marker.

6.2 Recommendations for further work

6.2.1 Investigating the potential of Bac5 as a novel therapy using THP-1 cells

6.2.1.1 Effect of Bac5 treatment on intracellular replication and intercellular spread of *M. marinum* during infection

Investigations into the effect of Bac5 treatment on bacterial burden of infected THP-1 cells could be performed by CFU plating following treatment with the exogenous HDP. Experiments could also be performed to determine whether Bac5 affects intercellular spread of *M. marinum* and/or the intracellular replication of the bacilli. Such experiments could be performed by microscopy of THP-1 cell samples over time using fluorescently labelled strains of *M. marinum* with the number of bacilli per THP-1 cell and number of infected cells as measures of intracellular replication and intercellular
spread, respectively. We believe that immunomodulation by the peptides could lead to a reduction in bacterial replication and dissemination, which would recommend these HDPs as suitable treatments for tuberculous disease.

6.2.1.2 Effect of Bac5 treatment on host gene expression
Experiments undertaken by our collaborator Sam Willcocks demonstrated increased expression of IL-8 and iNOS by the Bac5 A459 cell line compared to the parent A459 cell line upon challenge with mycobacteria and TNF-α. This could be investigated further to determine whether Bac5 also leads to increased iNOS and IL-8 expression in THP-1 cells. To this end, supernatants were collected from the THP-1 cell experiments presented in Chapter 4 for later ELISA testing of the iNOS and IL-8 expression levels by Sam Willcocks. Increased expression of these genes would indicate that Bac5 treatment could lead to neutrophil recruitment and activation in the human host, which would be expected to be beneficial to the host during infections due to the resulting increase in the clearance of the infecting bacilli.

6.2.1.3 Investigation of the mechanism of Bac5 signalling
It would be interesting to investigate the mechanism of Bac5 signalling during immunomodulation as it is currently unknown. Previous studies have investigated the signalling mechanisms of the human cathelicidin LL-37 and an IDR peptide (IDR-1002) derived from a member of the bovine cathelicidin family, the bactenecins. These studies have demonstrated the involvement of Gi-coupled receptors, the MAPKs MEK-1 and p38, PI3K, and the NF-B signalling pathway\[^{[19, 34, 76, 209, 215, 221, 223, 293, 369]}\]. Microtubule assembly and actin polymerisation were also found to be involved, suggesting that intracellular uptake of the peptides using cytoskeletal reorganisation and endocytic uptake are required for the peptide actions\[^{[221]}\]. These studies indicate that there is an overlap between the signalling pathways used by synthetic and natural HDPs, thus Bac5 signalling may involve the same receptors and signalling pathways. Experiments to determine the mechanism of Bac5 signalling could therefore be performed using the same inhibitors as the above studies to target individual signalling molecules, such as with pertussis toxin against Gi-proteins, with the pathways required for Bac5 signalling determined by monitoring the expression of a gene upregulated by Bac5 using qRT-PCR.
6.2.2 Investigating the potential of Bac5 as a novel therapy using zebrafish

6.2.2.1 Studies using the zebrafish embryo

6.2.2.1.1 Investigation of additional macrophages recruited by Bac5 during *M. marinum* infections

During zebrafish embryo infection experiments presented in Chapter 4, increased leukocyte recruitment to the *M. marinum* infection site was seen following Bac5 injections but the additional recruitment did not lead to increased bacterial killing. To further investigate these effects of Bac5 the state of infiltrating macrophages and the chemokines induced by Bac5 could be determined. Recruitment of resting macrophages benefits the infecting bacilli during very early stages of zebrafish embryo infection with *M. marinum*[^41] and also at a later stage of early infection once granuloma formation has occurred[^345]. Antibody staining against iNOS, present in macrophages with microbicidal activity, could be used to determine whether the additional macrophages recruited to the infection site following Bac5 treatment of zebrafish embryos were of benefit to the host or infecting bacilli. Chemokines of particular interest for qRT-PCR quantification are the neutrophil chemokine IL-8 and macrophage chemokine MCP-1. IL-8 was differentially expressed in the Bac5 A459 cell line (Sam Willcocks, unpublished data). MCP-1 was differentially expressed in THP-1 cells, PBMCs and a murine infection model following treatment with an IDR peptide derived from a bactenecin family member (IDR-1002)[^221]. The zebrafish orthologue of MCP-1 was recently identified[^41], enabling the assessment of its expression levels. Also of interest is the extracellular matrix remodelling enzyme MMP-9, which demonstrated increased expression following Bac5 treatment during experiments presented in Chapter 4 and was involved in the mechanism for macrophage recruitment by a bacterial secreted protein, ESAT-6 after granuloma formation[^345].

