Mycobacterial Resuscitation
Promoting Factors: Roles and
Mechanisms in Infected
Macrophages

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Abstract

Members of the *Mycobacterium tuberculosis* complex are the causative agents of tuberculosis, a major global health threat to human populations. The majority of infected individuals harbour a latent infection where bacteria persist in a non-replicating, dormant state. Resuscitation promoting factors (Rpfs) are secreted proteins whose peptidoglycan hydrolyzing abilities have been correlated with the reactivation of dormant bacteria; however, the precise molecular mechanisms underlying this remain poorly understood. The individual roles, localisation and expression patterns of *Mycobacterium marinum* Rpf homologues during macrophage infection were investigated by immunofluorescence using custom Rpf-specific polyclonal antibodies and confocal microscopy.

We found Rpfs to associate to the polar ends of bacteria, along the lengths of the bacterial surface and free in the cytoplasm of infected macrophages suggesting a role for these proteins in apical extension and peptidoglycan biosynthesis. No distinct patterns of Rpf localisation were observed in relation to phagosomal mycobacteria. Microscopy based semi-quantification of Rpf expression levels during macrophage infection, and analysis of *in vitro* grown bacteria from logarithmic phase by flow cytometry identified that only a subset of mycobacteria produce Rpf at detectable levels.

Significant protein sequence homology between RpfA and *Listeria monocytogenes* ActA suggested that RpfA could play a similar role in actin polymerisation and bacterial motility in the macrophage cytoplasm as ActA. However, under the conditions tested, ΔrpfA *M. marinum* did not show any differences to wildtype during infection of murine macrophages. Recombinant ActA was demonstrated to have muralytic activity, a previously uncharacterised property of this protein suggesting a function beyond the polymerisation of host cell actin.
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Last but never least; thank you to my little family. Mama and my big brother; love you, this is for you!
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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ADC</td>
<td>Albumin Dextrose Catalase</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
</tr>
<tr>
<td>Amp</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>Arp</td>
<td>Actin Related Protein</td>
</tr>
<tr>
<td>BCG</td>
<td>bacille Calmette-Guerin</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon Dioxide</td>
</tr>
<tr>
<td>CA</td>
<td>Central and Acidic</td>
</tr>
<tr>
<td>CD</td>
<td>Circular Dichroism</td>
</tr>
<tr>
<td>C-di-AMP</td>
<td>Cyclic di-Adenosine Monophosphate</td>
</tr>
<tr>
<td>CLC</td>
<td>Carp Leukocyte Culture</td>
</tr>
<tr>
<td>CRB</td>
<td>Cambridge Research Biochemicals</td>
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<tr>
<td>CV</td>
<td>Column Volumes</td>
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<tr>
<td>DIG</td>
<td>Digoxigenin</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Media</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DosR</td>
<td>Dormancy Survival Regulon</td>
</tr>
<tr>
<td>DUF</td>
<td>Domain of Unknown Function</td>
</tr>
<tr>
<td>ECACC</td>
<td>European Collection of Cell Cultures</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>ESX</td>
<td>ESAT-6 Secretion System</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence Associated Cell Sorting</td>
</tr>
<tr>
<td>F-actin</td>
<td>Filamentous Actin</td>
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</table>
FCS  Foetal Calf Serum
His-tag  Histidine Tag
G-actin  Monomeric Actin
G + C  Guanine and Cytosine
GSP  General Secretion Pathway
HIV  Human Immunodeficiency Virus
IFN-γ  Interferon-γ
IgG  Immunoglobulin G
IPTG  Isopropyl β-D-1-thiogalactopyranoside
LA  Lysogeny Agar
LB  Lysogeny Broth
mAGP  Mycolyl-Arabinogalactan-Peptidoglycan
MDR-TB  Multidrug Resistant Tuberculosis
MOI  Multiplicity of Infection
mRNA  Messenger RNA
Mtb  Mycobacterium tuberculosis
MUF tri-NAG  4-methylumbelliferyl β-D-N,N’,N’’-triacetylchitotrioside
N  Normality
NaF  Sodium Fluoride
NAG  N-Acetyl Glucosamine
NAM  N-Acetyl Muramic Acid
Ni-NTA  Nickel Nitrilotriacetic Acid
NPF  Nucleation Promoting Factor
NRP  Non-Replicating Persister
N-WASP  Neural-WASP
ORF  Open Reading Frame
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>PAGE</td>
<td>Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>PBP1</td>
<td>Penicillin-Binding Protein 1</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline Solution</td>
</tr>
<tr>
<td>RD1</td>
<td>Region of Difference 1</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>RipA</td>
<td>Rpf Interacting Protein A</td>
</tr>
<tr>
<td>RNA-seq</td>
<td>RNA Sequencing</td>
</tr>
<tr>
<td>Rpf</td>
<td>Resuscitation Promoting Factor</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>S2P</td>
<td>Site-two Protease</td>
</tr>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>TEV</td>
<td>Tobacco Etch Virus</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour Necrosis Factor</td>
</tr>
<tr>
<td>tri-NAG</td>
<td>N,N',N''-triacetylchitotriose</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated Region</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>WASP</td>
<td>Wiskott-Aldrich Syndrome Protein</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
<tr>
<td>WH-2</td>
<td>WASP Homology-2</td>
</tr>
<tr>
<td>XDR-TB</td>
<td>Extensively Drug Resistant Tuberculosis</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl β-D-galactopyranoside</td>
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1 Introduction
1.1 Tuberculosis and the Current Situation

In 1882, Robert Koch discovered that the causative agent of the ancient disease tuberculosis (TB) was the acid fast bacillus, *Mycobacterium tuberculosis* (*Mtbc*). To this day, tuberculosis remains a major global health problem and is the leading cause of death by a curable infectious disease. Although the global rate of new TB cases has been in steady decline for a decade, the World Health Organisation (WHO) still estimated 8.6 million new incidences of TB and 1.3 million deaths as a result of this disease in 2012 (WHO 2013; Figure 1), with the majority occurring in Asia and Sub-Saharan Africa.

![Figure 1: Estimated Global TB Incidence Rates, 2012. Dark blue areas correspond with the highest estimated number of new TB cases. The highest TB incident rates are found in Asia and Africa (WHO Global Tuberculosis Report, 2013).](image)

The TB epidemic is complicated by the Human Immunodeficiency Virus (HIV) epidemic. Currently it is estimated that a third of the 40 million people living with HIV/ Acquired Immunodeficiency Syndrome (AIDS) are also co-infected with *Mtbc* and of the 1.3 million deaths caused by TB in 2012, 320 000 were in HIV positive individuals. Up to
14% of the new incident cases in 2012 were among people living with HIV, the majority of which were again concentrated in Sub-Saharan Africa. Globally, this region accounts for 75% of HIV associated TB (Figure 2). The HIV positive sub-population is up to 50 times more likely to develop active TB in a given year (WHO 2013). These individuals are not only at increased risk from reactivation of latent TB infection (Godfrey-Faussett & Ayles 2003) but are more susceptible to rapid progression of the disease following new infection or re-infection (Getahun et al. 2010). In the last decade, HIV infection has emerged as the single biggest risk factor for the development of TB (WHO 2013).

The emergence of multidrug resistant TB (MDR-TB) in the 1990’s, which is defined as resistance to at least the front line anti-TB drugs isoniazid and rifampicin, and more recently extensively drug resistant TB (XDR-TB) has exacerbated the TB epidemic even further. With XDR-TB being defined as resistance to front-line and second-line anti-TB drugs, these two forms of the disease do not respond to standard treatment regimens and require treatment with drugs that are less effective, more toxic and more
expensive for up to two years (WHO 2010). An estimated 38% of the 450 000 people infected with MDR-TB and XDR-TB died in 2012, and with nearly 4% of new TB cases, and 20% of re-infection cases thought to be MDR-TB, it is clear that these forms of TB are currently a massive threat to tuberculosis control (WHO 2013; Espinal 2003).

1.2 Mycobacteria

The majority of bacteria belonging to the phylum *Actinobacteria* are characterised by a gram-positive cell wall and a high guanine and cytosine (G + C) content in their genomic deoxyribonucleic acid (DNA) (Gao & Gupta 2012). *Mycobacterium* belong to this phylum and have a G + C content in excess of 60% (Gao & Gupta 2012; Li et al. 2005; Stinear et al. 2008; Cole et al. 1998).

Mycobacteria are characterised by a cell wall rich in unusual lipids, mycolic acids and polysaccharides that collectively facilitate their survival within macrophages, mediate resistance to antibiotic agents and contribute to the virulence and hardiness of this family (Cole et al. 1998). The mycobacterial cell wall is composed of two distinct layers: the inner cell wall core, known as the mycolyl-arabinogalactan-peptidoglycan (mAGP) complex, that lies adjacent to the cell membrane and is composed of a layer of peptidoglycan covalently attached to arabinogalactan followed by mycolic acids; and the outer segment containing short- and long-chain fatty acid lipids amongst which a number of cell wall proteins including phosphatidylinositol mannosides, lipomannans and lipoarabinomannans (Brennan 2003; Hett & Rubin 2008) are dispersed. Although generally classified as gram-positive organisms due to their thick peptidoglycan layer, lack of a true outer membrane (Hett & Rubin 2008) and phylogenetic analysis (Fu & Fu-Liu 2002), mycobacteria cannot be differentiated by Gram staining. As a result of this stain not penetrating the lipid rich outer cell wall, and in reference to their staining abilities, these organisms are often described as being ‘acid-fast’ (Bhatt et al. 2007) where the organism is able to resist decolourisation by acidic alcohol after being stained with carbol fuchsin (Reynolds et al. 2009).
In humans, TB is mostly caused by *Mtb* and *Mycobacterium africanum* (de Jong et al. 2010). Together, they form a part of the *Mtb* complex- a genetically related group of six *Mycobacterium* species that cause TB in humans and other organisms (Brosch et al. 2004). The remaining members that compose this group are *Mycobacterium canettii*, a rarely encountered mycobacterium that infects humans (Cosma et al. 2003); *Mycobacterium microti*, which infects voles; *Mycobacterium bovis*, the aetiological agent of TB in cattle and other mammals; and *M. bovis* bacille Calmette-Guerin (BCG), an attenuated variant of *M. bovis* commonly used as a vaccine (Cole 2002).

These rod shaped, aerobic bacteria can naturally be divided into two classes: slow-growers and fast-growers that generally form colonies that appear matt and creased (Fregnan & Smith 1962). Generation times vary from up to 24 hours in the case of members of the *Mtb* complex (Cole, 2002) to 3 hours in the case of *Mycobacterium smegmatis* (Smith 2003), a non-pathogenic bacteria often used to study mycobacterial processes.

### 1.2.1 *Mycobacterium marinum*

Of importance to the work described here is *Mycobacterium marinum*, which, although not part of the *Mtb* complex, has been observed to cause ‘fish TB’ during the natural infection of zebrafish (van der Sar et al. 2004). With a faster generation time of 12 hours, *M. marinum* is a close genetic relative of *Mtb* sharing 85 % nucleotide identity, and 3000 orthologs with an average amino acid identity of 85 % (Stinear et al. 2008). Key genes encoding proteins essential for the virulence and pathogenicity of *Mtb* are also conserved in *M. marinum* including the PE/PPE, ESX (Stinear et al. 2008) and Resuscitation promoting factor (Rpf) families. Although the genome of *M. marinum* is 50% larger than that of *Mtb*, the similarities of these two organisms is highlighted by the fact that genes encoding virulence determinants in *Mtb* can complement orthologous *M. marinum* genes (Cosma et al. 2006; Bhatt et al. 2007; Volkman et al. 2004). This smaller genome is thought to be a result of reductive evolution to facilitate the restricted survival of *Mtb* in its niche host, whilst the larger
genome of *M. marinum* is required due to its ability to survive in the environment, humans and a range of ectothermic hosts (Brosch et al. 2001).

### 1.3 Pathogenesis of Tuberculosis

*Mtb* is spread by aerosolized droplets that are inhaled by uninfected individuals allowing the tubercle bacilli to enter the lungs. Often the infection remains in the lungs as pulmonary TB however cases of extrapulmonary TB where the bacteria are disseminated throughout the body are increasing, particularly in the immunocompromised population (Yang et al. 2004). Upon entry into the lungs, the bacteria are phagocytosed by alveolar macrophages; it is within these cells that the bacteria are able to replicate in a membrane bound phagosome as a direct result of their ability to inhibit phagosomal maturation, fusion with lysosomes and acidification of the organelle (Vergne et al. 2005). In the majority of cases, the host immune response is capable of eliminating or controlling mycobacterial infection by mounting a strong inflammatory response (Figure 3)- macrophages, monocytes and neutrophils congregate loosely to form the early granuloma (Russell et al. 2010); the complement system is activated to enhance recognition of the pathogen by macrophages (Knechel 2009); dendritic cells that have come in contact with bacteria migrate to the lymph nodes to begin priming naive T cell lymphocytes (Tailleux et al. 2002) and tumour necrosis factor (TNFα) and interferon γ (IFN-γ) is released inducing phagosome maturation and the production of reactive nitrogen and oxygen species (Schnappinger et al. 2003; Voskuil et al. 2011).

As the protective cell-mediated response is mounted and lymphocytes are activated; macrophages in the loose granuloma differentiate into foamy macrophages, multinucleated giant cells and epithelioid macrophages; become surrounded by a fibrous cuff of collagen and adopt the organised structure of the mature granuloma that is characteristic of TB infection (Guirado & Schlesinger 2013). As infection progresses, there is an accumulation of caseous, necrotic cell debris within the centre of the granuloma where *Mtb* is still able to persist. Increasing damage to the lung
tissues occurs resulting in the granuloma being broken down to release bacteria into the lung cavity and supporting effective transmission of the organism once again (Russell et al. 2009) (Figure 3).

![Figure 3: Life Cycle of Mtb](image)

**Figure 3: Life Cycle of Mtb.** Upon initial exposure to aerosolized *Mtb*, alveolar macrophages phagocytose the bacteria and the innate and adaptive immune systems are activated resulting in either complete clearance of the bacteria, bacterial replication and active TB disease or containment of the infection in the form of a granuloma. Secondary TB can develop years post-initial infection when containment is breached, resulting in the release and reactivation of persistent bacteria and the same possible outcomes as initial infection (adapted from Young et al. 2008).

Approximately 10% of individuals initially infected with *Mtb* will succumb to active disease if the host immune response is unable to eliminate the bacterial load, or contain the infection (Chan & Flynn 2004). The remainder that have managed to successfully control the infection are described as being latently infected whereby the bacteria are present in a non-replicating state with low metabolic activity, and the host is asymptomatic and not contagious (Gengenbacher & Kaufmann 2012). Bacteria that have transitioned to this dormant state are often referred to as non-replicating persisters (NRP) (Wayne & Sohaskey 2001). They are characterised by a thickened, more impermeable cell wall that arises as a result of increased crosslinking of the
peptidoglycan strands in the cell wall in response to the stresses of the granuloma (Gupta et al. 2012; Hett & Rubin 2008) which includes hypoxia (Wayne & Sohaskey 2001), nutrient stress, exposure to reactive oxygen and nitrogen species and lytic enzymes (Schnappinger et al. 2003). An interesting feature of *Mtb* infection is that induction of the adaptive immune response is significantly delayed when compared to other intracellular human pathogens such as *Listeria* (Zenwicz & Shen 2008), however, it is unclear if this delay is significant in the establishment of latent infection (Gupta et al. 2012).

*Mtb* is capable of reactivating from this dormant state any number of years after initial infection to result in active secondary tuberculosis. The primary risk of reactivation is weakening of the host immune system which can be a result of diabetes, obesity, alcoholism, co-infection with HIV/AIDS (Barry et al. 2009) or anti-tumour necrosis factor (TNF) treatment of rheumatoid arthritis or chronic obstructive pulmonary disease (Keane 2004).

### 1.4 Modelling TB Infection

*Mtb* is an obligate human pathogen; its slow growth and requirement for special laboratory facilities (category 3) and training of researchers make studying it in its natural environment difficult and time consuming. The uses of cell lines, animal models and closely related species to *Mtb* have been crucial in developing our understanding of *Mtb* infection. The attenuated *M. bovis* BCG, a slow growing category 2 pathogen and *M. smegmatis*, a fast growing, non-virulent saprophytic species have been extensively used.

Rabbits, guinea pigs and non-human primates are often used to model TB infection, however the most commonly used animal model remains the mouse due to the relative ease of manipulation of the animals and availability of mutant strains and reagents (Flynn 2006). This model has been used to study extra-pulmonary TB (Kong et al. 2009), immune responses, drug and vaccine candidates (Hanif et al. 2011). Both the
acute and chronic stages of infection can be observed although there are limitations. A true latent infection is difficult to model (Scanga et al. 1999) and although the infection is controlled, it is also progressive with bacterial numbers increasing over time. Crucially, the granuloma that develops in mice lacks the characteristic structure observed in human TB infection (Ramakrishnan 2012). Human lungs will contain a spectrum of granuloma types during infection, from a cavity to necrotic to solid to caseous and this heterogeneity is typically not observed during infection of mice with *Mtb*. The use of non-human primates is advantageous because of the formation of caseating necrotic granulomas, the ability to model HIV/AIDS and *Mtb* coinfection and to some extent, latent disease (Iii et al. 2003; Walsh et al. 1996), however due to cost and biocontainment, use of this model is limited.