6.2.2.1.2 Investigation of the effect of Bac5 on host gene expression

During zebrafish embryo infection experiments presented in Chapter 4, trends for the modulation of zebrafish immunity gene expression were found following Bac5
injections. The reduction in immunity gene expression observed at 24 hpi in particular is worthy of further investigation. These effects could be due to control of excess immunomodulation by Bac5, a feature of HDP action\cite{50, 195}, or enhancement of wound healing by Bac5, which other HDPs and IDR peptides have been shown to be capable of in murine and porcine models\cite{147, 194}. Detection of increased expression of anti-inflammatory genes, such as IL-10 and IL-1RA, by qRT-PCR would indicate that it was immunomodulation by Bac5 to control excess inflammation which had occurred.

We proposed that additional resting macrophages recruited to the infection site in zebrafish embryos following Bac5 treatment at early infection stages are not activated upon arrival at the site due to a lack of activator molecules. It is important to confirm this hypothesis by further experiments to determine the baseline expression levels, and any effects of Bac5 on expression, of key activator molecules of the Th1 response, IL-1 and IFNγ, in our zebrafish embryo experiments by qRT-PCR.

6.2.2.1.3 Investigation of cytotoxicity of Bac5 in the zebrafish embryo
During zebrafish embryo infection experiments presented in Chapter 4, trends for reduced health of zebrafish treated with Bac5 were observed. It will be important to determine whether there are detrimental effects on zebrafish embryos of treatment with Bac5 at these early developmental stages and/or at such early stages of infection. The cytotoxicity of Bac5 could be investigated by assessing the effects on zebrafish health of varying doses of Bac5. Future experiments could include quantification of zebrafish health by scoring according to the parameters identified in the sequential injection experiments: loss of buoyancy control, loss of interest in feeding and epithelium weakening over the infection site.

6.2.2.1.4 Investigation of the effects of Bac5 on M. marinum dissemination during zebrafish embryo infections
One technique for infection progression parameter quantification presented in Chapter 3 was the Bacterial Distance Distribution protocol, which could be used to determine the amount and degree of bacterial dissemination during zebrafish embryo infections following Bac5 treatment. We hypothesise that Bac5 treatment would benefit the host
during mycobacterial infections by leading to enhanced containment of the infection. However, it was not possible to assess dissemination of bacteria following Bac5 treatments as very few zebrafish demonstrated dissemination of infection during our experiments. This could be attributed to the timing of the infections or the bacterial strain used. Therefore, to test our hypothesis zebrafish embryos could be locally infected to the HBV at an earlier developmental stage (30 hpf) or with a different bacterial strain and bacterial dissemination quantified over time following Bac5 treatment to the HBV. In addition, bacterial dissemination could also be quantified following systemic infections via caudal vein injection, with Bac5 also administered systemically via the caudal vein, either as co-injections with *M. marinum* or as a later treatment.

6.2.2.1.5 *Investigation of the mechanism of Bac5 signalling*

Following determination of the mechanism of Bac5 signalling in THP-1 cells as described in Section 6.2.1.3, the *in vivo* mechanism could be confirmed by treatment of zebrafish embryos with kinase inhibitors as in recent cardiovascular studies. In addition, morpholinos targeting signalling pathways or cytoskeleton-associated genes of interest could be used. In both cases, involvement of the target could be confirmed by Bac5 treatment followed by monitoring of leukocyte recruitment to the injection site or the expression of a gene targeted by Bac5 using qRT-PCR.

6.2.2.2 *Studies using the zebrafish adult*

6.2.2.2.1 *Investigating the effect of Bac5 on infection progression, against latent infection and as prophylaxis*

During zebrafish embryo infection experiments presented in Chapter 4, we observed no lasting effects on *M. marinum* infection progression following Bac5 treatment. To determine whether Bac5 treatment may be beneficial to the zebrafish host during mycobacterial infections if delayed until later stages of the infection or when the adaptive immune system has developed, experiments using treatment of zebrafish adults during chronic infections could be performed and the results compared to those obtained with the embryo model. In addition, Parikka *et al.* have recently described the
development of a latent tuberculosis model in the zebrafish adult which could be used to determine the effects of Bac5 treatment on latent infections\textsuperscript{[244]}. If Bac5 is able to cause immunomodulation which leads to the targeting of latent bacilli this would be an extremely significant step in the fight against TB disease. Furthermore, prophylaxis would be particularly beneficial in the prevention of TB disease in humans, as household contacts are exposed to \textit{M. tuberculosis} for months or even years when living with a person infected with TB. Determination of the effects of pre-, co- and post-treatment of Bac5 on its protective efficacy in the chronic and latent infection models is therefore of interest. Infection progression in the zebrafish adult could be monitored through assessing zebrafish survival, the immune response by histopathology and analysis of gene expression in organs such as the liver, and bacterial burden by CFU plating organs such as the liver or spleen. Drug administration to the zebrafish adult could be performed by bathing in a solution of the drug, oral administration or by intraperitoneal injections.