*M. marinum* is increasingly being used to model TB infection. Beyond the strong genetic similarity to *Mtb*, this organism is also capable of replicating within cultured mammalian cells such as the often used J774 adherent macrophage line from *Mus musculus* (Ramakrishnan & Falkow 1994), the Carp Leukocyte Culture (CLC) cell line established from the peripheral blood mononuclear cells of the common carp (El-etr et al. 2001) and a number of model organisms including the amoeba *Dictyostelium discoideum* (Arafah et al. 2013), *Drosophila melanogaster* (Dionne et al. 2003) and its natural host *Danio rerio*, the zebrafish (van der Sar et al. 2004). Like *Mtb*, *M. marinum* has been demonstrated to reside in a non-acidic intracellular compartment that is indistinguishable when observed in cultured mammalian cell lines (Barker et al. 1997). It is an opportunistic pathogen of humans, most commonly affecting the hand and extremities where the temperature is low enough to support *M. marinum* growth. Infection causes the formation of dermal granulomas that are pathologically indistinguishable to those caused by *Mtb* (MacGregor 1995). Further advantages arise from the lower biohazard classification of *M. marinum* meaning it does not require specialized training or facilities for handling.

The zebrafish-*M. marinum* infection model is particularly attractive due to the granulomatous TB like disease that can be observed. The bacteria cause similar caseating necrotic granuloma’s, and zebrafish possess an innate and an adaptive immune system composed of macrophages, T- and B-cells that allow for close
monitoring of host-pathogen interactions *in vivo* (Swaim et al. 2006). Not only is the zebrafish a genetically tractable organism, but sequencing of its genome identified that approximately 70% of human genes have clear orthologues in zebrafish (Howe et al. 2013). The embryos are optically transparent allowing for real time observations of the early stages of infection (Davis et al. 2002). Their development is rapid, *ex-utero* and require no ethical approval until 6 days post fertilisation by which point they are capable of independent feeding (Fleming 2007).

Use of the zebrafish model is limited by the unavailability of antibodies and reagents for immune analysis although this is currently in development (Lawson & Wolfe 2011). The granulomas caused by *M. marinum* seem to contain few lymphocytes in comparison to the numbers observed in humans (Swaim et al. 2006) and lung pathology cannot be observed. Zebrafish contain a novel family of leukocyte regulators, the novel immune-type receptor genes, that have no orthologues in humans (Yoder et al. 2004) highlighting an inherent problem associated with modelling infectious diseases.

**1.4.1 Intracellular Movement of *M. marinum***

It is well recognised that many bacterial pathogens such as *Listeria monocytogenes*, *Shigella flexneri* and *Burkholderia pseudomallei* are able to polymerise host actin to facilitate their intracellular spread- a key factor in the virulence of these organisms. *M. marinum* has been observed to escape the phagosome and enter the cytoplasm of bone-marrow derived macrophages where it is able to polymerise host cell actin and form short, highly-crosslinked actin tails that allow the bacteria to become motile and spread intracellularly in a manner similar to that displayed by *L. monocytogenes* (Stamm et al. 2003) (Figure 4a). Cytosolic entry of *Mtb*, but not the formation of actin tails, has also been observed by electron microscopy (McDonough et al. 1993; van der Wel et al. 2007). Both organisms seem to require a functional region of difference 1 (RD1) gene that encodes ESX-1 in order to escape the phagosome.
An extremely interesting development was the observation that *M. marinum* could spread intracellularly in the amoeba *Dictyostelium* via an F-actin based structure that the authors called an ejectosome, and that the formation of this ejectosome required an intact ESX-1 secretion system (Hagedorn *et al.*, 2009) (Figure 4b). These authors also reported that *Mtb* is able to form ejectosomes in the same manner as *M. marinum* after translocation in *Dictyostelium*.

![Figure 4: M. marinum Polymerises Host Actin to Form Actin Tails and Ejectosomes.](image)

**Figure 4:** *M. marinum* Polymerises Host Actin to Form Actin Tails and Ejectosomes. (A) CLC macrophages infected with *M. marinum* expressing GFP (green) show actin tails that have been stained for F-actin using Alexafluor Phalloidin in red (taken from Stamm *et al.* 2003). (B) GFP expressing *M. marinum* (green; black arrowhead) was used to infect *Dictostelium*. F-actin was stained for using Phalloidin (red). Bacteria can clearly be seen to span the membrane of the cell via an ejectosome (white arrowhead) (taken from Hagedorn *et al.* 2009).

Actin exists in two forms- as monomeric (G) actin and filamentous (F) actin that is formed from a specific arrangement of G actin. In order for actin polymerisation to occur, first a nucleation step is necessary whereby the first monomers are attached to F actin. This step is kinetically unfavourable in comparison to elongation which is predicted to occur 10 times faster than nucleation, and therefore requires activation by the Arp2/3 complex. This complex is formed by 5 subunits and the actin related proteins (Arp) 2 and 3 that are structurally similar to F actin, and itself requires activation by members of the Wiskott-Aldrich syndrome protein (WASP) family. The WASP family are part of a larger family of cellular proteins named NPF’s or nucleation promoting factors. WASP is able to bind monomeric actin via a C-terminal WASP homology region (WH2) and at the same time bind the Arp2/3 complex via a C-terminal central and acidic domain (CA) to induce a conformational change in the
Arp2/3 complex resulting in its activation and the polymerisation of a new actin filament at an angle akin to a Y-branched structure (Lambrechts et al. 2008).

Like *M. marinum*, *L. monocytogenes* is also able to escape the host phagosome. ActA has been found to induce actin polymerisation and thus aid intracellular movement of the bacteria within the cytosol (Domann et al. 1992). It is asymmetrically distributed on the cell’s surface, localizes specifically to the site of actin comet tail formation (Kocks et al. 1993), and functions as a mimic of the WASP family of proteins by interacting directly with the Arp2/3 complex, as indicated by its ability to form actin tails in fibroblasts lacking neural-WASP (N-WASP) proteins (Lommel et al. 2001). It is thought that *M. marinum* does not secrete an NPF mimic, and polymerises actin by a different mechanism- by the recruitment of cellular WASP proteins to the site of actin tail formation in a similar fashion to IcsA from *Shigella flexneri* (Figure 5). Currently, the mycobacterial protein responsible for this recruitment remains unidentified (Stamm et al. 2005).

**Figure 5: Proposed Mechanisms of Actin Polymerisation by Intracellular Pathogens.** *L. monocytogenes* ActA functions like a WASP mimic to directly interact with the Arp2/3 complex and actin monomers, via the central and acidic (CA) region and WH2 (WASP homology-2) domain to induce polymerisation of actin. The proposed mechanism of actin nucleation in *M. marinum* is thought to be similar to that of IcsA from *Shigella flexneri* whereby N-WASP is directly recruited to the bacterial surface allowing binding of G-actin and the Arp2/3 complex. The protein involved in actin polymerisation in *M. marinum* is currently unidentified. (Adapted from Stevens et al. 2006)
1.5 Secreted Bacterial Proteins

It is clear that *Mtb* is a highly adapted, very successful pathogen and this is in part due to the large number and variety of proteins that it is able to secrete. A number of secretion pathways exist to support this including the conserved general secretion (sec) pathway, twin-arginine transporter (Tat) pathway and one of five ESX secretion systems (Champion & Cox 2007). Many of these secreted proteins have been shown to be essential for the virulence and pathogenicity of this organism having been implicated in cell wall processes and lipid metabolism (Målen et al. 2007), respiration and metabolism, regulation and signal transduction (Cole et al. 1998), promoting survival of the organism within the macrophage and immune evasion (Forrellad et al. 2013).

Although much progress has been made in understanding the host-pathogen interactions during infection with *Mtb*, and the roles proteins play, approximately 59% of exported proteins identified in this organism have no confidently annotated function (McCann et al. 2011). Many are thought to be involved in immunogenicity and inducing the host immune response such as those belonging to the CFP-10/ESAT-6 family of proteins that are expressed during infection and are also involved in zinc and iron uptake (Maciag et al. 2007) or the glycine rich PE/PPE family of proteins that are coded for by nearly 10% of the *Mtb* genome and are thought to modulate innate immune responses (Sampson 2011). Others have been implicated in key biological processes such as the reactivation of NRP cells by RpfS (Mukamolova et al. 1998; Mukamolova et al. 2006). With a third of the global population thought to be latently infected, this large reservoir of infection highlights the urgent need to understand mycobacterial persistence and the precise mechanisms of reactivation from dormancy (Young et al. 2009).
1.5.1 Resuscitation Promoting Factors

The founding member of this family is the single, secreted and essential Rpf from the ubiquitous bacterium, Micrococcus luteus (Mukamolova et al. 1998) however it has since been observed that multiple homologues of this protein are widespread among the Actinobacteria species (Schroeckh & Martin 2006) with five homologues existing in Mtb (RpfA-E) and four (RpfA, B, C and E) having been annotated in the closely related M. marinum. The presence of multiple homologues of these genes in Mycobacterium leprae which is thought to possess the minimal genome required for mycobacteria to be pathogenic suggests an important physiological role for Rpf (Error! Reference source not found.).

1.5.1.1 Biological Effects

When first identified, the secreted M. luteus Rpf was found to increase the viable cell count of dormant M. luteus, induce growth of extensively washed cells and reduce reactivation time at picomolar concentrations. Interestingly these growth promoting effects have cross-species activity and the M. luteus Rpf has been found to stimulate the growth of various mycobacterial species including Mtb, M. bovis BCG and M. smegmatis (Mukamolova et al. 1998; Mukamolova et al. 2002) highlighting the conservation of function between these proteins. Additionally, RpfS have been observed to be muralytic enzymes, whose cell wall digesting abilities have been correlated with their growth stimulating and resuscitation effects (Mukamolova et al. 2006).

The functional hierarchy of the Mtb RpfS can most clearly be observed in various infection models and during resuscitation from dormancy models. Whereas, individual or double deletions of the RpfS from Mtb were found to also be dispensable for growth in vitro and in vivo (Downing et al. 2004; Tufariello et al. 2004), triple deletion mutants have been observed to be differentially attenuated in a mouse model of infection and to resuscitate poorly (Downing et al. 2005) indicating a certain degree of functional
specialization. For example, deletion of only \textit{rpfB} in \textit{Mtb} is sufficient in itself to delay mycobacterial reactivation in mice in comparison to the Erdman strain- a phenotype that is corrected upon complementation of this gene (Tufariello et al. 2006). Disruption of \textit{rpfB} alongside \textit{rpfA} identified that this mutant was significantly delayed in its ability to reactivate in a mouse model of infection. Again, this phenotype is not observed when the \textit{rpfB} gene remains undisrupted. Additionally, this work identified that growth of the \textit{ΔrpfAB} mutant in activated and resting macrophages was significantly lower in comparison to the Erdman wild type (Russell-Goldman et al. 2008) however it should be noted that complementation of these genes did not reverse this poor growth phenotype. Interestingly, this mutant altered the immune response of the host and induced higher levels of TNF-α and interleukin-6 in comparison to the Erdman wildtype in infected macrophages that could be corrected by complementation with \textit{rpfA} and \textit{rpfB}. Double knockouts of other combinations of Rpfs did not display these reactivation defects suggesting a functional importance for RpfB over the other homologues that exist.

The construction of a quintuple mutant that was lacking all Rpfs in \textit{Mtb} identified that all of these proteins are collectively dispensable for growth \textit{in vitro} however the strain displays delayed colony formation when plated onto Middlebrook 7H11 agar that could be corrected by the complementation by either \textit{rpfB} or \textit{rpfE}. This same study also showed that multiple Rpf deletion mutants that were specifically lacking \textit{rpfB} or \textit{rpfE} were exquisitely sensitive to the detergent, sodium dodecylsulphate (Kana et al. 2008). Taken together, these observations again, tentatively highlight the potential significance of RpfB above the other Rpfs of \textit{Mtb}.  

Figure 6: Multiple Sequence Alignment of Mycobacterial Rpfs. Aligned amino acid sequences of Rpf homologues from *M. marinum* (Mm), *Mtb* (Mt) and *M. leprae* (Ml) using ClustalW (available at the European Bioinformatics Institute server, www.ebi.ac.uk) and viewed in Jalview. Colours represent aliphatic/hydrophobic residues in pink (ILVAM), aromatic residues in orange (FWY), positive in red (KRH), negative in green (DE), hydrophilic residues in blue (STNQ), proline or glycine in purple (PG) and cysteine in yellow (C). Conservation refers to extent of conservation of the physical-chemical properties of the amino acids; quality refers to the extent of sequence homology. The higher the bar, the more similar the amino acid properties are, or the higher the extent of homology. Consensus refers to the dominant conserved amino acid in the alignment.
Figure 6 cont.: Multiple Sequence Alignment of Mycobacterial RpfS. Aligned amino acid sequences of Rpf homologues from *M. marinum* (Mm), *Mtbc* (Mtbc) and *M. leprae* (Ml) using ClustalW (available at the European Bioinformatics Institute server, www.ebi.ac.uk) and viewed in Jalview. Colours represent aliphatic/hydrophobic residues in pink (ILVAM), aromatic residues in orange (FWY), positive in red (KRH), negative in green (DE), hydrophilic residues in blue (STNQ), proline or glycine in purple (PG) and cysteine in yellow (C). Conservation refers to extent of conservation of the physico-chemical properties of the amino acids; quality refers to the extent of sequence homology. The higher the bar, the more similar the amino acid properties are, or the higher the extent of homology. Consensus refers to the dominant conserved amino acid in the alignment. The conserved catalytic glutamine (E), adjacent to a cysteine residue is highlighted with a blue arrow.
Figure 6 cont.: Multiple Sequence Alignment of Mycobacterial Rfps. Aligned amino acid sequences of Rpf homologues from *M. marinum* (Mm), *Mtb* (Mtb) and *M. leprae* (Mi) using ClustalW (available at the European Bioinformatics Institute server, www.ebi.ac.uk) and viewed in Jalview. Colours represent aliphatic/hydrophobic residues in pink (ILVAM), aromatic residues in orange (FWY), positive in red (KRH), negative in green (DE), hydrophilic residues in blue (STNQ), proline or glycine in purple (PG) and cysteine in yellow (C). Conservation refers to extent of conservation of the physico-chemical properties of the amino acids; quality refers to the extent of sequence homology. The higher the bar, the more similar the amino acid properties are, or the higher the extent of homology. Consensus refers to the dominant conserved amino acid in the alignment.
1.5.1.2 Gene Expression

Further evidence supporting that the Rpf homologues of *Mtb* may have slightly different and specialised functions arises from research into the transcription and regulation of these genes as gene expression profiling of individual Rpf deletions in *Mtb* results in some upregulation of the remaining genes (Downing et al. 2004).

The expression profiles of all 5 Rpf homologues in *Mtb* show that all of these proteins are expressed during early exponential growth in mouse lungs (Tufariello et al. 2004). Expression of these genes *in vitro* is induced in a growth phase dependant manner and these levels change in reaction to a number of different stresses (Gupta et al. 2010) indicating that these proteins may serve individual functions. It was found that all of the Rpf s are expressed in the early stages of resuscitation from non-culturability however *rpfC* was consistently more highly expressed compared to the other homologues at all stages of growth. The expression profiles from this study suggest that *rpfC* and *rpfD* may be required during stationary phase and persistence, whilst *rpfB* and *rpfE* may be required during active growth. It is interesting to note from this body of work that *rpfE* was highly expressed under low oxygen and acid stress conditions, and to a lesser extent *rpfD*, that are reminiscent of those found within the phagosome indicating a possible role for this protein whilst *Mtb* is in the phagosome and/or subjected to hypoxia and acid stress. Additionally, the upregulation of *Mtb rpfD* and *E* in response to acidification and an increasing concentration of Cl⁻ (a feature of phagosome maturation) has been independently confirmed by Tan et al. (2013).

*RpfA* has been observed to be negatively regulated by a homologue of the transcriptional regulator- cAMP receptor protein that is thought to bind the promoter region of this gene and repress its expression (Rickman et al. 2005). Characterisation of *rpfA* from *Streptomyces coelicolor* by in silico analysis and its adjacent 5’ untranslated region has identified a riboswitch thought to be the ydaO element (Haiser et al. 2009; Barrick et al. 2004). The ydaO motif has been shown to bind cyclic diadenosine monophosphate (c-di-AMP) which induces the conformational change that results in the repression of transcription of downstream genes (Nelson et al. 2013; Block et al. 2010). This specific motif is associated with cell wall hydrolases,
transporters involved in remodelling of the bacterial cell wall and osmotic shock responses (Barrick et al. 2004) implicating a role for \textit{rpfA} in osmotolerance or protection. Hatzios et al. confirmed this in 2013 when they identified \textit{rpfA} as one of 100 genes to be significantly upregulated when \textit{Mtb} was exposed to sodium chloride.

Microarray analysis has identified a role for the alternative sigma factor SigD of \textit{Mtb} in the positive regulation of \textit{rpfC} only (Raman et al. 2004). The SigD regulon has been shown to be upregulated during nutrient stress and starvation, possibly implying that RpfC may be required for growth and survival during low nutrient conditions (Betts et al. 2002). There is further evidence that this \textit{rpf} may be negatively regulated by the MprAB two-component system of \textit{Mtb} (Pang et al. 2007) which regulates the expression of many genes in the sigD and the dormancy survival regulons (dosR). The latter is overexpressed in bacteria experiencing dormancy inducing conditions and is responsive to hypoxia (Bacon et al. 2004) and nitric oxide stress (Voskuil et al. 2003). A homologue of a site-two protease (S2P) that is involved in membrane composition in \textit{Mtb} has also been found to be a positive regulator of \textit{rpfC} (Makinoshima & Glickman 2005). The observation that a number of different bacterial systems are able to influence expression of \textit{rpfC}, particularly those involved in the stress response and dormancy further implicates RpfC as necessary for survival during such environments.

\subsection*{1.5.1.3 Protein Structure and Function}

This family of proteins are characterised by a conserved 70 amino acid Rpf domain flanked by extra N- and C- terminal domains that differentiate these proteins from each other and are predicted to have a single disulphide bond. RpfA in \textit{Mtb} and \textit{M. marinum} is characterised by proline-alanine rich repeats following the Rpf domain. In both organisms, RpfB is characterised by the presence of multiple domains of unknown function 348 (Pfam: DUF348) and a G5 domain that appears to be widely found amongst proteins involved in biofilm formation and cell wall degradation (Bateman et al. 2005).
A lipophilic anchor can be found on the N-terminus of *Mtb* RpfB suggesting that this protein may be cell-wall associated (Mukamolova et al. 2002) however, it is absent in RpfB from *M. marinum* (Figure 7). The remaining Rpfs, including all of those in *M. marinum*, are likely to be secreted due to the presence of predicted N-terminus secretion signals (http://www.cbs.dtu.dk/services/SignalP). The precise function that these differences may assign to the individual proteins, if any, is unknown, however, they may contribute to the proposed specialised functions of the individual Rpfs.

The ability of lysozyme and Rpf to hydrolyse peptidoglycan has previously been demonstrated using zymograms, and the fluorogenic substrate 4-methylumbelliferyl-β-D-N,N′,N″-triacyctethylchitotrioside (MUF tri-NAG), a model analogue of bacterial peptidoglycan that when cleaved at the β-1-4-glycosidic bond releases MUF which can be monitored fluorescently (Telkov et al. 2006; Mukamolova et al. 2006). Structural predictions of the conserved Rpf domain suggested that this domain formed a c-type lysozyme like fold (Cohen-Gonsaud et al. 2004). The presence of such a fold was confirmed by NMR of the conserved domain from *Mtb* RpfB (Figure 8a). This structure was shown to possess a peptidoglycan binding site similar to that found in lysozyme and lytic transglycosylases, both of which are capable of binding tri-NAG (N,N′,N″-
triacetylchitotriose), a trimer of N-acetyl glucosamine (NAG) resulting in cleavage of one of the sugars that forms peptidoglycan. It is thought that the mechanism of binding to tri-NAG is also conserved between lysozyme and Rpf (Cohen-Gonsaud et al. 2005).

More recently, the crystal structure of a truncated form of RpfB (residues 194-362) was solved at 1.8 Å (Ruggiero et al. 2009) corroborated the presence of a lysozyme like fold. The authors observed a comma-like shape to this construct that was composed from the globular, negatively charged, conserved catalytic domain and further identified an associated elongated structure, the glycine rich G5 domain, which could be involved in anchoring the protein to the mycobacterial cell wall from where it can exert its function (Figure 8b). The G5 domain is composed of a novel superfine-structure formed from two triple-stranded β-sheets (β-triple helix- β) connected by a central motif. Interestingly, interaction modelling of this structure with the accepted model of bacterial peptidoglycan structure indicates that the adhesive properties of the G5 domain allow it to interact with the cross-linking peptides of the cell wall. This facilitates the correct positioning of the catalytic domain to be able to

![Figure 8: Structure of the Rpf Domain from Mtb RpfB. (A) Structural imposition of the C-terminal residues of RpfB from Mtb, specifically 274-362 (red) and c-type lysozyme. Note the same position of the catalytic glutamate (position Glu292 in RpfA and Glu35 in lysozyme) and the presence of a GXXQ turn that is conserved among soluble lytic transglycosylases, Rpf, C- and G-type lysozyme. (Taken from Cohen-Gonsaud et al. 2005). (B) Crystal structure of the catalytic Rpf domain and the associated G5 domain (residues 194-362) composed of a β-triple helix- β, forming a comma-like shape (adapted from Ruggiero et al. 2009).](image-url)
cleave the β-1-4-glycosidic bond of the NAM-NAG polymer supporting the idea that RpfB may be cell surface attached however this model remains to be experimentally verified.

Protein crystallography has been used to solve the structure of the Rpf domain in *Mtb* RpfE (residues 98-172). It was found that the domain adopts the characteristic Rpf fold however the catalytic cleft is narrower, and the area surrounding the catalytic glutamate is more positively charged compared to the previously described negatively charged binding pocket (Mavrici et al. 2014). These differences, which are also conserved in the Rpfs from *M. leprae* suggest that the Rpfs may be capable of discriminating between modifications in peptidoglycan and are likely to contribute to the presumed functional differences of the Rpf homologues.

The presence of a key glutamic acid residue (Glu292) in the Rpf domain that is also conserved in lysozyme and in two lytic transglycosylases provides further evidence for the enzymatic function of Rpfs. In theoretical proposals of the mechanism of action of Rpf, this residue is thought to be indispensible for activity (Squeglia et al. 2013) however it should be noted that mutation of this catalytic residue suppresses the resuscitation effects of this protein but does not completely abolish muraletic activity (Cohen-Gonsaud et al. 2005; Mukamolova et al. 2006).

Rpf interacting protein A (RipA) is a secreted, cell wall associated endopeptidase that was first identified in a yeast two-hybrid screen to interact with *Mtb* RpfB (Hett et al. 2007). This protein is essential for normal cell division and growth as its deletion results in arrested growth and the formation of long branched cells that are not characteristic of *M. smegmatis* growth (Hett et al. 2008), a phenotype that is reversible upon induction of ripA expression. RipA has been found to interact with *Mtb* RpfE and function synergistically with RpfB to hydrolyze cell wall peptidoglycan *in vitro*. Both proteins are capable of digesting peptidoglycan and have been demonstrated to localize to the septa of logarithmic phase bacteria (Hett et al. 2007; Hett et al. 2008) indicating that some of the Rpfs may work in a complex to digest the mycobacterial
cell wall and resuscitate from dormancy. This activity appears to be modulated by the peptidoglycan synthesizing enzyme penicillin-binding protein 1 (PBP1) whose depletion under a tetracycline-inducible promoter system results in the formation of short cells that are poorly separated. Addition of PBP1 inhibits the synergistic hydrolysis of FITC-labelled cell wall substrates by the RipA-RpfB complex. The PBP1-RipA interaction occurs at the same C terminal residues as RipA-RpfB suggesting competition between RpfB and PBP1 for binding (Hett et al. 2010).

The recent crystal structure of RipA has identified that the active site of the catalytic domain is physically obstructed by another domain whose function is not known (Pfam: PB015164) suggesting that this protein likely requires activation by proteolysis prior to being able to exert its hydrolytic function (Ruggiero et al. 2010). Dysregulation of RipA causes it to behave as an autolysin, with lethal consequences, suggesting that RipA activation is tightly regulated post-transcriptionally (Chao et al. 2013) although the precise mechanisms are yet to be identified. Interestingly, in Mtb, the two component system, MtrAB, which localises to the bacterial cell wall, septa and poles, has also been implicated in expression of ripA (Plocinska et al. 2012).

The exact mechanism of how the muralytic activity of RpfS results in resuscitation from dormancy is currently unknown. Initially, due to their potent growth promoting abilities, the RpfS were thought to act as cytokines (Mukamolova et al. 1998) however a receptor has yet to be identified. One hypothesis is that Rpf action is able to overcome the physical restraint of a highly crosslinked peptidoglycan cell wall that exists during dormancy resulting in the resumption of bacterial growth and cell division (Ravagnani et al. 2005). An alternative mechanism may be that muropeptides, small fragments of the peptidoglycan cell wall, are released during hydrolysis and act as signalling molecules to promote resuscitation (Keep et al. 2006). It is predicted that the muropeptides released are capable of binding serine/threonine protein kinases that are found on the bacterial surface which results in an intracellular signalling cascade and thus a bacterial response that may ultimately be resuscitation (Kana & Mizrahi 2010; Shah et al. 2008).
An attractive model has been proposed whereby during latent infection, which is dominated by dormant bacilli, some active bacilli remain to ‘scout’ the environment. If it is not conducive for replication, the bacteria die, with a small pool of scouts remaining. Once conditions are favourable, the scouts release Rpfs to reactivate the remainder of the dormant population resulting in the development of secondary disease (Votyakova et al. 1994; Gengenbacher & Kaufmann 2012). The detection ofRpfs in infected human tissues (Davies et al. 2008) and the presence of large numbers of Rpf-dependent cells in sputum samples from TB patients has already been confirmed (Mukamolova et al. 2010). As such, there remain many questions that need to be addressed, and importantly, experimentally verified, in order to elucidate the precise mechanism by which Rpfs resuscitate dormant bacteria.

1.5.1.4 Immunology

*Mtb* secretes an extensive amount of proteins that may be key to the development of a new vaccine. The current approved vaccine for tuberculosis is the BCG that is prepared from an attenuated strain of *M. bovis* and has been in use for many years although questions have arisen regarding its efficacy in adults against pulmonary tuberculosis and its inability to protect from reactivated tuberculosis (Andersen & Doherty 2005).

As a result of the important role that Rpfs play in the maintenance and reactivation of *Mtb* infection and the urgent requirement for a novel vaccine candidate, there have been a number of studies looking into the protective abilities of Rpfs or strains deficient in their production. Mice immunized with full length *Mtb* purified Rpf proteins displayed both a humoral and T–cell response with the exception of those injected with RpfC (Yeremeev et al. 2003) and quadruple Rpf mutants in *Mtb* that retained either RpfE or RpfB were found to be similar to BCG in immunogenicity and their ability to induce protective immunity in mice (Kondratieva et al. 2011; Romano et al. 2012; Lee et al. 2014). The use of a subunit vaccine consisting of RpfE fused with ESAT-6 yielded a robust immune response in mice (Xin et al. 2013) and when all of the
Mtb Rpfs, with the exception of RpfE, were tested against classical Mtb antigens such as heat shock protein (hsp) and the ESAT-6/CFP-10 complex ESX-A/ESX-B, they were found to be significantly more immunogenic. This implicates Rpfs as promising candidates for new vaccines (Kassa et al. 2012) or for immunodiagnosis of TB (Chegou et al. 2012). Clearly the fact that RpfA and, in particular, RpfD are able to induce the production of antigen-specific cytokines that can be detected years after initial infection in individuals that have not suffered from secondary tuberculosis (Commandeur et al. 2011; Riaño et al. 2012) indicates that we may be able to harness these proteins to boost the immune response toward reactivating mycobacteria and control TB infection through inhibition of resuscitation. The increased inflammatory response to RpfA and D, in comparison to those seen in pulmonary TB patients, has led to attempts to use IFN-γ responses to these proteins to aid the identification of LTBI (Huang et al. 2013).

There is also some evidence that Rpfs can also be targeted by a class of low molecular weight inhibitors, or NPT (2-nitrophenylthiocyanate) compounds that inhibit resuscitation from dormancy (Demina et al. 2009) which may represent a novel way of inhibiting reactivation in latently infected individuals.

### 1.5.2 Actin Assembly-Inducing Protein

We have recently identified that RpfA shares over 50% sequence homology to ActA at the protein level. A multiple sequence alignment of RpfA from Mtb and M. marinum against ActA from L. monocytogenes (Figure 9) also shows that the invariant catalytic glutamate that is important for the muralytic activity of Rpfs and other lytic transglycosylases is conserved between these three proteins.

The many parallels between the intracellular life cycles of L. monocytogenes and M. marinum are striking. Rpfs are present in both organisms (Pinto et al. 2013), both pathogens form similar actin tails to facilitate their motility, and the similarity between RpfA and ActA suggests an important and precise role for RpfA in mycobacterial
intracellular movement via actin tail formation. Additionally, this information may infer that ActA could be a bifunctional protein with a muralytic function that warrants further investigation.
Figure 9: Multiple Sequence Alignment of RpfA from Mtb and M. marinum and ActA from L. monocytogenes. Absolutely conserved residues are highlighted in red, and similarly grouped amino acids are in yellow. The blue arrow highlights the catalytic glutamate that is conserved in all 3 proteins. (Mt, Mtb; Mm, M. marinum; Lm, L. monocytogenes).
1.6 Aims and Objectives

The aim of this project was to gain insight into the mechanism of action and roles of the Rpf homologues to better understand mycobacterial pathogenesis and resuscitation from dormancy. To this end, ΔRpfA, B and E from *M. marinum* were characterised during infection. Additionally, we sought to decipher the possible role of RpfA in actin tail formation and hypothesised that ActA from *L. monocytogenes* may have muralytic activity.

The specific objectives were to:

- Generate recombinant *M. marinum* Rpf and *L. monocytogenes* ActA for use in *in vitro* based assays designed to test muralytic activity;
- Begin to elucidate the specific roles of individual Rpfs in a panel of over-expressing strains and deletion mutants by characterising the effect of Rpf deletion on survival within macrophages;
- Use recombinant Rpf to raise polyclonal Rpf-specific antibodies for use in immunofluorescence based studies to characterise the expression and localisation patterns of Rpfs during infection in a murine macrophage/ *M. marinum* model;
- Monitor ΔrpfA *M. marinum* for actin tail and ejectosome formation.
2 Materials and Methods
2.1 Reagents and Media

2.1.1 Reagents

All reagents used were purchased from Fisher Scientific UK Ltd, or Sigma-Aldrich Company Ltd unless otherwise stated. All antibodies were obtained from Invitrogen, Life Technologies unless otherwise stated.

2.1.2 Media

Luria broth (LB) was prepared by dissolving 10 g/L tryptone, 5 g/L yeast extract and 5 g/L sodium chloride in distilled water and autoclaved prior to use. Luria agar (LA) was prepared as for LB with the addition of agar to 1.5 %.

7H9 Middlebrook broth, and 7H10 Middlebrook agar was prepared according to the manufacturers specifications. Both were supplemented with 10 % Albumin-Dextrose-Catalase (ADC) immediately prior to use. ADC was prepared by dissolving 50 g/L Bovine serum albumin, 20 g/L D-glucose and 8.5 g/L sodium chloride in water prior to filter sterilisation.

Dulbecco’s Modified Eagle’s Medium (DMEM) was supplemented with 10 % foetal calf serum and 2 mM L-glutamine for maintenance of J774 macrophages.

2.2 Bacterial Strains

2.2.1 Stocks

Frozen stocks for all strains were prepared by mixing 800 μl of mid-log phase bacteria with 400 μl 75 % sterile glycerol in a 1.5 ml cryogenic tube (VWR) and stored at -80 °C.
2.2.2 Bacterial Strains and Cultivation

A list of the bacterial strains used in this work can be found in Table 1. Starter cultures of *E. coli* were inoculated by resuspending bacterial cells scraped from the frozen glycerol, or from a singly colony from LA plates in 5 ml LB. Cultures were incubated overnight at 37 °C, with shaking at 200 rpm.

*M. marinum* M starter cultures were inoculated as above in 7H9 containing all supplements and any antibiotics that are required and incubated at 32 °C for 2 weeks, without shaking.

*L. monocytogenes* EGD-e was cultured in LA or LB and incubated at 32 °C without shaking for 2 days.