### 6.2.2.2 Investigating the effectiveness of Bac5 as an adjuvant

Several recent studies have demonstrated that HDPs and IDR peptides possess adjuvant activities. As we urgently require new treatments for TB infections, the adjuvant capabilities of Bac5 could be investigated in the adult zebrafish infection model using infection progression monitoring as described above. Investigations could begin by testing the effects of Bac5 and CpG, as this compound has been used in successful adjuvant combinations with other peptides in several studies\textsuperscript{[150, 167]}.

### 6.2.2.3 Studies using transgenic zebrafish

#### 6.2.2.3.1 Investigating the effectiveness of Bac5 as prophylaxis

Should Bac5 be found to lack cytotoxic properties in zebrafish embryo experiments outlined above, transgenic zebrafish which constitutively express Bac5 in various cell types could be generated for future studies. This would provide a model for the effects of gene therapy in humans and permit the assessment of the effects of Bac5 on zebrafish and protection of zebrafish from infections throughout development. The study of transgenic zebrafish embryos, using the techniques to monitor infection progression
described in Chapter 3, could be used to inform on the suitability of Bac5 as prophylaxis in immunocompromised individuals. In addition, the study of infected transgenic zebrafish adults, as described for non-transgenic zebrafish above, could be used to inform on the suitability of Bac5 as prophylaxis in healthy hosts. In addition, feeding of transgenic zebrafish embryos to non-transgenic adults to determine the effect of Bac5 as a co- or post-treatment, or as prophylaxis, could be performed as described by Lin et al. in experiments determining the effects of the HDP lactoferricin\textsuperscript{[181]}. 

6.2.2.3.2 Investigating the effects of Bac5 on other infections and inflammatory conditions

HDPs such as Bac5 play a role in infection control and inflammatory conditions. It would therefore be of interest to study the effects of Bac5 on other infections as well as inflammatory conditions and inflammation in the transgenic zebrafish. The Mostowy group study various infections in the zebrafish model, including with \textit{Shigella flexneri}\textsuperscript{[212]}. The Dallman group study inflammation at mucosal surfaces, including the gill and gut. The group have shown that zebrafish are a useful model in which to study the inflammatory effects of high cholesterol diet (HCD) on the mucosal epithelium of the zebrafish gut\textsuperscript{[262]}. In addition, the gills of zebrafish are an alternative model for the effects of irritants, such as smoke or silica, on the respiratory epithelium and demonstrate similar immunopathology to human respiratory illnesses\textsuperscript{[261]}. Further studies using the models which have been developed by the Mostowy and Dallman groups would determine whether Bac5 can protect the host against other infections or control harmful inflammation and protect the host against its damaging effects.

6.2.3 Investigating the importance of selected TCSs on virulence

6.2.3.1 Generation of TCSs mutants for further study

Attempts to construct targeted TCSs operon deletion mutants in \textit{M. marinum} by recombineering and sucrose counter-selection using the pSMT100 system were not successful, as described in Chapter 5. Construction of the TCSs mutants could proceed using the pNIL/pGOAL system\textsuperscript{[247]} for sucrose counter-selection. Constructed TCSs operon deletion strains could then be investigated in terms of their virulence in further
studies, using the zebrafish tools now in place as described in Chapter 3. Identification
of TCSs which are involved in mycobacterial virulence would be significant as these
TCSs would be attractive targets for novel therapies to treat TB disease.
References


254


Appendices

Appendix 1- Maps for plasmids used during this study

Figure A1. Mycobacteria-adapted recombineering vector pYUB854. Empty pYUB854 showing the restriction enzyme sites available for cloning the flanking regions on either side of the hygromycin resistance cassette to cloning sites 1 and 2.

Figure A2. Mycobacteria-adapted recombineering vector pJV53.
Figure A3. Sucrose counter-selection vector pSMT100.
Figure A4. Plasmid used to generate *M. marinum* DsRed strain, pMSP12::DsRed2. The derivative pGFPHYG2 was used to generate *M. marinum* GFP strain. pGFPHYG2 was generated from pMSP12::DsRed2 through substitution of *gfpmut3* for *dsred2*, and disruption of the kanamycin resistance gene by insertion of a hygromycin resistance cassette. Plasmid map taken from Addgene, both plasmids sourced from Addgene (pMSP12::DsRed2 = plasmid # 30171, pGFPHYG2 = plasmid # 30173).
Appendix 2 - Icy protocol scripts