*M. luteus* Fleming strain 2665 (NCTC) was grown in LA or LB and incubated at 37 °C, shaking at 200 rpm for 2 days.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> DH5α</td>
<td>For DNA propagation and cloning</td>
<td>Bioline</td>
</tr>
<tr>
<td><em>E. coli</em> BL21 (DE3)</td>
<td>Protein expression host</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>WT <em>M. marinum</em> M</td>
<td>Human isolated WT <em>M. marinum</em></td>
<td>Dr. Monica Hagedorn, University of Geneva</td>
</tr>
<tr>
<td>WT <em>M. marinum</em> M dsRed</td>
<td>As above; constitutively expressing dsRed from pSMP12:dsRed2</td>
<td>Dr. Monica Hagedorn, University of Geneva (originally engineered by Dr. Lalita Ramakrishnan, University of Washington)</td>
</tr>
<tr>
<td>pMV_GFP_rpfA M. marinum M</td>
<td>Over-expressing RpfA from pMV261</td>
<td>Dr. Galina Mukamolova, University of Leicester.</td>
</tr>
<tr>
<td>ΔrpfA::rpfA M. marinum M</td>
<td>ΔrpfA complemented with rpfA ligated into pRBexint</td>
<td>This work</td>
</tr>
<tr>
<td>ΔrpfA::rpfA M. marinum M dsRed</td>
<td>As above; over-expressing dsRed2 from pSMP12</td>
<td>This work</td>
</tr>
<tr>
<td>ΔrpfA M. marinum M</td>
<td>Unmarked deletion of RpfA from WT <em>M. marinum</em> M</td>
<td>This work</td>
</tr>
<tr>
<td>ΔrpfA M. marinum M dsRed</td>
<td>As above; over-expressing dsRed2 from pSMP12</td>
<td>This work</td>
</tr>
<tr>
<td>ΔrpfB M. marinum M</td>
<td>Unmarked deletion of RpfB from WT <em>M. marinum</em> M</td>
<td>This work</td>
</tr>
<tr>
<td>ΔrpfBM. marinum M dsRed</td>
<td>As above; over-expressing dsRed2 from pSMP12</td>
<td>This work</td>
</tr>
<tr>
<td>ΔrpfE M. marinum M</td>
<td>Unmarked deletion of RpfE from WT <em>M. marinum</em> M</td>
<td>This work</td>
</tr>
<tr>
<td>ΔrpfE M. marinum M dsRed</td>
<td>As above; over-expressing dsRed2 from pSMP12</td>
<td>This work</td>
</tr>
<tr>
<td>ΔrpfAE M. marinum M</td>
<td>Unmarked deletion of RpfA and RpfE from WT <em>M. marinum</em> M</td>
<td>This work</td>
</tr>
<tr>
<td>ΔrpfAE M. marinum M dsRed</td>
<td>As above; over-expressing dsRed2 from pSMP12</td>
<td>This work</td>
</tr>
<tr>
<td><em>L. monocytogenes</em> EGD-e</td>
<td>Rabbit isolated WT <em>L. monocytogenes</em> EGD-e</td>
<td>Dr. Sarah Glenn, University of Leicester</td>
</tr>
<tr>
<td><em>M. luteus</em> Fleming strain 2665</td>
<td>WT strain</td>
<td>Dr. Galina Mukamolova, University of Leicester</td>
</tr>
</tbody>
</table>

Table 1: Table of Strains. Description and origins of the strains used in this work
2.3 Molecular Biology

2.3.1 Polymerase Chain Reaction

PCR was employed for amplification of DNA for generation of over-expression or deletion constructs, and for strain confirmation by colony PCR. A list of primers is described in Table 2.

Amplification of DNA for cloning was performed using the high fidelity Platinum Taq DNA polymerase (Invitrogen). Reaction conditions to amplify DNA were: 1 x HiFi Buffer, 1.5 mM magnesium chloride, 0.25 μM forward primer, 0.25 μM reverse primer, 0.2 mM dNTP (each), < 500 ng DNA, 2 units per reaction Platinum Taq DNA polymerase and sterile, RNAse and DNAse free water. DMSO was used as required at 10 % v/v.

The reaction cycle consisted of an initial denaturation at 94 °C for 2 min, followed by 30 cycles of a denaturation step at 94 °C for 30 sec; an annealing step at approximately 60 °C (5 °C less than the melting temperature of the primers used); an extension step at 72 °C for 1 min/kb; and a final extension step at 72 °C for 5 min.

Colony PCR to confirm deletion mutants was performed using GoTaq DNA Polymerase (Promega). The DNA template was obtained by resuspending M. marinum colonies in 100 μl DNAse and RNAse free water, heating samples to 95 °C for 20 min, centrifuging for 5 min and using 1 μl of the resulting supernatant. Reaction conditions were similar to those described for Platinum Taq PCR reactions with the following differences: 1 x Go Taq Reaction Buffer, 1.25 units/reaction of polymerase and no addition of magnesium chloride.

The same reaction cycle was used as for amplification using Platinum Taq polymerase with denaturation steps performed at 95 °C. Samples were stored at 4 °C until analysis by agarose gel electrophoresis.
### A.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Restriction Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔrpfA::rpfA F</td>
<td>rpfA</td>
<td>5’-gat ACT AGT atg gga cgt cac cgt-3’</td>
<td>SpeI</td>
</tr>
<tr>
<td>ΔrpfA::rpfA R</td>
<td>rpfA</td>
<td>5’-cga GTT AAC tca ggc gaa gac ggt cgg-3’</td>
<td>HpaI</td>
</tr>
<tr>
<td>pET15b::rpfA F</td>
<td>rpfA</td>
<td>5’-cac gtt CAT ATG gcc acc ggc gaa tgg gac-3’</td>
<td>Ndel</td>
</tr>
<tr>
<td>pET15b::rpfA R</td>
<td>rpfA</td>
<td>5’-gtg atg GGA TCC tca ggc gaa gac ggt cgg ctg-3’</td>
<td>BamHI</td>
</tr>
<tr>
<td>pET15b::rpfB F</td>
<td>rpfB</td>
<td>5’tac atg CCA TGG cgt cca aga ccg tga cat tga-3’</td>
<td>Ncol</td>
</tr>
<tr>
<td>pET15b::rpfB R</td>
<td>rpfB</td>
<td>5’-act cat CTC GAG tca gtt gcc cgg ctc ccc ctc-3’</td>
<td>Xhol</td>
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<tr>
<td>pET15b::rpfE F</td>
<td>rpfE</td>
<td>5’-cga tgg CAT ATG gac ggc ggc tgg gac cgg aat-3’</td>
<td>Ndel</td>
</tr>
<tr>
<td>pET15b::rpfE R</td>
<td>rpfE</td>
<td>5’-agt gat GGA TCC cta ggc ggc tgg gcc gca ga-3’</td>
<td>BamHI</td>
</tr>
<tr>
<td>pET15b::Tev::actA F</td>
<td>actA</td>
<td>5’-cta ata CAT ATG gcg aca gat agc gaa gat-3’</td>
<td>Ndel</td>
</tr>
<tr>
<td>pET15b::Tev::actA R</td>
<td>actA</td>
<td>5’-atc GGA TCC tta att att ttt ct taa ttg-3’</td>
<td>BamHI</td>
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</tbody>
</table>

### B.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer Sequence</th>
<th>Restriction Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔrpfA FR1 F</td>
<td>5’-gcc AAG CTT ccc tgt ttc ggc act cgg tga-3’</td>
<td>HindIII</td>
</tr>
<tr>
<td>ΔrpfA FR1 R</td>
<td>5’-gat GGT ACC tcc act atc agt tgt act ccg-3’</td>
<td>KpnI</td>
</tr>
<tr>
<td>ΔrpfA FR2 F</td>
<td>5’-tga GGT ACC acc gtc ttc gcc tga gac agg gtc-3’</td>
<td>KpnI</td>
</tr>
<tr>
<td>ΔrpfA FR2 R</td>
<td>5’-gcg GCG GCC GCA ccc gtt acc ttc gcg cgc gga-3’</td>
<td>NotI</td>
</tr>
<tr>
<td>ΔrpfB FR1 F</td>
<td>5’-ccg AAG CTT act atc gct gca acc tgc ga-3’</td>
<td>HindIII</td>
</tr>
<tr>
<td>ΔrpfB FR1 R</td>
<td>5’-tga GGT ACC ggc gcc tgg gtc tgt gat-3’</td>
<td>KpnI</td>
</tr>
<tr>
<td>ΔrpfB FR2 F</td>
<td>5’-ttt GGT ACC cag att cca cgt cct ctc atc-3’</td>
<td>KpnI</td>
</tr>
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<td>ΔrpfB FR2 R</td>
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<td>NotI</td>
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<tr>
<td>ΔrpfE FR1 F</td>
<td>5’-act AGT ACT gag cga aac gcc agc tac gcg ccc-3’</td>
<td>Scal</td>
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<td>HindIII</td>
</tr>
<tr>
<td>ΔrpfE FR2 R</td>
<td>5’-ata GGT ACC cga ctg aat tga gga gaa gtt-3’</td>
<td>KpnI</td>
</tr>
</tbody>
</table>

Table 2: Primers Used in This Study. **(A)** Gene specific primers for amplification of Rpfs for complementation or generation of protein expression constructs. **(B)** Primers used to amplify the flanking regions (FR) for deletion of Rpfs. All restriction sites are italicized.
### C.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer Sequence</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔrpfA Test F</td>
<td>5’-ctc acg taa cgg aac gat aac-3’</td>
<td>582 bp</td>
</tr>
<tr>
<td>ΔrpfA Test R</td>
<td>5’-tgg ctg ccg atc atc gcg agg-3’</td>
<td></td>
</tr>
<tr>
<td>ΔrpfB Test F</td>
<td>5’-cgt gct gga cga agt tct gt-3’</td>
<td>262 bp</td>
</tr>
<tr>
<td>ΔrpfB Test R</td>
<td>5’-gct agg cca aca gcc gat tc-3’</td>
<td></td>
</tr>
<tr>
<td>ΔrpfE Test F</td>
<td>5’-aga ccc caa aca aga tga ga-3’</td>
<td>220 bp</td>
</tr>
<tr>
<td>ΔrpfE Test R</td>
<td>5’-cag caa ttc cta act gga ca-3’</td>
<td></td>
</tr>
</tbody>
</table>

Table 2 cont.: Primers Used in This Study. (C) Primers used to confirm rpf gene deletion. All restriction sites are italicized.

#### 2.3.2 Gel Electrophoresis

1 % agarose gel was prepared by dissolving 1 % w/v Agarose in Tris-Acetate-EDTA (40 mM Tris base, 20 mM Acetic acid, 1mM EDTA) buffer. 0.5 μg/ml ethidium bromide was added to visualise DNA by exposure to UV light. Electrophoresis was carried out at 85 V for 45 min and visualised using a gel doc system.

#### 2.3.3 DNA Purification

DNA from 5 ml or 50 ml bacterial culture was purified for cloning following PCR using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer’s instructions. DNA was eluted using 60 μl or 200 μl RNAse and DNAse free water (Sigma), respectively, depending on the amount of culture used.

DNA was purified by gel extraction if contaminating bands were present following PCR or digestion using QIAquick Gel Extraction kit (Qiagen) according to the manufacturer’s instructions. DNA was eluted using 100 μl RNAsed/ DNAse free water (Sigma).
2.3.4 Restriction Digest

DNA digestions were performed using enzymes, the recommended buffers and according to the instructions supplied by New England Biolabs. Diagnostic digestions were performed using 500 ng DNA, in a final volume of 10 μl. 3 μg DNA was used for molecular cloning digestions, in a final volume of 50 μl. Reaction conditions for both types of digestions were 1x NEBuffer, 10 units restriction enzyme, 1x BSA if required, DNA and RNAse/DNAse free water to final volume. Digestions were incubated at 37 °C for 3 hours.

2.3.5 Ligation

T4 DNA Ligase (Promega) was used to ligate DNA according to the manufacturer’s instructions. Reaction conditions were 100 ng vector DNA, 150 ng insert DNA, 1x Ligase buffer, 1 unit T4 DNA ligase and water to a final volume of 5 μl, followed by overnight incubation at room temperature and transformation into E. coli.

2.3.6 Transformation

Alpha-select Competent E. coli cells (Bioline) were used for all cloning steps. BL21 Competant E. coli (New England Biolabs) were used for protein expression. Cells were thawed on ice, 3 μl purified DNA was added and incubated for 5 mins. The bacteria were heat-shocked at 42 °C for 45 sec and placed immediately back on ice. 1 ml sterile LB was added and incubated at 37 °C for 1 hr, with shaking, prior to plating on LA containing the required antibiotic for plasmid selection.
2.3.7 Plasmid Extraction

5 ml *E. coli* cultures were grown in LB, overnight at 37 °C with shaking, with the appropriate antibiotic for plasmid purification using GenElute Plasmid Miniprep kit (Sigma Aldrich) according to the manufacturer’s protocol. Plasmid was eluted in 60 μl DNAse and RNAse free water.

50 ml cultures were used for larger scale plasmid purification using QIAfilter Plasmid Midi Kit (Qiagen). Cultures were grown as above, and the extraction performed according to the manufacturer’s protocol. Plasmid was eluted in 100 μl DNAse and RNAse free water.

2.3.8 Preparation of *M. marinum* Competent Cells

100 ml *M. marinum* in 7H9 was grown to OD$_{600}$= 0.6-0.8 and harvested by centrifugation at 2000 x g for 20 min. The supernatant was removed and the bacterial pellet was washed 3 times at room temperature using 10 % sterile glycerol. The pellet was concentrated during each wash by using less glycerol (first wash, 80 ml; second wash, 40 ml; third wash, 10 ml) and the finally resuspended in 1 ml. Competent cells were stored at room temperature for a maximum of 2 hours.

2.3.9 Electroporation of Mycobacteria

3 μg concentrated and purified DNA was resuspended in 3 μl DNAse and RNAse free water and incubated with 400 μl competent *M. marinum* cells at room temperature for 10 min. The cells containing DNA were transferred to a 2 mm long-electrode electroporation cuvette (Protech International) before being transformed at 2500 V, 1000 Ω and 2500 μF. The electroporated cells were transferred to 2 ml 7H9 and allowed to recover overnight at 32 °C in a static incubator. 200 μl of cells were then
plated onto 7H10 containing the appropriate antibiotic for selection. 200 μl of competent cells that were not electroporated with DNA were plated as a negative control.

2.4 Protein Biochemistry

2.4.1 Protein Expression Trials

Expression trials were performed to confirm protein expression, and optimise the conditions required for large scale protein production. Starter cultures of *E. coli* expressing the recombinant protein were grown over night and 500 μl used to inoculate 5 ml LB. Cultures were grown until mid-log phase (OD$_{600}$ = 0.6-0.8) at 37 °C before adding 0.5 mM or 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) to the cultures to induce protein expression. Post-induction temperatures of 37 °C or 18 °C were tested for 4 hr or overnight and the samples normalised to equal cell density at the end of the expression trial. BugBuster 10x Protein Extraction Reagent (EMD Millipore) was used to the manufacturer’s instructions to lyse the cells and remove contaminating DNA prior to preparation for analysis by SDS-PAGE.

2.4.2 Protein Analysis by SDS-PAGE

20 μl of the protein sample was combined with 10 μl 200 mM dithiothreitol (DTT) and 10 μl 4x NuPAGE LDS sample buffer (Life Technologies) and heated to 75 °C for 10 min. Samples were loaded onto 4-12 % acrylamide gradient pre-cast NuPage Bis-Tris gels (Life Technologies) and electrophoresis carried out in MES-SDS Running Buffer (Life Technologies) at a constant 200 V for 35 min. 5 μl Novex Sharp Pre-Stained Protein Markers (Invitrogen) was used as a protein marker for electrophoresis and Western blotting. Proteins were visualised on gels by Coomassie staining. 2.5 g/L Coomassie Brilliant Blue was dissolved in 40 % v/v methanol and 10 % acetic acid. Gels were
incubated at room temperature in this solution for 1 hr prior to destaining in 40 % v/v methanol, 10 % acetic acid solution until protein bands became visible.

2.4.3 Western Blot

Proteins were separated by SDS-PAGE as above; after electrophoresis, the gel was placed on a nitrocellulose membrane, with 5 sheets of Whatman filter paper (GE Healthcare Life Sciences) on either side. All membranes and filter paper had been equilibrated in transfer buffer (25 mM tris base, pH 8.3, 192 mM glycine and 20 % v/v methanol) prior to assembly in a semi-dry blotter (Cleaver Scientific Ltd). The transfer was performed at 20 V for 1 hr, and following disassembly, the membrane was blocked in 5 % w/v milk in PBS for 30 min. The membrane was washed in PBS and incubated overnight at 4 °C in primary antibody- mouse anti-polyHis (1:3000 dilution; Sigma) or sheep anti-Rpf antibody (as previously described by Mukamolova et al. 2002). The membrane was washed three times in PBS containing 0.1 % Tween 20 (PBST) and incubated in secondary antibody-anti-mouse or anti-rabbit antibody conjugated to alkaline phosphatase (1:10000 dilution) in 2 % w/v milk in PBS, for 1 hr at room temperature with constant agitation. Following washing with PBST, 1 tablet of BCIP/NBT substrate (Sigma) was dissolved in 10 ml deionised water and the membrane incubated in this solution, in the dark, until bands were visible. The reaction was stopped by washing with water.