Figure A5. Icy FPC protocol Block 2 (Script Block v1). Generates Microsoft Excel output file path from input folder path (Input 1).

```java
importClass( java.io.File );
importClass( Packages.icy.file.FileUtil );
dir = FileUtil.getDirectory( input1.getAbsolutePath() );
fileName = FileUtil.getFileName( input1.getAbsolutePath() );
FileUtil.createDir( dir + "/save" );
output1 = new File( dir + "/save/" + fileName + "_excel.xls" );
```

Figure A6. Icy FPC protocol Block 4 (Script Block v1). Generates binary image output file path from input folder path (Input 1).

```java
importClass( java.io.File );
importClass( Packages.icy.file.FileUtil );
dir = FileUtil.getDirectory( input1.getAbsolutePath() );
fileName = FileUtil.getFileName( input1.getAbsolutePath() );
FileUtil.createDir( dir + "/save" );
output1 = new File( dir + "/save/" + fileName + "_binary.tif" );
```

// USER PARAMETERS //

// which channel to use when detecting bacteria
// NB: channels are ordered as they appear in the Inspector panel
// Also, the first channel index is 0, not 1
detectionChannel = 2
// detection scale (corresponds to the spot size):
detectionScale = 2
// detection sensitivity:
detectionSensitivity = 50
// precision of the distance histogram (size of the bars in pixels)
histogramPrecision = 10
// MAIN CODE //

// 1) make sure an image is available
sequence = getSequence()

if (sequence == null) {
    MessageDialog.showMessageDialog("No opened image");
    throw "No opened image"; // stops the script and say why
}

// 2) Extract the channel and the regions of interest
rois = sequence.getROI2Ds()
numberOfROIs = rois.size()

// 2.b) Extract the channel containing the bacteria (if need be)
if (sequence.getSizeC() > 1) {
    // replace the original sequence by the extracted channel
    sequence = SequenceUtil.extractChannel(sequence, detectionChannel)
    // don't forget to put the ROI on the extracted channel
    for (i = 0; i < numberOfROIs; i++) {
        sequence.addROI(rois.get(i));
    }
}

// 2.a) Make sure there are 3 ROIs
if (numberOfROIs != 3) {
    MessageDialog.showMessageDialog("Wrong number of ROIs (found " +
    numberOfROIs + ", expected 3)");
    throw "Wrong number of ROIs (found " + numberOfROIs + ", expected
    3)";
    // stops the script and say why
}

// 2.b) Isolate them one by one
for (i = 0; i < 3; i++) {
    roi = rois.get(i)
    if (roi instanceof ROI2DPoint) {
        injection = roi.getPosition2D()
    } else {
        name = roi.getName()
        if (name.contains("yolk")) {
            yolk = roi
        } else {
            fish = roi
        }
    }
}

// 3) Subtract the yolk from the fish roi
fishName = fish.getName()
fish = fish.getSubtraction(yolk)
fish.setName(fishName)

// 4) Detect bacteria inside the fish
// 4.a) create the spot detector toolbox with appropriate parameters
detector = new UDWTWavelet()
scaleParameters = [0, 0, 0, 0, 0]
scaleParameters[detectionScale - 1] = detectionSensitivity
// 4.b) detect spots
detector.detect(sequence, false, true, scaleParameters)
bacteria = detector.getDetectionResult();
```java
numberOfBacteria = bacteria.size()
println("Found " + numberOfBacteria + " bacteria in " + fish.getName() + ".")

// 5) Measure the distance distribution
histogram = []
println("List of distances to the injection site (in pixels):")
for (i = 0; i < numberOfBacteria; i++) {
    // 5.a) retrieve the current bacterium
    spot = bacteria.get(i)
    // 5.b) retrieve its mass center
    center = spot.getMassCenter().asPoint2D()
}

// 5.c) Calculate the distance to the injection site
distance = center.distance(injection)
// 5.d) Output the distance to the script console (for verification)
println(distance)
// 5.e) Accumulate the distance in the histogram
bin = Math.floor(distance / histogramPrecision) | 0
if (histogram[bin] == undefined) histogram[bin] = 0
histogram[bin] = histogram[bin] + 1
println("")

// 5.7) Print out the histogram to the script console
println("Histogram (bin size = " + histogramPrecision + " pixels):")
for (i = 0; i < histogram.length; i++) {
    nb = histogram[i]
    if (nb == undefined) nb = 0
    println(i + " | " + nb)
}
```

**Figure A7. Bacterial Distance Distribution script used to generate the Icy protocol.** 1) Loads the original image file and sets Spot Detector settings. 2) Extracts fish, yolk sac and injection site regions of interest (ROI). 3) Subtracts the yolk sac ROI from the fish ROI. 4) Runs the Icy Spot Detector, designed by Fabrice de Chaumont. 5) Outputs the distances between the detections and the injection site along with a distance histogram to the script console. Script was provided by Dr Alexandre Dufour.
importClass(Packages.plugins.kernel.roi.roi2d.ROI2DPoint)