2.5 Macrophage Infection

2.5.1 Maintenance of J774 Macrophages

Macrophages were maintained in DMEM supplemented with foetal calf serum and L-glutamine and incubated statically at 37 °C with 5 % carbon dioxide. Macrophages were passaged twice a week by scraping the cells off the bottom of a 75 cm² flask,
transferring the cells into a falcon for centrifugation at room temperature, 1000 x rpm for 5 min and removing the spent media. Macrophages were resuspended in 10 ml of fresh media that had been warmed to 37 °C and new flasks seeded at $2 \times 10^5$.

Macrophage viability was determined by incubating cells with 1:1 Trypan Blue (Sigma) and counting cells on a haemocytometer by light microscopy.

**2.5.2 Infection of J774 Macrophages with *M. marinum***

24 hours prior to infection with *M. marinum*, macrophages were seeded in 6 well plates containing sterile coverslips. Coverslips were sterilised by washing in 100 % ethanol, flaming the remaining ethanol away and being transferred to a sterile well. Macrophages were scraped as above to remove them from the base of the flask, counted using a haemocytometer and light microscope, and 2 ml seeded at $1 \times 10^5$ in each well. Macrophages were infected 24 hours later upon adhering to coverslips and reaching a density of $2 \times 10^5$.

Only strains expressing dsRed where used for macrophage infection to allow observation by microscopy. 100 ml flasks containing 7H9+ADC+Tween+Kanamycin and any additional antibiotic that was required were inoculated with 2 ml of starter culture and grown to mid-log phase ($OD_{600} = 0.6-0.7$). Bacteria were harvested by centrifugation for 10 min at 2700 x g, washed three times with sterile PBS, pH 7.4 (Oxoid) and finally resuspended in 10 ml PBS. This culture was passaged through a 26 gauge needle (Harvard Apparatus) 3 times to break up bacterial clumps and aggregates. Any remaining clumps were removed by centrifugation of the culture at 100 x g for 1 min. The resulting single cell suspension of mycobacteria was counted using a haemocytometer and added to macrophages at an MOI (multiplicity of infection) of 0.5. Following 4 hours of infection, macrophages were washed with DMEM to remove extracellular bacteria, and incubated for 48 hours in fresh media, prior to processing slides for confocal microscopy.
3 Generation, Purification and Characterisation of RpfB and ActA
3.1 Introduction

To address the objectives of this project, a custom polyclonal Rpf specific antibody needed to be produced. This chapter describes the high level production and purification of *M. marinum* RpfB from *E. coli* that was required for immunisation and antibody production. Circular dichroism (CD) spectroscopy was used to determine that purified RpfB was folded, and the muralytic activity of the protein was confirmed using two commonly used methods (Mukamolova et al. 2006; Pinto et al. 2013) that monitor digestion of *M. luteus* cell wall in a zymogram and MUF tri-NAG, a fluorogenic lysozyme substrate.

As described in detail in 1.5.2, previous work in our laboratory has identified significant amino acid sequence homology and conservation of a key catalytic residue between *M. marinum* RpfA and the well characterised actin assembly-inducing protein of *L. monocytogenes*, leading us to propose that ActA may have Rpf-like activity. To investigate this, soluble recombinant ActA was purified from *E. coli*; CD was used to record the secondary structure of the protein and determined it to be folded. ActA was found to hydrolyse *M. luteus* cell wall, which could be improved by the addition of manganese, and MUF tri-NAG thus confirming our initial hypothesis.

3.2 Methods

3.2.1 Bacterial Strains, Cell lines and Media

DH5α and BL21 (DE3) *E. coli* were used for cloning and protein expression, respectively. Both were grown at 37 °C in LB or LA supplemented with the appropriate antibiotic at 50 μg/ml.

All restriction enzymes and their buffers were obtained from New England Biolabs; all solutions and antibiotics described were obtained from Sigma unless otherwise stated.
Monoclonal anti-polyHis primary antibody and secondary Anti-Mouse IgG conjugated with Alkaline Phosphatase were obtained from Sigma.

3.2.2 Generation of Protein Expression Constructs

To generate pET15b:rpfB, rpfB lacking the signal sequence (cleavage site predicted to be at amino acid residues 23-24 by signalP: http://www.cbs.dtu.dk/services/SignalP/) was amplified from M. marinum M genomic DNA using primers described in Table 2a, and the high fidelity polymerase PlatinumTaq was amplified from M. marinum M genomic DNA using primers described in Table 2a. The PCR product was digested with NcoI and XhoI to facilitate ligation dependent cloning. Successful ligation of the PCR product was determined by plasmid isolation from E. coli transformed with this plasmid, and digestion with NcoI and XhoI to release the insert. Constructs containing the right sized insert were sent for sequencing by GATC Biotech, and upon confirmation that there were no PCR mutations, the construct was transformed into BL21 (DE3) E. coli for protein expression trials and purification. These steps were repeated using primers that amplify rpfA and rpfE to facilitate cloning into pET15b-Tev (a derivative of pET15b differing only in the presence of a TEV (Tobacco Etch Virus) protease cleavage site instead of thrombin) that would produce N-terminally His-tagged RpfA and RpfE.

ActA that lacked the signal sequence (predicted to be at residues 29-30) was amplified using PlatinumTaq from a boiled sample of L. monocytogenes EGD-e using primers described in Table 2, which introduced the restriction sites Ndel and BamHI for cloning. Following digestion of the PCR product and pET15b-Tev with these enzymes; the digested PCR product and vector backbone were purified and ligated followed by transformation into DH5α E. coli (Bioline) competent cells. Colonies were grown to isolate plasmid and the presence of the insert was established by digestion of the purified plasmid; the correct clone was confirmed using DNA sequencing and used for transformation into BL21 (DE3) E. coli competent cells for protein expression and purification.
3.2.3 Expression Conditions

Protein expression trials as described in 2.4.1 were performed to identify the conditions for optimal expression of recombinant *M. marinum* RpfB from pET15b:rpfB and *L. monocytogenes* ActA from pET15b-Tev:actA. Large scale protein production was carried out by inoculating 500 ml LB containing 50 μg/ml ampicillin with 2.5 ml of overnight starter culture that had been resuspended in fresh LB. Large scale cultures were grown at 37 °C until they reached an OD$_{600}$ of 0.6-0.7 and expression was induced with 0.5 mM IPTG.

Following induction, RpfB was expressed for 4 hr at 37 °C and ActA expressed overnight at 18 °C prior to harvesting cells at 4000 x g for 20 min at 4 °C (Beckman JA 25.5 rotor, Beckman Coulter Avanti J-30I centrifuge).

3.2.4 Purification of RpfB

RpfB was detected in the insoluble fraction and was therefore purified from inclusion bodies. Following large scale expression and centrifugation to harvest the bacteria, the supernatant was removed and the bacterial pellet resuspended in buffer containing 50 mM Tris hydrochloride, 2 mM EDTA, 0.1 % (w/v) Triton X-100, pH 8. The cells were lysed by passing through the French press (SLM-AMINCO Spectronic Instruments) resulting in a viscous suspension. Further treatment by French pressing resulted in shearing of the DNA present- identified by loss of viscosity and a homogenous suspension (total passage 3-5 times).

To obtain the insoluble fraction containing RpfB, the sample was centrifuged at 4 °C at 10000 x g for 20 min (Beckman JA 25.5 rotor, Beckman Coulter Avanti J-30I centrifuge) and the supernatant removed. The pellet was washed in buffer containing 50 mM Tris hydrochloride, 10 mM DTT, 10 mM EDTA, 0.5 % v/v Triton X-100, pH 8.75 three times, being homogenised and centrifuged for 20 mins, at 8000 x g at 4 °C between each wash. The resultant inclusion bodies were resolubilised in resolubilisation buffer (50 mM Tris hydrochloride, 11 mM DTT, 5 M guanidine hydrochloride, pH 8.75) for 30 mins
whilst being rocked at room temperature. The denaturant was dialysed into 25 mM sodium phosphate, 100 mM sodium chloride, pH 7.5 overnight, with a buffer change occurring 3 times. Precipitated material and protein that had not resolubilised was removed by centrifugation of the sample and filtration of the supernatant through a 0.2 μm syringe filter (Fisher Scientific). Low and high molecular weight contaminants were removed by gel filtration (GE Healthcare Superdex 75) using an AKTA FPLC system (Amersham Pharmacia Biotech). The appropriate peaks were analyzed by SDS-PAGE and pooled together for use. ProtParam (http://web.expasy.org/protparam/) was used to predict the molecular weight (35744.5 Da) and extinction coefficient (40575 M⁻¹ cm⁻¹) to facilitate calculations of protein concentration using the absorbance of the sample at 280 nm.

3.2.5 Purification of ActA

Soluble, N-terminally His-tagged ActA was purified as follows: the bacterial pellet was resuspended in 30 ml binding buffer (50 mM sodium phosphate, 300 mM sodium chloride, 30 mM imidazole, pH 7.5); cells were lysed and DNA sheared as described in 3.2.4. Insoluble material was removed by centrifugation at 10000 x g, the supernatant filtered using a 0.2 um Whatman filter and the soluble material loaded onto a 5 ml Ni-NTA column (Qiagen) that had been equilibrated with binding buffer for purification on an AKTA FPLC system (Amersham Pharmacia Biotech). Following a 10 column volume (CV) wash step with the binding buffer, the protein was eluted with a linear gradient of 50 mM sodium phosphate, 300 mM sodium chloride, 500 mM imidazole, pH 7.5 and the appropriate fractions were analyzed on a NuPAGE SDS-PAGE. A constant flow rate of 2 ml/min was maintained during affinity chromatography.

The sample was further purified by gel filtration using a Superdex 75 column (GE Healthcare). Buffer containing 50 mM NaPO₄, 300 mM NaCl₂, pH 7.5 was used to equilibrate the column and for the purification. A flow rate of 1 ml/min was maintained, the appropriate fractions pooled and analysed by SDS-PAGE. As above, predictions of molecular weight (67193.8 Da) and extinction coefficient (13980 M⁻¹ cm⁻¹).
were used to enable calculations of protein concentration using the absorbance of
the sample at 280 nm.

3.2.6 Circular Dichroism of Purified Protein

Purified, recombinant protein samples were dialysed against buffer containing 100 mM sodium fluoride, 25 mM sodium phosphate, pH 7.5 and spectra were recorded between 190 nm – 260 nm at room temperature using a Chirascan Plus Spectrometer (Applied Photophysics).

Spectra of the buffer were first obtained, followed by the sample at room temperature. A denaturation curve was then performed to test for cooperative unfolding by heating the sample from 15 °C to 80 °C, and recording spectra between 190 nm – 260 nm every degree. Spectra were analysed using Pro-Data Viewer (Applied Photophysics) or Spectra Manager (Jasco).

3.2.7 Zymography to Test for Muralytic Activity

A single colony of M. luteus was used to inoculate 100 ml LB and grown until OD$_{600}$=2-2.5. 15 ml of this starter culture was used to inoculate 500 ml LB and grown to OD$_{600}$=2. Cells were harvested by centrifugation for 20 min at 2000 x g, the supernatant removed and the pellet washed in 100 ml deionised water. Following centrifugation as before, the pellet was resuspended in 50 ml deionised water and 5 ml aliquots prepared in 30 ml universal tubes. Each aliquot was centrifuged to remove the supernatant, freeze dried under vacuum and stored at -80 °C.

A 12 % polyacrylamide gel containing 0.2 % w/v of lyophilised M. luteus cell wall was used for zymography. Protein samples were prepared as described in 2.4.2, prior to electrophoresis at 200 V for 1 hr. The gel was washed briefly in water at room temperature, cut into strips containing the protein being tested and renatured overnight at 37 °C. The renaturation buffers tested were 25 mM Sodium Citrate at pH
4.5, 5, 5.5 and 6, 25 mM Sodium Phosphate at pH 6.5, 7 and 7.5, and Tris Hydrochloride at pH 7.5, 8, 8.5 and 9. All renaturation buffers were prepared with the addition of 0.2 % w/v Triton X100. When required, up to 1 mM manganous chloride or up to 5 mM magnesium sulphate was added to the renaturation buffer.

Following renaturation, gels were stained with 0.1 % w/v methylene blue dissolved in 0.01 % (w/v) potassium hydroxide for 2 hours at room temperature, and destained with water until clearance bands were visible.

3.2.8 Hydrolysis of MUF tri-NAG

The protein sample was filter sterilised using a 0.2 μm filter syringe that had been washed in 5 ml sterile PBS to ensure hydrolytic activity was not due to contaminants in the preparation. The sterility of the sample was confirmed by streaking 10 μl onto LA and incubated at 37 °C overnight. The buffer used was 25 mM sodium phosphate, pH 6.5, and the assay was performed in black 96 well plates (Greiner Bio One). Per well, 180 μl of buffer containing 1.8 μl 600 μM MUF tri-NAG was added to 10 μl of purified, sterile protein and mixed. 10 μl of freshly prepared lysozyme at 1 mg/ml was used as a positive control, and negative controls consisted of protein that had been boiled at 100 °C for 15 min. The plate was covered in foil to prevent evaporation and incubated at 32 °C overnight to allow the reaction to occur. 10 μl of 10 N sodium hydroxide was added to stop the reaction and fluorescence measured at excitation 364 nm / emission 448 nm using a Varioskan Flash plate reader (Thermo Scientific) to assess umbelliferone release.
3.3 Results and Discussion

3.3.1 Generation of Recombinant RpfB and ActA

Constructs were initially generated to express untagged RpfA, RpfB and RpfE in *E. coli*, however a high protein yield after induction of protein expression was obtained with RpfB only. Attempts to purify large amounts of folded N-terminally His-tagged *M. marinum* RpfA and RpfE were unsuccessful due to poor expression of unfolded protein; therefore purified RpfB was used for immunisation and antibody production.

To address the hypothesis regarding the possible muralytic activity of ActA a construct was generated to produce an N-terminally His-tagged ActA protein in *E. coli*.

![Figure 10: Protein Expression Trials of Recombinant Proteins.](image)

RpfB was found to be produced in large amounts in inclusion bodies after 4 hours at 37 °C, as demonstrated by the clear band of over-expressed protein that is not present before induction (Figure 10a). ActA was produced in substantial amounts when IPTG-induced cultures were incubated overnight at 18 °C. Analysis of samples by SDS-PAGE
(Figure 10b) did not show any obvious expression of protein so a Western blot was performed (Figure 10c) using a poly-histidine-specific antibody. The blot confirmed that soluble ActA was being expressed, however the presence of smaller sized bands suggested partial degradation of the protein. BL21 (DE3) *E. coli* transformed with pET15b or pET15b-Tev were used as a negative control during expression trials.

Both proteins migrate unusually on SDS-PAGE; RpfB is predicted to be 35 kDa and appears to be 40 kDa whilst ActA is predicted to be 67 kDa and is significantly larger on SDS-PAGE, running at 80 kDa. Interestingly, this behaviour has been reported a number of times in the literature (Footer et al. 2008; Smith et al. 1996; Pistor et al.)
and has been attributed to the proline rich domains of ActA (Niebuhr et al. 1997). Mycobacterial RpfS have also been described to migrate higher than their actual size on SDS-PAGE (Ruggiero et al. 2009) and, like ActA, RpfA is particularly rich in proline, supporting further our hypothesis that there may be some conservation of function between these two proteins.

RpfB was purified out of inclusion bodies and low and high molecular weight contaminants removed by gel filtration where two peaks were apparent- a small higher molecular weight peak that is likely to represent large, incorrectly folded or aggregates of RpfB, and a main peak containing the protein of interest. Gel filtration resulted in a very clean preparation of RpfB that lacked any low or high molecular weight contaminants (Figure 11) after fractions A12-B12 had been pooled together, however, the protein seemed to be susceptible to proteolysis that was associated with long term storage and high concentrations of the protein.

Prior to sending RpfB to CRB for immunisation and antibody production it was important to confirm the identity of the protein purified. Using the online bioinformatic tool Expasy ProtParam, the theoretical intact mass of the protein purified was obtained by inputting the actual amino acid sequence of RpfB (35744 Da) obtained from DNA sequencing of the construct. The experimentally determined mass of RpfB was obtained by electrospray mass spectrometry and was found to be 35743 Da (Figure 12) confirming that the protein purified was RpfB lacking its N-terminal signal sequence.

A two-step process was also employed to purify ActA. First, affinity chromatography was used to purify the His-tagged protein from other soluble proteins. The degradation products that were visible during expression trials (Figure 10b), and possibly some additional non-specific proteins were also bound to the column during this step (Figure 13Error! Reference source not found.a) therefore gel filtration was performed to remove low molecular weight contaminants. The fractions from the main peak (Figure 13b; yellow arrow) were pooled together resulting in a homogenous preparation of ActA that lacked any contaminating proteins or products of degradation.
Figure 12: Identification of the Intact Mass of RpfB as Determined by Electrospray Mass Spectroscopy. The experimentally confirmed mass of 35742 Da is in agreement with the predicted mass of this protein (35774 Da).
It is generally accepted that a recombinant protein that is correctly folded is more likely to exhibit qualities attributed to its native form such as enzymatic activity; it was therefore important to determine the secondary structure of purified, recombinant RpfB and ActA. Additionally, it is essential that polyclonal antibodies are raised against a correctly folded protein that has not undergone proteolysis to ensure specificity towards the epitopes that would allow recognition of the native protein.

### Figure 13: FPLC Traces and SDS-PAGE Analysis of Purification of ActA.

(A) FPLC trace of the soluble fraction loaded onto a Ni-NTA column and below, analysis of the protein loaded on the column (lane 1), the flow through (X1, X3 and X4) and protein from the eluted peak (A9, A11, B3, B5, B9 and B12). (B) FPLC trace of gel filtration of ActA and below, analysis of the pooled eluate after Ni-NTA loaded on the column (lane 1), flow through before (X1) and after (X3) elution of the protein and the various protein peaks- A5, A10, B2, B5, C3 and D2. Both the peak and bands representing full length ActA are highlighted in yellow.

#### 3.3.2 Secondary Structure Determination of RpfB and ActA by Circular Dichroism
CD was used to address the state of both proteins. The spectra of RpfB showed a mixed alpha-helix, beta-sheet structure (Figure 14a) that agrees with the current structural data available for the conserved Rpf domain and surrounding regions (Ruggiero et al. 2009; Cohen-Gonsaud et al. 2005). The protein was found to unfold as the temperature of the sample was increased to 80 °C indicating that, prior to denaturation, RpfB existed as a compact, well-folded structure (Figure 14b). This cooperative unfolding can be observed by the characteristic sigmoidal curve that forms when spectra is monitored at 206 nm during denaturation (Figure 14c); this data also indicates that *M. marinum* RpfB is stable until 35 °C.

Figure 14: Spectra and Thermal Denaturation of RpfB by CD Spectroscopy. (A) Average of 15 spectra recorded of RpfB (at 0.03 mg/ml), taken at room temperature. (B) Thermal denaturation from 15 °C to 95 °C. (C) Cooperative unfolding of RpfB as temperature is increased at 206 nm. The midpoint for RpfB unfolding is reached at 40 °C.
There is currently no structural information available on ActA, however, analysis of the amino acid sequence of this protein using jpred, a secondary structure prediction program supports that ActA is predominantly α-helical, with some β-sheet (Figure 15). The spectra obtained for ActA shows the characteristic ‘double–dip’ profile of a predominantly α-helical protein, with the second peak being somewhat flattened due the presence of some β-sheet (Figure 16a). This is in agreement with our predictions of the secondary structure of ActA.

The thermal stability of ActA was assessed by following changes in the spectrum as the temperature was increased to 80 °C in a step wise manner (Figure 16b). ActA was found to cooperatively unfold indicating that the protein has secondary structure that is lost upon denaturation (Figure 16c) and is therefore folded. ActA appears to be stable until approximately 34 °C- a feature that likely reflects the ability of L. monocytogenes to replicate optimally at temperatures reaching 37 °C (Gründling et al. 2004).

Figure 16: Characterisation of Recombinant ActA by CD Spectroscopy. (A) Recorded spectra of ActA at room temperature, from 190 nm-260 nm indicating a predominantly helical structure, with the presence of some beta sheet. (B) Denaturation curve of ActA. Spectra were obtained from 190 nm-260 nm as the sample was heated from 15 °C-80 °C. The change in structure of the protein as the temperature is increased indicates cooperative unfolding of the sample. (C) Characteristic sigmoidal curve of spectra obtained at 208 nm during thermal melt. ActA begins to unfold at approximately 34 °C, reaching its midpoint at 42 °C.
3.3.3 Investigation of Muralytic Activity of Recombinant RpfB and ActA

Following confirmation that RpfB and ActA were folded, two well described methods were used to test for muralytic activity. The first, zymography, is a qualitative electrophoretic technique that is dependent on the successful renaturation of the protein of interest in a range of buffers (sodium citrate, sodium phosphate and tris hydrochloride) and pH’s (4.5-9) to observe clearance, or hydrolysis, of the lyophilised bacterial cell wall within the SDS-PAGE. Fresh preparations of RpfB and ActA were both found to be able to hydrolyse *M. luteus* cell wall following renaturation, as observed by the appearance of a clearance band (Figure 17) in buffer containing 25 mM sodium phosphate, pH 6.5.

![Figure 17](image)

**Figure 17: RpfB and ActA Have Muralytic Activity in a Zymogram.** (A) Purified RpfB can be seen on the SDS-PAGE on the left; the same protein preparation was run in a zymogram and renatured in buffer containing 25 mM NaPO₄, pH 6.5. (B) Purified ActA can be seen on the SDS-PAGE and the same preparation was run in a zymogram renatured in buffer containing 25 mM sodium phosphate, pH 6.5 with varying concentrations of MnCl₂ for 48 hours at 37°C. A yellow arrow highlights the clearance band in (A) and (B).

Many bacterial enzymes require metal ion co-factors (Waldron & Robinson 2009); whilst addition of MgSO₄ or MnCl₂ to renaturation buffers had no effect on RpfB activity, the activity of ActA was seen to improve, as judged by the extent of clearance, when renatured in buffer containing manganese (Figure 17b). This effect was dose
dependent— as the concentration of MnCl₂ increased from 0.5 mM to 5 mM, so did the visibility of the clearance band. Manganese is a key bacterial trace element, and has been implicated as a cofactor for a number of metalloenzymes, in the detoxification of ROS and in the stabilization of bacterial peptidoglycan (Jakubovics & Jenkinson 2001). It is not unknown for lytic transglycosylases to also require a cofactor for their activity (van Asselt & Dijkstra 1999). The role of manganese as a cofactor for lytic transglycosylases has not been widely reported although its use in enzyme assays investigating Rpf activity have been previously described (Hett et al. 2008).

<table>
<thead>
<tr>
<th></th>
<th>RLU/μM</th>
<th>BOILED</th>
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<tbody>
<tr>
<td>ActA</td>
<td>0.83 ± 0.05</td>
<td>0.01 ± 0.00</td>
</tr>
<tr>
<td>RpfB</td>
<td>2.6 ± 0.02</td>
<td>0.02 ± 0.02</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>2.82 ± 0.25</td>
<td>2.05 ± 0.57</td>
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Table 3: Hydrolysis of MUF tri-NAG by RpfB and ActA. Release of umbelliferone is expressed as relative light units (RLU) per μM of purified protein assayed. Activity values are obtained from the average of 5 technical replicates (3 for boiled samples); all values have been corrected for background fluorescence obtained from pure buffer (0.11±0.01 RLU); ± denotes standard deviation. The experiment was repeated twice; representative data are shown.

Zymograms are a very qualitative method of assessing the enzymatic activity of a protein, and judging the clearance bands can be subjective however this is a good preliminary indication that ActA is able to digest bacterial peptidoglycan. In order to more rigorously test this murlatory activity, and gain some insights into the potency and specificity of this action, a second, more quantitative method was utilised. The ability of both proteins to digest MUF tri-NAG, an artificial substrate for lysozyme and peptidoglycan muramidases, was tested. Specific cleavage of the β 1-4 glycosidic bond between NAM and NAG results in the release of the fluorescent product 4-methylumbelliferone and therefore provides a direct measurement of murlatory activity.
Freshly purified preparations of recombinant RpfB and ActA, and lysozyme were used in this assay, and all samples filter sterilised prior to addition to remove any bacterial contaminants that may result in a false positive result. Again, ActA demonstrated muralytic activity (Error! Reference source not found.3) however this was considerably less than RpfB and the lysozyme control. Thermal denaturation of the recombinant proteins completely abolished their activity in this assay, however, heat treated lysozyme consistently and reproducibly retained most of its muralytic activity. The ability of hen egg white lysozyme to refold correctly and retain activity after denaturation is a well described phenomenon (Noritomi 2011).

The capacity of ActA to produce a clearance band in zymograms indicates it is able to cleave one of the many available bonds found in bacterial peptidoglycan. Further testing of this hydrolytic activity using MUF tri-NAG suggests that although ActA is able to cleave the same bond as Rpfs, it may not be the optimal substrate for this protein as it appears to be cleaved with less efficiency.

Although the muralytic activity of ActA has not been previously demonstrated, it is not the first additional function to be attributed to this protein. As well as its role in actin polymerisation, ActA has also been implicated in facilitating bacterial escape from the phagosome (Poussin & Goldfine 2010), entry into epithelial cells (Suárez et al. 2001), protection from the host autophagic response (Yoshikawa et al. 2009) and mediation of cell attachment by binding of host cell heparan sulfate receptors (Alvarez-Dominguez et al. 1997). Deletion of actA in L. monocytogenes reduces cell aggregation resulting in a natural single-cell suspension which consequently inhibits biofilm formation (Travier et al. 2013).

3.4 Conclusions

Supplying good quality protein for antibody production is key for the synthesis of specific and sensitive polyclonal antibodies. Confirmation that recombinant RpfB is folded, cooperatively unfolds and is active in muralytic assays indicates that the
protein is likely to be in its native conformation and the polyclonal antibodies generated will reflect that by being sensitive and specific towards natively produced *M. marinum* RpfB.

The work described in this chapter has addressed the hypothesis that ActA from *L. monocytogenes* may have muralytic activity. The presence of this activity was confirmed using two independent methods. A number of functions have previously been attributed to ActA suggesting a dynamic role for this protein in *Listeria* infection, motility and biofilm formation. Functional equivalents of RpfS in this organism have recently been described (Pinto et al. 2013) suggesting that this enzymatic activity may be more crucial to adhesion to cells and the disassembly of these aggregations and biofilms than resuscitation from dormancy.

Additionally, whilst previous studies have confirmed the essentiality of the catalytic glutamate for the muralytic activity of lysozyme (Grütter et al. 1983) and some lytic transglycosylases (Thunnissen et al. 1995), mutation of this residue appears to only attenuate the muralytic activity of RpfS (Mukamolova et al. 2006). The effect of mutating this residue in ActA remains to be determined and investigation of this would shed light on the mechanism by which ActA is able to digest bacterial peptidoglycan.
4 Purification and Characterisation of Anti-Rpf Antibodies
4.1 Introduction

Multiple approaches have been described to facilitate the study of protein localisation within cells including cell fractionation procedures followed by detection of proteins in various fraction (Koul et al. 2001; Beatty & Russell 2000), fluorescence microscopy to observe expression of proteins fused to fluorescent tags (Cowley & Av-Gay 2001), electron microscopy of proteins fused to non-fluorescent tags (Cascioferro et al. 2007) and the use of protein specific antibodies (Banu et al. 2002).

Reporter fusions to proteins have occasionally been found to influence the localisation patterns of proteins (Margolin 2012), and cell fractionation studies can only provide a population view of subcellular protein localisation. Immunofluorescence is mainly limited by the potential of non-specific recognition of the antibodies used, bleaching of the fluorophores and that tissues need to be fixed and permeabilised which can introduce artifacts (Wilson and Bacic 2012). We opted to produce antibodies sensitive to Rpf that allow microscopy based studies of Rpf expression and localisation. Particularly, this approach facilitates the study of Rpfs at the single cell, population level, and across all different stages of macrophage infection. A further advantage is that the use of these antibodies in combination with specific stains allows information to be gained on Rpf localisation in the context of specific processes such as mycobacterial compartmentalisation to the phagosome and ejectosome formation.

Little is known about the post-translational modifications of Rpfs, however, glycosylation of the protein surface of Rpf2 from Corynebacterium glutamicum (Mahne et al. 2006) has been reported and suggests that these alterations cannot yet be excluded to occur on mycobacterial Rpfs. Due to the possibility of epitope occlusion on Rpfs as a result of protein-protein interactions (Read et al. 2009; Leder et al. 1994) and/or post-translational modifications, polyclonal antibodies were raised. In this situation, although monoclonal antibodies may offer higher specificity to Rpfs, there is some risk of the antibody failing to bind the protein due to epitope occlusion of the single epitope that it has been raised to recognise. The lack of specificity to a single epitope allows polyclonal antibodies to withstand small changes to antigenic
determinants without losing affinity to their target and is the premise for raising polyclonal antibodies against RpfB.

This chapter describes the production and characterisation of a polyclonal antibody that was raised against recombinant, purified *M. marinum* RpfB. The specificity and sensitivity of this antibody to RpfB, mycobacterial and macrophage lysates was investigated by blotting and ELISA (Enzyme-Linked Immunosorbent Assay). A previously generated polyclonal anti-Rpf domain antibody (Mukamolova et al. 2002) was also included in this analysis. Both antibodies were found to recognise *M. marinum* RpfB with differing sensitivities, and showed minimal non-specific recognition of culture supernatants, infected and uninfected J774 macrophages. By using two antibodies with different affinities for RpfS, we hope to shed more light on the individual behaviours of these proteins during the infection process.

### 4.2 Methods

#### 4.2.1 Bacterial Strains, Cell Lines and Media

DsRed expressing *M. marinum* M was used as a WT. Remaining strains were generated as described in 5 and maintained in 7H9 Middlebrook broth, supplemented with ADC, Tween 80 and Kanamycin at 50 μg/ml.

J774 A.1 murine macrophages were maintained in DMEM supplemented with 10 % FCS and 2 mM L-glutamine at 37 °C and 5 % CO₂. Further detail can be found in 2.5.1.

Anti-Rabbit and Anti-Sheep secondary antibodies conjugated with AlexaFluor 488 for flow cytometry were obtained from Life Technologies. Anti-Rabbit IgG and Anti-Sheep IgG conjugated to Alkaline Phosphatase for ELISA and western blotting were obtained from Sigma.
4.2.2 Production of Anti-RpfB Antibodies

Polyclonal antibodies 5222 and 5223 were produced by Cambridge Research Biochemicals using their custom polyclonal antibody production service. 1.5 mg of purified, recombinant *M. marinum* RpfB was provided at 0.5 mg/ml in PBS for immunisation of two rabbits using Titremax adjuvant. During a 77 day immunisation schedule, pre-immune sera was supplied prior to immunisation and test bleeds provided at day 21, 35, 49 and 63. Terminal bleeds were provided at day 77 and the antibodies purified and characterised as described in 4.2.3, 4.2.4 and 4.2.6.

4.2.3 Purification of Anti-RpfB Polyclonal Antibodies (Batch 5222) from Sera

A pre-packed FliQ Protein A column (Generon) was equilibrated with 10 column volumes (CV) of filtered, degassed PBS. 5 ml of polyclonal sera was diluted with an equal amount of PBS and centrifuged at 5000 rpm for 10 min to remove any cell debris. The supernatant was loaded onto the column and the flow through collected prior to washing the column with 10 CV PBS. The bound antibodies were eluted using 5 CV 100 mM sodium citrate, pH 2.8, and 1 ml fractions were collected into tubes containing 450 μl 1 M Tris hydrochloride, pH 9. The eluted fractions were analysed on an SDS-PAGE, pooled together and aliquoted for long term storage at -80 °C.

4.2.4 Dot Blots to Determine Affinity to *M. marinum* RpfB and RpfA

3 μl of purified recombinant RpfB was pipetted directly onto a nitrocellulose membrane and allowed to air dry. The total protein amounts spotted were: 810 μg, 81 μ, 8.1 μg, 810 ng, 81 ng and 8.1 ng. Once dry, the membranes were blocked for 30 mins using 5 % milk dissolved in PBS, washed three times with PBS + 0.05% Tween (PBST), and incubated with a dilution of 5222 anti-RpfB antibody in 1 % milk in PBST for 1 hour at room temperature. The membranes were further washed with PBST and
incubated with a 1:5000 dilution of the secondary anti-rabbit alkaline phosphatase conjugated antibody at room temperature for one hour. Following another wash step the membranes were exposed to the alkaline phosphatase substrate (1 BCIP/NBT tablet dissolved in 10 ml water; Sigma) and the reaction stopped when appropriate by washing the membrane with water.

4.2.5 Sample Preparation for ELISA and Western Blotting

10 ml mid-log phase bacterial cultures (OD_{600}=0.7) were harvested by centrifugation at 5000 x g for 10 min. The supernatant was transferred to a fresh tube and stored on ice until use. Bacteria were resuspended in 1 ml PBS, lysed by sonication (30 sec on and off for 3 min) and stored on ice.

Macrophages were infected as described in 2.5.2, collected by scraping cells, centrifuging at 1000 x g for 5 min, removing the supernatant and lysing the remaining macrophage pellet in 1 ml RIPA buffer (50 mM tris hydrochloride, 150 mM sodium chloride, 10 mM magnesium chloride, 0.1 % SDS). Samples were stored on ice until use.