// Input
sequence = input0

// 2) Extract the channel and the regions of interest
rois = sequence.getROI2Ds()
numberOfROIs = rois.size()

// 2.a) Make sure there are 3 ROIs
if (numberOfROIs != 3) {
    throw "Wrong number of ROIs (found " + numberOfROIs + ", expected 3)";
    // stops the script and say why
}

// 2.b) Isolate them one by one
for (i = 0; i < 3; i++) {
    roi = rois.get(i)
    name = roi.getName()
    if (name.contains("injection")) {
        injection = roi
    } else if (name.contains("yolk")) {
        yolk = roi
    } else {
        fish = roi
    }
}

// Output
output0 = new Array(fish)
output1 = new Array(yolk)
output2 = new Array(injection)

Figure A8. Icy Bacterial Distance Distribution protocol Block 14 (Extract Fish/Yolk/Injection ROIs). Extracts fish, yolk sac and injection site regions of interest (ROI) based on their ROI name from an Icy sequence (image, Input 0). First output (Output 0) = fish ROI data; second output (Output 1) = yolk sac ROI data; third output (Output 2) = injection site ROI data.
importClass(Packages.icy.roi.ROIUtil)
importClass(Packages.icy.util.XLSUtil)
importClass(Packages.java.awt.geom.Point2D)

// Input
fileName = input0
bacteria = input1.getROIs()
injection = input2[0].getPosition2D()
histogramPrecision = input3 // Size of the bars in pixels

// Create Excel workbook
wkbk = XLSUtil.createWorkbook(fileName)
wksht = XLSUtil.createNewPage(wkbk, "Spot Detector")

// Process
XLSUtil.setCellString(wksht, 0, 0, "Number of Bacteria")
numberOfBacteria = bacteria.size()
XLSUtil.setCellNumber(wksht, 1, 0, numberOfBacteria)

// 5) Measure the distance distribution
histogram = []
XLSUtil.setCellString(wksht, 0, 2, "List of distances to the injection site (in pixels):")
for (i = 0; i < numberOfBacteria; i++) {
    // 5.a) retrieve the current bacterium
    spot = bacteria.get(i)

    // 5.b) retrieve its mass center
    center = ROIUtil.getMassCenter(spot)

    // 5.c) Calculate the distance to the injection site.
    distance = center.distance(injection)

    // 5.d) Output the distance to Excel
    XLSUtil.setCellNumber(wksht, 1, 2 + i, distance)

    // 5.e) Accumulate the distance in the histogram
    bin = Math.floor(distance / histogramPrecision) | 0
    if (histogram[bin] == undefined) histogram[bin] = 0
    histogram[bin] = histogram[bin] + 1
}

// 5.7) Print out the histogram
XLSUtil.setCellString(wksht, 3, 0, "Histogram (bin size = " + histogramPrecision + " pixels):")
XLSUtil.setCellString(wksht, 3, 2, "Bin")
XLSUtil.setCellString(wksht, 4, 2, "Frequency")
for (i = 0; i < histogram.length; i++) {
    nb = histogram[i]
    if (nb == undefined) nb = 0
    XLSUtil.setCellNumber(wksht, 3, 3 + i, nb)
    XLSUtil.setCellNumber(wksht, 4, 3 + i, nb)
}

// Save Excel workbook
XLSUtil.saveAndClose(wkbk)
Figure A9. Icy Bacterial Distance Distribution protocol Block 26 (Export to Excel). Calculates the distances between the Wavelet Spot Detector readings and the injection site. Outputs the calculated distances and distance histogram to a specified Microsoft Excel file. Input 0 = Microsoft Excel output file path; Input 1 = sequence containing Wavelet Spot Detector readings as ROIs (with yolk sac region subtracted); Input 2 = Injection site ROI; Input 3 = Histogram bin size (bin size of 10 pixels used during this PhD).

```java
importClass(Packages.icy.roi.ROIUtil)
importClass(Packages.java.util.ArrayList)
importClass(Packages.java.awt.geom.Point2D)
importClass(Packages.plugins.fab.spotDetector.DetectionSpot)
importClass(Packages.plugins.fab.spotDetector.GlobalDetectionToken)
importClass(Packages.plugins.fab.spotDetector.ROISaver)
importClass(Packages.plugins.fab.spotDetector.Point3D)

// Input
inputSequence = input0
detectionSequence = input1

// Global Detection Token
gdt = new GlobalDetectionToken()
gdt.inputSequence = inputSequence

// Populate detection result from ROIs
rois = detectionSequence.getROI2Ds()
gdt.detectionResult = new ArrayList()
for (i = 0; i < rois.size(); i++) {
    ds = new DetectionSpot()
    roi = rois.get(i)
    mc_2D = ROIUtil.getMassCenter(roi)
    mc_3D = new Point3D(mc_2D.getX(), mc_2D.getY(), 0.0)
    ds.points = new ArrayList()
    ds.points.add(mc_3D)
    gdt.detectionResult.add(ds)
}

// Output
output0 = gdt
```