These samples were normalised to 400 μg/ml using Quick Start Bradford protein assay according to the manufacturer’s instructions (Bio-Rad).

4.2.6 ELISA to Determine Sensitivity of Antibodies to RpfB, Mycobacterial and Macrophage Lysates

Purified RpfB or the samples described in section 4.2.5 were adhered to a 96 well microtitre plate overnight at 4 °C. Excess protein was removed by washing with PBS solution, pH 7.4 (Oxoid) three times and blocked for 2 hr with 100 μl 5% bovine serum albumin (BSA) in PBS. After further washing with PBS, 100 μl of the anti-RpfB polyclonal antibodies diluted in 1 % BSA was added to every sample and incubated overnight. The plate was again washed with PBS three times, and samples were
incubated with 50 μl of a 1:5000 dilution of the secondary antibody conjugated to alkaline phosphatase for 2 hours. Wells were again washed with PBS and exposed to 50 μl room temperature para-Nitrophenylphosphate (pNPP) solution (Sigma). Absorption at 405 nm was measured using a microplate reader (BioRad).

4.3 Results and Discussion

4.3.1 Purification of Anti-RpfB Antibodies

Purified, recombinant *M. marinum* RpfB was supplied to CRB for immunisation of rabbits to obtain two batches of anti-RpfB antibodies (5222 and 5223). A dot blot was performed to establish the affinity of both batches to recombinant RpfB. Whilst both antibodies recognised as little as 81 ng of purified RpfB, due to the pre-immune serum of batch 5223 recognising large amounts of RpfB (81 μg and more), polyclonal antibodies obtained from batch 5222 were purified for use in immunofluorescence (Figure 18).

![Dot Blots of Anti-RpfB Polyclonal Antibodies Prior to Purification](image)

*Figure 18: Dot Blots of Anti-RpfB Polyclonal Antibodies Prior to Purification.* The sensitivity of two batches of antibody (5222 and 5223) was tested against varying dilutions of purified, recombinant RpfB. Pre-immune sera and final bleeds were used at a 1:1000 dilution.
Anti-RpfB antibodies (5222) were purified using a Protein A column, eluted using a low pH buffer and the relevant fractions containing the antibodies was analysed by SDS-PAGE. The heavy (~50 kDa) and light chains (~30 kDa) of the purified immunoglobulin G (IgG) are visible on the gel due to being reduced and denatured (Figure 19). A homogenous preparation of the antibody was obtained following purification and aliquoted to avoid numerous freeze-thaw cycles.

Figure 19: Protein A Purification of Anti-RpfB 5222 Antibodies and Analysis of Fractions by SDS-PAGE. Anti-RpfB antibody was purified from the serum of rabbit number 5222 using a protein A column. The antibody was eluted using a low pH buffer and individual fractions analysed by SDS-PAGE. A yellow arrow highlights the antibody peak and protein on an SDS-PAGE. The green trace represents pH change.
4.3.2 Characterisation of Anti-Rpf and Anti-RpfB Antibodies

Alongside the anti-RpfB antibody that was raised in this work against recombinant *M. marinum* RpfB (described in chapter 3), another antibody was also used to characterise Rpf expression during macrophage infection. This was a polyclonal antibody raised against the Rpf domain of *M. luteus* Rpf which has been reported to recognise all five of the *Mtb* Rpf s (Mukamolova et al. 2002) and will be referred to as the anti-Rpf antibody hereon.

A.

![Graph A](image)

B.

![Graph B](image)

**Figure 20: Minimum Detection of Rpf Specific Antibodies to RpfB.** The sensitivity of the (A) anti-RpfB batch S222 and (B) anti-Rpf polyclonal antibodies characterised against varying concentrations of purified, recombinant RpfB by ELISA.
The affinity of both antibodies to \textit{M. marinum} RpfB was characterised by ELISA. Both antibodies were used at a 1:1000 dilution resulting in a final concentration of 2.5 μg/ml. A positively correlated dose dependent response is seen as the concentration of RpfB increases (Figure 20). With the anti-RpfB antibody recognising as little as 5 pM purified protein (Figure 20a) at this dilution, and the anti-Rpf recognising from 6 nM RpfB (Figure 20b), it is clear that the latter is approximately 1000-fold less sensitive to recombinant RpfB. This difference in affinity to \textit{M. marinum} RpfB is likely due to the anti-Rpf antibody being raised to a polypeptide from a different species, in this case, the \textit{M. luteus} Rpf domain. We could not obtain high enough yields of folded RpfA or RpfE to directly test the specificity of the anti-RpfB antibody to other \textit{M. marinum} Rpfs.

![Figure 21: Validation of Antibodies by Western Blotting.](image)

The ability of both antibodies to recognise Rpfs was tested in ELISA and Western blots against culture grown WT \textit{M. marinum}, spent culture supernatant, uninfected and WT infected macrophages. The anti-RpfB antibody raised in this project was found to show a minimal response in the western blot, recognising only the positive control of purified RpfB and not reacting with any other proteins on the blot (Figure 21a). The anti-Rpf antibody was again found to have less affinity to the positive control, but was able to detect two proteins present in the \textit{in vitro} grown WT \textit{M. marinum} sample (Figure 21c, lane 1).
Although these proteins have not been formally identified in this study, they are likely to be mycobacterial Rpf s as cross-reactivity of the anti-Rpf antibody has previously been described (Mukamolova et al. 2002). The size of the proteins suggests it could be
either RpfB or RpfA (also 38 kDa) or a processed form of the protein and both have previously been detected in *Mtb* culture supernatant and cell envelope fractions (de Souza et al. 2011; personal communication). Western blots are not always the optimal methodology to investigate the specificity of polyclonal antibodies. The inherent nature of SDS-PAGE’s means that the anti-RpfB antibody, which was raised against a folded protein, and is thus likely to recognise conformational epitopes, is being tested against a sample that has been denatured and reduced, and is unlikely to resemble the native state of the protein (O’Hurley et al. 2014; Marx 2013). Alternatively, the currently unknown detection limit of this antibody against other Rpfs, which are also likely to be dilute in the samples tested, may be another reason why these proteins remain undetected by Western.

An ELISA was performed using serial dilutions of the same samples. Both antibodies showed a minimal response to all of the samples except high concentrations of RpfB suggesting that both are specific to Rpfs (Figure 22). Again, the anti-RpfB antibody showed a significantly stronger response to purified, recombinant RpfB than the anti-Rpf antibody (*p* value=0.0002). No significant difference was found between the absorbances obtained when anti-Rpf or anti-RpfB antibodies were used on WT *M. marinum* (*p* value=0.12), culture supernatant (*p* value=0.46), uninfected (*p* value= 0.8) or infected (*p* value= 0.5) macrophage lysates.

### 4.4 Conclusions

The successful production of a custom polyclonal anti-RpfB antibody by immunisation of rabbits with recombinant, *M. marinum* RpfB; and characterisation of its affinity to mycobacterial Rpfs alongside a previously generated antibody raised against the Rpf domain of *M. luteus* Rpf is described in this chapter. The results indicate that both antibodies are able to detect mycobacterial Rpfs, and do not elicit a significant response against other mycobacterial or macrophage released proteins that to an extent that would compromise microscopy based studies of Rpf expression and localisation. The anti-RpfB antibodies were found to be significantly more sensitive to
recombinant *M. marinum* Rpf, recognising as little at 5 pM protein, than the anti-Rpf antibodies (which could detect RpfB from 6 nM). In these experiments, the cross-reactivity of the anti-RpfB antibody to other Rpfs cannot be excluded as purified and folded Rpf homologues apart from RpfB could not be obtained. This antibody would thus require further characterisation using other recombinant Rpfs.

Together, the use of antibodies with different affinities to different Rpf homologues should allow further insight into the localisation and expression patterns of this protein family during the infection process.
5 Generation and Initial Characterisation of Rpf Deletion Mutants
5.1 Introduction

The construction of a panel of single and double Rpf deletion mutants in *M. marinum* was a key step in allowing the individual roles of RpfS to be investigated. This chapter reports the use of a well described cloning methodology developed in *Mtb* (Parish & Stoker 2000) that was adapted to generate unmarked single and double in-frame deletion mutants of RpfS in *M. marinum*. Additionally, no antibiotic resistance marker is left in the chromosome when using this technique, allowing for the introduction of a dsRed expressing plasmid to facilitate observation of *M. marinum* deletion mutants by microscopy. The successful deletion of these genes was confirmed by PCR and Southern blotting before their phenotypes *in vitro* and during macrophage infection could be assessed.

The *in vitro* growth of these strains, in optimal liquid media for mycobacteria, illustrated that the deletion of *rpfA*, *rpfE*, *rpfAE* or over-expression of *rpfA* in *M. marinum* did not result in any growth differences compared to WT. These strains were also characterised for their ability to replicate during infection of J774 murine macrophages. All strains displayed a growth profile that was similar to WT *M. marinum*. Interestingly, Δ*rpfA* *M. marinum* produced more aggregated cultures during growth *in vitro*. A mutant of *M. marinum* containing an unmarked deletion of *rpfB* was also generated; although it could not be properly characterised due to time constraints, the strain did not appear to aggregate or growth differently to WT.

5.2 Methods

5.2.1 Bacterial Strains, Cells Lines and Reagents

All restriction enzymes and appropriate buffers were obtained from New England Biolabs; all solutions and antibiotics described were obtained from Sigma unless otherwise stated.
Alpha Select Gold Efficiency *E. coli* (Bioline) was used for all cloning procedures. The bacteria were grown at 37 °C in LB or LA supplemented with the appropriate antibiotic at a concentration of 50 μg/ml unless otherwise stated.

WT *M. marinum* M background was used for the generation of Δrpf deletion mutants. All *M. marinum* strains were grown in 7H9 liquid media or on 7H10 Middlebrook agar supplemented with ADC. DsRed expressing strains were maintained with the addition of kanamycin. All *M. marinum* strains were grown statically at 32 °C for up to 14 days.

J774 A.1 murine macrophages (91051511, ECACC) were maintained in DMEM supplemented with 10 % (v/v) FCS and 2 mM L-glutamine at 37 °C and 5 % CO₂. Further detail can be found in 2.5.1.

### 5.2.2 Generation of Constructs for Deletion of Rpf's

The cloning strategy for the generation of Rpf deletion mutants is outlined in Figure 23 and information on the primers used for generating ΔrpfA, ΔrpfB, ΔrpfE and ΔrpfAE is provided in Table 2.

Equal sized fragments of the flanking regions either side of the gene to be deleted were amplified by PCR using the high fidelity polymerase Platinum *Taq* (Life Technologies). Restriction sites were introduced into primer sequences to facilitate cloning into the p2NIL plasmid. The flanking regions were individually cloned into pGEM-T Easy (Promega). The presence of the insert was confirmed by digestion with EcoRI, and insert specific enzymes, and positive clones sent for sequencing. Upon confirmation that there were no mutations in the fragments, each fragment was then individually ligated into the manipulation vector p2Nil and the resultant plasmids transformed into *E. coli*. Plasmids obtained by purification from individual colonies were screened for the correct insertion of both fragments by restriction digest to release the fragments. At the final stage, the PacI cassette encoding a number of selection markers such as hygromycin resistance, SacB and LacZ (*hyg*, *lacZ*, *P_{hsp60-sacB}* ) was cloned into the p2Nil plasmid containing both fragments. Gene deletions were
confirmed by PCR using diagnostic primers (Table 2) and GoTaq DNA Polymerase (Promega).

**Figure 23: Overview of Cloning Strategy for Generation of Deletion Mutants.** The flanking regions of the gene to be deleted were amplified and cloned into the manipulation vector p1Nil or p2Nil. The marker gene cassette containing resistance genes to hygromycin, sucrose and the lacZ gene was excised from pGoal19 by digestion with PacI and cloned into the manipulation vector to make the final deletion construct that was electroporated into *M. marinum* (adapted from Parish & Stoker 2000).
5 μg of the construct was then electroporated into competent *M. marinum* as described in 2.3.8 and 2.3.9, and single crossover clones were selected by plating onto 7H10+ 50 μg/ml kanamycin+ 50 μg/ml x-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) and incubated at 32 °C for 2-3 weeks. Blue, kanamycin resistant colonies were grown in 7H9 medium, to OD<sub>600</sub>=1.5, without antibiotic to allow the second crossover to occur. Following incubation for up to 3 weeks, the cultures were plated on 7H10+ 2 % w/v sucrose+ x-gal to select for double crossover clones. White kanamycin sensitive colonies were screened for gene deletion by PCR using diagnostic and gene specific primers. The deletion was confirmed by Southern hybridisation. Finally, the confirmed deletion mutants were electroporated with 5 μg pSMP12::dsRed to make the strain suitable for fluorescence microscopy.

### 5.2.3 Isolation of *M. marinum* Genomic DNA

Genomic DNA was isolated by enzymatic lysis of cultures as previously described by Belisle & Sonenberg 1998. 20 ml of mid log-phase *M. marinum* was harvested by centrifugation and washed with 25 ml TE buffer (10 mM tris hydrochloride, pH 8, 1 mM EDTA) prior to freezing at -80 °C for a minimum of four hours to aid lysis. Once thawed, the pellet was resuspended in 1 ml TE buffer; 1 ml of 2:1 Chloroform: Methanol was added, shaken for 5 min and centrifuged at 2500 x g for 20 min at room temperature. The aqueous and organic layers were removed to leave the bacterial pellet which was air dried for 15 min to remove traces of solvents. The pellet was resuspended in 1 ml TE buffer, vortexed, and 100 μl of 1 M tris hydrochloride at pH 9 added. 11 μl of 100 μg/ml lysozyme was added and the sample incubated at 37 °C for 16 hours.

The following day, 110 μl of 10 % SDS and 11 μl of 10 mg/ml proteinase K (Sigma) was added to the samples, mixed carefully and incubated at 55 °C for 3 hours. An equal volume of 25:24:1 phenol/chloroform/isoamyl alcohol was added and the sample rocked for 30 min prior to centrifugation for 30 min. The aqueous layer was transferred into a new eppendorf and residual phenol removed by adding an equal volume of 24:1 chloroform/isoamyl alcohol and mixing for 5 min. The sample was
centrifuged at 12000 x g for 30 min and as previously, the aqueous layer was transferred to a fresh eppendorf. DNA was precipitated by addition of 11 μl 3 M sodium acetate, pH 5.2 and 1 ml isopropanol (Sigma). The sample was gently mixed and allowed to incubate at 4 °C for a minimum of one hour. Following incubation, the preparation was centrifuged for 30 min and the resultant pellet washed in 30 μl 70 % ice cold ethanol. The pellet was centrifuged again; the remainder of the ethanol removed and allowed to air dry. The DNA was dissolved in 200 μl TE buffer by rocking at 4 °C overnight. Following resuspension, the genomic DNA was stored at -20 °C until use.

5.2.4 Southern Blotting to Confirm Gene Deletion in *M. marinum*

Digoxigenin (DIG) high prime DNA labelling and detection starter kit II (Roche) was used for Southern blotting according to the manufacturer’s directions. The region surrounding and including the gene to be deleted was analysed by restriction digest to identify the most suitable enzyme to generate genomic fragments that would allow confirmation of the deletion of *rpfA* or *rpfE* (Figure 24a and b, respectively).

![Figure 24: Probe Design for Southern Blot](image)

Schematic representation of Southern blotting to confirm deletion of a gene. Restriction sites are highlighted in red. Following digestion and probing, WT genomic DNA will yield a larger sized band (lane 1) than the fragment (dashed bracket) that will be visualised in the deletion mutant (lane 2) due to this gene no longer being present.
Briefly, 1.3 μg of extracted genomic DNA was digested for 5 hours in a final volume of 20 μl. A 1 % (w/v) agarose gel was run at 45 V for up to 6 hours and 5 μl DIG labelled DNA marker (Roche) was loaded. Following separation of DNA, the gel was depurinated twice for 7 min in 0.25 N hydrochloric acid at room temperature and briefly rinsed in deionised water. The gel was then denatured in 0.5 M sodium hydroxide, 1.5 M sodium chloride for 15 min. This was repeated a second time and the gel was the neutralised in 0.5 M tris hydrochloride, 3 M sodium phosphate for 30 min. At this stage the transfer was set up as described by Brown (2001) to ensure DNA was transferred onto a positively charged nylon membrane (Hybond, GE Healthcare) for blotting. 20x SSC (saline sodium citrate; 2 M sodium chloride, 0.3 M sodium citrate, pH 7) buffer was used as the transfer solution and capillary transfer was left overnight. Both the wick and nylon membrane were equilibrated in this buffer prior to assembly.