Figure A10. Icy Bacterial Distance Distribution protocol Block 27 (Create Detection Token). Recreates the global detection token used by the Spot Detector plugin to interface with Spot Detector code in subsequent blocks. Input 0 = original input sequence (image); Input 1 = sequence containing Wavelet Spot Detector readings as ROIs (with yolk sac region subtracted); Output 0 = global detection token.
Figure A11. Icy Bacterial Distance Distribution protocol Block 29 (Export with ROIs & Detections).
Uses Icy Spot Detector plugin code to mark detection sites and outputs the image file with detections marked as ROIs. Input 0 = image output file path; Input 1 = Spot Detector global detection token.
Appendix 3 - Zebrafish images from Bac5 sequential injection experiments

<table>
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<tr>
<th>Mock 48hpi</th>
<th>Bac5 48hpi</th>
<th>Mock 48 &amp; 96hpi</th>
<th>Bac5 48 &amp; 96hpi</th>
</tr>
</thead>
</table>

*lysC:GFP; M. marinum*
lysC:GFP; *M. marinum*
Figure A12. Effect of injection of bactenecin-5 (Bac5) to the hindbrain ventricle after 48 hours of *M. marinum* infection on zebrafish neutrophil recruitment at 72hpi. Two dpf *casper Tg(LysC:GFP)* zebrafish embryos were injected into their hindbrain ventricle with approximately 220CFU *M. marinum*. Zebrafish embryos were live-imaged using a fluorescence stereomicroscope 24hpi, individual bacterial burdens calculated using the Icy FPC protocol, and embryos were separated into four groups of equal infection burden. Two groups were treated with Mock or 10ng Bac5 at 48hpi and two groups were treated with repeated doses of Mock or 10ng Bac5 at 48hpi and 96hpi. Zebrafish embryos were live-imaged using a fluorescence stereomicroscope and z-stack images of the hindbrain region acquired at 72hpi and 120hpi. Fluorescence overlay images of the hindbrain region of zebrafish embryos at 72hpi are shown for one representative experiment. At this time point, both Mock injected groups and both Bac5 injected groups have been treated identically, and quantification of the data was performed for the two pooled groups (Mock injection at 48hpi and Bac5 injection at 48hpi). White line represents zebrafish head outline as seen in brightfield images. Scale bar 100 µm. Experiment was performed three times. Sample size (n): 20, 20, 20, 19.
lysC:GFP; *M. marinum*
lysC:GFP; M. marinum
Figure A13. Effect of injection of bacterenecin-5 (Bac5) to the hindbrain ventricle after 48 hours of *M. marinum* infection on zebrafish neutrophil recruitment at 120hpi. Two dpf casper Tg(LysC:GFP) zebrafish embryos were injected into their hindbrain ventricle with approximately 220CFU *M. marinum*. Zebrafish embryos were live-imaged using a fluorescence stereomicroscope 24hpi, individual bacterial burdens calculated using the Icy FPC protocol, and embryos were separated into four groups of equal infection burden. Two groups were treated with Mock or 10ng Bac5 at 48hpi and two groups were treated with repeated doses of Mock or 10ng Bac5 at 48hpi and 96hpi. Zebrafish embryos were live-imaged using a fluorescence stereomicroscope and z-stack images of the hindbrain region acquired at 72hpi and 120hpi. Fluorescence overlay images of the hindbrain region of zebrafish embryos at 120hpi are shown for the same representative experiment as in Figure A12. In this experiment zebrafish were maintained in groups, and therefore zebrafish images do not correspond between the two figures. White line represents zebrafish head outline as seen in brightfield images. Scale bar 100 μm. Experiment was performed three times. Sample size (n): 20, 18, 19, 17.
Appendix 4 - Protein sequence alignments and genomic views for TCSs

Figure A14. Coding sequence amino acid alignments and genomic context views of KdpD/E TCS proteins in *M. tuberculosis* and *M. marinum*. (A-B) Coding sequence alignments of TCSs proteins to be studied, created using ClustalX2. Mtb = *M. tuberculosis* sequence, and Mmar = *M. marinum* sequence. * = fully conserved residue, : = residue conserved within a group of residues highly similar in their properties, . = residue conserved within a group of residues moderately similar in their properties. (C) Genomic context view of KdpD/E operon, created in Geneious. The *M. tuberculosis* sequence is shown first. Yellow = TCSS genes, blue = homologous genes between the two organisms, red = different genes between the two organisms. For clarity, the gene name prefix “MMAR_” has been omitted from the *M. marinum* genomic view.
Figure A15. Coding sequence amino acid alignments and genomic context views of MtrA/B TCSs proteins in *M. tuberculosis* and *M. marinum*. (A-B) Coding sequence alignments of TCSs proteins to be studied, created using ClustalX2. Mtb = *M. tuberculosis* sequence, and Mmar = *M. marinum* sequence. * = fully conserved residue, : = residue conserved within a group of residues highly similar in their properties, . = residue conserved within a group of residues moderately similar in their properties. (C) Genomic context view of MtrA/B operon, created in Geneious. The *M. tuberculosis* sequence is shown first. Yellow = TCSS genes, blue = homologous genes between the two organisms, red = different genes between the two organisms. For clarity, the gene name prefix “MMAR_” has been omitted from the *M. marinum* genomic views. The red bar indicates the position in the *M. tuberculosis* sequence of the additional gene cluster in the *M. marinum* sequence.
Figure A16. Coding sequence amino acid alignments and genomic context views of MprA/B TCS proteins in *M. tuberculosis* and *M. marinum*. (A-B) Coding sequence alignments of TCS proteins to be studied, created using ClustalX2. Mtb = *M. tuberculosis* sequence, and Mmar = *M. marinum* sequence. * = fully conserved residue, : = residue conserved within a group of residues highly similar in their properties, . = residue conserved within a group of residues moderately similar in their properties. (C) Genomic context view of MprA/B operon, created in Geneious. The *M. tuberculosis* sequence is shown first. Yellow = TCS genes, blue = homologous genes between the two organisms, red = different genes between the two organisms. For clarity, the gene name prefix "MMAR_" has been omitted from the *M. marinum* genomic views. The red bars indicate the position in the *M. marinum* sequence of the additional gene clusters in the *M. tuberculosis* sequence.
Figure A17. Coding sequence amino acid alignments and genomic context views of NarL/Rv0845 TCSs proteins in *M. tuberculosis* and *M. marinum*. (A-B) Coding sequence alignments of TCSs proteins to be studied, created using ClustalX2. Mtb = *M. tuberculosis* sequence, and Mmar = *M. marinum* sequence. * = fully conserved residue, : = residue conserved within a group of residues highly similar in their properties, . = residue conserved within a group of residues moderately similar in their properties. (A) Coding sequence alignment of NarL in both species. (B) Coding sequence alignment of Rv0845 in both species and the second Rv0845 homologue in *M. marinum*, MMAR_4783. (C) Genomic context view of NarL/Rv0845 operon, created in Geneious. The *M. tuberculosis* sequence is shown first. Yellow = TCSS genes, blue = homologous genes between the two organisms, red = different genes between the two organisms. For clarity, the gene name prefix "MMAR_" has been omitted from the *M. marinum* genomic views. The red bars indicate the positions in the *M. tuberculosis* sequence of the additional gene clusters in the *M. marinum* sequence.
Figure A18. Coding sequence amino acid alignments and genomic context views of PdtaR and PdtaS TCSs proteins in *M. tuberculosis* and *M. marinum*. (A-B) Coding sequence alignments of TCSs proteins to be studied, created using ClustalX2. Mtb = *M. tuberculosis* sequence, and Mmar = *M. marinum* sequence. * = fully conserved residue, : = residue conserved within a group of residues highly similar in their properties, . = residue conserved within a group of residues moderately similar in their properties. (C-D) Genomic context view of TCSs genes, created in Geneious. The *M. tuberculosis* sequence is shown first. Yellow = TCSs genes, blue = homologous genes between the two organisms, red = different genes between the two organisms. For clarity, the gene name prefix "MMAR_" has been omitted from the *M. marinum* genomic views. (D) The red bar indicates the position in the *M. marinum* sequence of the additional gene in the *M. tuberculosis* sequence.
Figure A19. Coding sequence amino acid alignments and genomic context views of PhoP/R TCS proteins in *M. tuberculosis* and *M. marinum*. (A-B) Coding sequence alignments of TCSs proteins to be studied, created using ClustalX2. Mtb = *M. tuberculosis* sequence, and Mmar = *M. marinum* sequence. * = fully conserved residue, : = residue conserved within a group of residues highly similar in their properties, . = residue conserved within a group of residues moderately similar in their properties. (C) Genomic context view of PhoP/R operon, created in Geneious. The *M. tuberculosis* sequence is shown first. Yellow = TCSs genes, blue = homologous genes between the two organisms, red = different genes between the two organisms. For clarity, the gene name prefix “MMAR_” has been omitted from the *M. marinum* genomic views. The red bars indicate the positions in the *M. tuberculosis* sequence of the additional gene clusters in the *M. marinum* sequence.
Figure A20. Coding sequence amino acid alignments and genomic context views of PrrA/B TCSs proteins in *M. tuberculosis* and *M. marinum*. (A-B) Coding sequence alignments of TCSs proteins to be studied, created using ClustalX2. Mtb = *M. tuberculosis* sequence, and Mmar = *M. marinum* sequence. * = fully conserved residue, : = residue conserved within a group of residues highly similar in their properties, . = residue conserved within a group of residues moderately similar in their properties. (C) Genomic context view of PrrA/B operon, created in Geneious. The *M. tuberculosis* sequence is shown first. Yellow = TCSs genes, blue = homologous genes between the two organisms, red = different genes between the two organisms. For clarity, the gene name prefix "MMAR_" has been omitted from the *M. marinum* genomic views. The red bar indicates the position in the *M. tuberculosis* sequence of the additional gene cluster in the *M. marinum* sequence.
Figure A21. Coding sequence amino acid alignments and genomic context views of RegX3/SenX3 TCSs proteins in *M. tuberculosis* and *M. marinum*. (A-B) Coding sequence alignments of TCSs proteins to be studied, created using ClustalX2. Mtb = *M. tuberculosis* sequence, and Mmar = *M. marinum* sequence. * = fully conserved residue, : = residue conserved within a group of residues highly similar in their properties, . = residue conserved within a group of residues moderately similar in their properties. (C) Genomic context view of RegX3/SenX3 operon, created in Geneious. The *M. tuberculosis* sequence is shown first. Yellow = TCSs genes, blue = homologous genes between the two organisms, red = different genes between the two organisms. For clarity, the gene name prefix “MMAR_” has been omitted from the *M. marinum* genomic views. The red bar indicates the position in the *M. marinum* sequence of the additional gene in the *M. tuberculosis* sequence.
Figure A22. Coding sequence amino acid alignments and genomic context views of TcrX/Y TCSs proteins in *M. tuberculosis* and *M. marinum*. (A-B) Coding sequence alignments of TCSs proteins to be studied, created using ClustalX2. Mtb = *M. tuberculosis* sequence, and Mmar = *M. marinum* sequence. * = fully conserved residue, : = residue conserved within a group of residues highly similar in their properties, . = residue conserved within a group of residues moderately similar in their properties. (C) Genomic context view of TcrX/Y operon, created in Geneious. The *M. tuberculosis* sequence is shown first. Yellow = TCSs genes, blue = homologous genes between the two organisms, red = different genes between the two organisms. For clarity, the gene name prefix “MMAR_” has been omitted from the *M. marinum* genomic views.
Figure A23. Coding sequence amino acid alignments and genomic context views of TrcR/S TCS proteins in *M. tuberculosis* and *M. marinum*. (A-B) Coding sequence alignments of TCS proteins to be studied, created using ClustalX2. Mtb = *M. tuberculosis* sequence, and Mmar = *M. marinum* sequence. * = fully conserved residue, : = residue conserved within a group of residues highly similar in their properties, . = residue conserved within a group of residues moderately similar in their properties. (C) Genomic context view of TrcR/S operon, created in Geneious. The *M. tuberculosis* sequence is shown first. Yellow = TCS genes, blue = homologous genes between the two organisms, red = different genes between the two organisms. For clarity, the gene name prefix "MMAR_" has been omitted from the *M. marinum* genomic views. The red bar indicates the position in the *M. marinum* sequence of the additional gene in the *M. tuberculosis* sequence.
Appendix 5 – Investigation of the growth and transformation efficiencies of Leicester and London *M. marinum* M strains

Figure A24. Growth and transformation efficiency at 28.5°C and 32°C of London and Leicester *M. marinum* M strains. (A-B) Growth of *M. marinum* M strains at 28°C (A) and 32°C (B). Quadruplicate experiments were performed with three biological replicates. Some replicates demonstrated contamination during the experiments and values were removed throughout. These experiments were performed by Yoon Jung Choi during an undergraduate project supervised by myself. Sample size in (A): London 10, Leicester 11. Sample size in (B): 12, 12. Repeated measures two-way ANOVA with Bonferroni’s post-test revealed no significant differences at any time points for both temperatures. (C) Transformation efficiencies of *M. marinum* M strains at 28°C and 32°C. Duplicate reactions were performed in three experiments, with one set of reactions displaying contamination. Sample size (n): 5 for all groups. Transformation efficiencies were calculated as the number of colonies obtained per transformation reaction divided by the quantity of DNA used in µg. One-way ANOVA with Bonferroni’s post-test revealed no significant differences.
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