The transfer was disassembled and the wells from the gel marked, in pencil, on the membrane as a guide. The membrane was ultraviolet (UV) cross-linked (UV Straterlinker 2400; Invitrogen) twice, using the automated cross-linking option and rinsed with water. Pre-hybridisation was performed by pre-heating DIG easy hyb buffer to 65 °C and submerging the membrane in this solution, also at 65 °C, with constant agitation for 30 min.

To DIG label the probe (DIG-high prime DNA labelling and detection starter kit II, Roche), 1 μg of DNA was resuspended in 16 μl, denatured at 95 °C for 5 min and chilled on ice. DIG high-prime solution was mixed and 4 μl mixed with the probe. The sample was incubated at 37 °C for 5 min and followed by incubation at 65 °C to inactivate the reaction. When not in use, the sample was stored at -20 °C.

The DIG-labelled DNA probe was denatured at 95 °C for 5 min, and cooled on ice prior to use. The probe was added to pre-heated DIG easy hyb buffer (3.5 ml/ 100 cm³ membrane) at a concentration of 25 ng/ml. At this point, the pre-hybridisation solution was removed and the freshly prepared hybridisation solution containing the DNA probe added to the membrane. This was incubated overnight at 65 °C with constant agitation.
Stringency washes to remove excess probe could be now be performed. The membrane was first washed twice in 5 min ample 2x SSC, 01 % SDS, and then twice for 15 min in 0.5x SSC, 0.1 % SDS. Both buffers were pre-warmed to 65 °C and washes were performed under constant agitation. The membrane was rinsed briefly in washing buffer (0.1 M maleic acid, 0.1 M sodium chloride, 0.3 % (w/v) Tween 20, pH 7.5) and blocked for 60 min in 60 ml 1 x blocking solution (10 x solution was diluted with washing buffer lacking Tween 20) at room temperature. 5 μl anti-DIG antibodies conjugated with alkaline phosphatase were added to 25 ml antibody solution and the membrane incubated in this for 30 min. Leftover antibody was removed by washing in 100 ml washing buffer, twice for 15 min at a time.

For immunological detection, the membrane was equilibrated in 20 ml detection buffer (0.1 M tris hydrochloride, 0.1 M sodium chloride, pH 9.5) for 5 min. Hybridized probes were detected by adding 200 μl NBT/BCIP stock solution to 10 ml detection buffer and incubating the membrane in this. The colorimetric reaction was stopped by washing the membrane with water when DNA bands were clearly visible.

5.2.5 Assessment of M. marinum Growth In Vitro

5 ml starter cultures of M. marinum were inoculated from glycerol stocks and allowed to grow until dense (OD_{600}=2). Larger 20 ml cultures were inoculated with appropriate volumes of starter culture to an optical density (600 nm) of 0.1 and their growth monitored by daily measurements of OD_{600} for 14 days. Cultures that had reached an optical density above 1 were diluted to allow for accurate measurements of density. The experiment was performed in triplicate with two biological replicates.

5.2.6 Quantification of Intracellular Bacteria by CFU Measurements

J774 murine macrophages were seeded into 96 well plates, infected as described in 2.5.2, and washed with PBS 4 hours post infection to remove extracellular bacteria.
CFU samples were prepared by washing wells with PBS, lysing macrophages with 100 μl 0.02 % (w/v) Triton X 100 and mixing the contents with a pipette tip. Three 10 μl spots of neat or serially diluted lysates were plated onto 7H10+ADC+kanamycin agar. Plates were incubated for 2-3 weeks, at 32 °C.

5.3 Results and Discussion

5.3.1 Generation of Rpf Deletion Mutants

Unmarked, in-frame deletions of rpfA, rpfE, rpfAE and rpfB were successfully generated as described above. Confirmation that a double crossover had occurred and the gene had been deleted was first obtained by colony PCR using diagnostic primers that bind in the flanking regions that lie outside of the gene of interest (Figure 25a) resulting in a significantly larger PCR product amplified from WT *M. marinum* where the gene is still present, than from the Rpf deletion strains.

*M. marinum* strains in which rpfA had been deleted yielded a band of 582 bp, and 1655 bp where the gene is still present (Figure 25b); strains in which rpfE was deleted from yielded a band of 250 bp, and 950 bp where it had not been (Figure 25c); and where rpfB had been deleted amplified a 300 bp band, or 1300 bp where the gene was still present (Figure 25d). As required, all negative controls did not contain any product.

A.

![Figure 25: Diagnostic PCR to Confirm Gene Deletion in *M. marinum*. (A) Schematic representation of strategy for diagnostic PCR. Primers in the flanking regions amplify the gene of interest and surrounding area. Deletion of the gene results in a PCR product that is significantly smaller than that of WT DNA.](image)
Southern hybridisation was performed using genomic DNA that had been purified from all the strains to confirm deletion of \( rpfE \) and \( rpfA \). The immunolabel digoxigenin (DIG),
a steroid, was used to label DNA probes to allow specific detection of DNA sequences through the use of DIG specific antibodies. The DNA region upstream of \textit{rpfA} and downstream of \textit{rpfE} was used to probe deletion of \textit{rpfA} and \textit{rpfE}, respectively. Each fragment was purified by gel extraction after excision from the construct with enzymes specific to the restriction sites introduced during amplification of DNA, and labelled with DIG prior to use.

The fragments visualised by Southern blotting are in agreement with the DNA sizes predicted in Figure 26a and b. WT and the deletion strains probed to check for \textit{rpfA} yielded the brightest bands at 4700 bp and 3500 bp, respectively, when genomic DNA had been digested with BamHI; and 4899 bp and 3700 bp when digested with EcoRI confirming deletion of this gene from \textit{ΔrpfA} and \textit{ΔrpfAE \textit{M. marinum}}. Although the blot is not clear enough to make a judgement on the bands that are expected in genomic DNA that was digested with Ncol and probed with FR2 to check for the deletion of \textit{rpfE}, the expected bands are visible at 4000 bp in WT genomic DNA and approximately 3200 bp for the \textit{rpfE} deletion (Figure 26c).

Collectively, the results of the PCR and Southern blot indicate that \textit{rpfA} and \textit{rpfE} have been successfully deleted from \textit{ΔrpfA}, \textit{ΔrpfE} and \textit{ΔrpfAE \textit{M. marinum}}. At this stage, deletion of \textit{rpfB} from \textit{M. marinum} has only been confirmed by PCR.
### A.

<table>
<thead>
<tr>
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<th>Observed</th>
<th>Expected</th>
<th>Observed</th>
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<tbody>
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<td>4700 bp</td>
<td>3540 bp</td>
<td>3500 bp</td>
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<tr>
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<td>4899 bp</td>
<td>3742 bp</td>
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### B.

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<th>Observed</th>
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<tbody>
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<td>-</td>
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<td>-</td>
</tr>
<tr>
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<td>4000 bpp</td>
<td>3203 bp</td>
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### C.

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<th>RpfE fr2</th>
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<td>EcoRI</td>
</tr>
<tr>
<td>marker</td>
<td>WT</td>
</tr>
<tr>
<td>4899bp</td>
<td>3639bp</td>
</tr>
</tbody>
</table>

**Figure 27: Southern Blot Analysis of Deletion Mutants.** The expected, and observed, DNA bands when checking for deletion of (A) *rpfA* by probing with fragment 1 (FR1) or (B) *rpfE* by probing with fragment 2 (FR2). (C) Purified flanking region (FR) 1 was used to probe genomic DNA that has been digested with BamHI and EcoRI for deletion of *rpfA*. To probe for deletion of *rpfE*, genomic DNA was digested with Ncol and NotI, and probed with purified flanking region (FR) 2 used in the construction of this deletion. Yellow arrows highlight the bands of interest.
5.3.2 Deletion of Rpfs Does Not Effect In Vitro Growth

The effects of Rpf deletion on *M. marinum* growth were investigated by cultivation of WT and mutant strains in 7H9 medium supplemented with ADC and Tween 80. Only the dsRed expressing variants of WT, ΔrpfA, ΔrpfE and ΔrpfAE *M. marinum* were investigated. Also included in this analysis was a strain that constitutively over-expresses rpfA alongside GFP from a *hsp60* promoter (pMV_GFP_rpfA). Expression of GFP was confirmed by fluorescence microscopy. Growth rate in 7H9 was monitored regularly over a period of 14 days by optical density measurements and CFU counts.

The growth curves shown in Figure 27a indicate that deletion of Rpfs, or over-expression of RpfA (pMV_GFP_rpfA) does not result in any growth impairment or difference suggesting that Rpfs are redundant for *M. marinum* replication *in vitro*. All strains were seeded at approximately OD$_{600}$ = 0.1 and grew to a maximum OD$_{600}$ of 2.5. As above, viable bacterial counts obtained through CFU measurements of the mutant strains showed a similar growth profile to WT *M. marinum* (Figure 27b). Such a lack of difference in lag phase and exponential growth from single and even certain double deletions of Rpfs during *in vitro* growth has previously been observed in Rpf deletion mutants generated in *Mtb* and is often attributed to the functional redundancy within this protein family (Downing et al. 2004; Tufariello et al. 2004).
Figure 28: *M. marinum* Growth *In Vitro* is Unaffected by Rpf Deletion. (A) OD$_{600}$ measurements (average of 3 biological replicates) and (B) CFU counts (average of 2 biological replicates; 3 technical replicates within each experiment) taken over the course of 14 days indicate that deletion of rpf genes, or over-expression of rpfA (pMV_GFP_rpfA) does not result in growth that is different from WT *M. marinum* when monitored in 7H9+ADC+Tween. Error bars indicate the standard deviation of the measurements.
5.3.3 Rpf Deletion Mutants Show No Differences in Replication in J774 Macrophages

The ability of *M. marinum* M to infect, survive and replicate with J774 murine macrophages is well documented (Adams et al. 2011; Ramakrishnan & Falkow 1994). To further characterise the effect of Rpf deletion or over-expression on *M. marinum*, the replication of these strains during infection of murine macrophages was monitored by CFU measurements over a 96 hour period. The deletion of *rpfA* resulted in clumpy *in vitro* growth of *M. marinum* in comparison to WT (Figure 28).

Macrophages were infected at an MOI of 0.5 and any extracellular bacteria removed after 4 hours of infection by washing with DMEM. The intracellular growth of the WT *M. marinum* M (WT) strain has been investigated multiple times throughout this project and was found to reproducibly increase by 2 logs over the course of 4 days (Figure 29a). Poor growth of the WT control (WT2) was observed when quantifying the growth of the remaining strains during macrophage infection (Figure 29), possibly due to using an early-log phase culture of WT *M. marinum*. The infection model used throughout this project requires the use of mid to high log-phase cultures for infection of macrophages; thus the intracellular replication of the Rpf mutants was compared to the profile of the reproducible WT control (WT).

![WT M. mar and ΔrpfA M. mar](image)

**Figure 29: Clumping of ΔrpfA M. marinum.** A homogenous culture of WT *M. marinum* demonstrates normal growth of this organism in 7H9 media. Deletion of *rpfA* results in bacterial aggregation and clumping.
Figure 30: Intracellular Replication of Rpf Deletion and Over-expression Strains. (A) Intracellular replication of WT *M. marinum* within J774 macrophages. WT2 represents the anomalous poor growth observed in this experiment; WT displays the reproducible growth of WT *M. marinum* previously observed and will serve as the control strain. (B) Growth kinetics of WT (light blue), ΔrpfA (red), ΔrpfE (green) and ΔrpfAE (purple). (C) Intracellular replication of WT, ΔrpfA and pMV_GFP_rpfA *M. marinum* in J774 macrophages. There are no statistically significant differences between the growth of any these strains in comparison to WT.
All of the deletion mutants tested replicated within murine macrophages. ΔrpfA, ΔrpfE and ΔrpfEA M. marinum grew to a final OD600 between 1 x 10^5 CFU/ml and 1 x 10^6 CFU/ml and demonstrated no statistical significant differences (ΔrpfA: p value= 0.09; ΔrpfE: p value= 0.14; ΔrpfEA: p value= 0.1 ) in growth or lag phase in comparison to WT M. marinum (Figure 29b). Over-expression of rpfA (pMV_GFP_rpfA) also did not result in any significant growth differences (p value= 0.14) with macrophages (Figure 29c).

The results obtained in this chapter support previously published literature describing the deletion of single Rpfs in Mtb as having no quantifiable effect on in vitro growth (Downing et al. 2005; Downing et al. 2004; Kana et al. 2008). Additionally these authors describe a similar clumping phenotype to ΔrpfA Mtb that does not impair replication highlighting the degree of conservation between these proteins, and suggesting that RpfA may have a role in peptidoglycan biosynthesis that cannot be compensated for by the remaining Rpfs.

5.4 Conclusions

The overall aim of this chapter was to generate a panel of Rpf deletion mutants and characterise the growth phenotypes that may arise as a result of Rpf over-expression or deletion. Strains in which rpfA or rpfE were deleted and a double knock out of rpfA and rpfE had been successfully produced and analysed for their ability to replicate in 7H9 media and during infection of J774 macrophages. A strain in which rpfA was over-expressed was also included. No significant differences in in vitro growth were observed, suggesting that rpfA and rpfE serve no critical function under these conditions that affect mycobacterial growth. Deletion of both of these genes or overexpression of rpfA also had no effect on the growth of these strains. This lack of growth defect has been previously described for Rpf mutants in Mtb by Downing et al. 2005 and the results displayed here are entirely consistent with this publication.

Whilst no obvious changes in bacterial replication in vitro or within J774 macrophages could be observed, deletion of ΔrpfA led to a clumping phenotype during in vitro growth (Figure 28), suggesting a key role for RpfA in the correct maintenance of the
bacterial cell wall. Due to the aforementioned (1.5.2) close relationship between this protein and *L. monocytogenes* ActA, and the clumping phenotype of Δ*rpfA* *M. marinum*, it may be possible to hypothesise that RpfA is involved in functions that ActA has been implicated in including cell adhesion, bacterial attachment and ultimately biofilm formation (Travier et al. 2013) although these remain untested in this work.

An *M. marinum* *rpfB* deletion strain was also generated, however due to delays in obtaining the correctly sequenced fragments required for recombination, could not be characterised for growth *in vitro* or during macrophage infection within the scope of this project. This strain is a key control for the microscopy based localisation studies of Rpfs when using the anti-RpfB antibody.
6 Localisation of Rpfs During Macrophage Infection
6.1 Introduction

There is relatively little information available on Rpfs at the single cell level during infection. Much of our current knowledge has been obtained from expression analysis of whole populations of WT or deletion strains that have been grown in vitro or in vivo, or from structural analysis of individual proteins. Whilst this information remains valuable, it is of importance to address the roles of Rpfs at the single-cell level.

A number of methods have been exploited to study individual bacteria; fluorescence activated cell sorting (FACS) can be used for screening of single cells within a population based on size, granularity, and is often dependent on specific cell-surface labelling of bacterial cells using antibodies or uptake of fluorescent stains (Mattanovich & Borth 2006; Kalisky & Quake 2011). Progress in high-throughput sequencing technology has made it possible to sequence single–cell transcriptomes by RNA sequencing (RNA-seq) from eukaryotic (Tang et al. 2009) and prokaryotic cells (Kang et al. 2011). A major technical advance in this field is the development of microfluidics devices that facilitate analysis, and observation, of gene expression by RNA-seq and microscopy of individual bacteria using one platform (Wakamoto et al. 2013; Saliba et al. 2014; Rusconi et al. 2014; Balaban et al. 2004; Golchin et al. 2012). The intracellular localisation of individual fluorescent reporter-fused bacterial proteins and their correlation with mRNA transcripts has also been studied; most interestingly, in individual cells, mRNA and protein levels for the same gene were found to be uncorrelated (Taniguchi et al. 2010).

The work described in this chapter aims to gain insight into the mechanism of Rpf action, localisation and expression at the single cell level and in the context of macrophage infection. Cell wall associated Rpf could be detected on in vitro grown bacteria by flow cytometry and microscopy using the anti-Rpf antibody. Control experiments using the anti-Rpfb antibody on macrophages infected with ΔrpfB M. marinum identified only background levels of fluorescence. Rpf patterns were found to be similar between the strains monitored in this work (WT, ΔrpfA, ΔrpfE and ΔrpfAE) with proteins found localised to the entirety of the bacterial envelope, as irregular flecks on the bacterial surface or as diffuse aggregates in the cytoplasm of infected
macrophages. Both antibodies identified Rpf localised to the cell poles and no specific association was observed between Rpf expressing cells and phagosomal mycobacteria or those forming ejectosomes. None of the strains were defective in their ability to reside in phagosomes. ΔrpfA M. marinum was tested specifically for its ability to form actin tails and ejectosomes during infection due to the similarities between M. marinum RpfA and L. monocytogenes ActA. No difference was observed between WT and ΔrpfA M. marinum in these processes. Lastly, manual semi-quantification of Rpf expression in infected macrophages, using an anti-RpfB and more general anti-Rpf antibody identified that a relatively small subset of infecting mycobacteria express Rpf.

6.2 Methods

6.2.1 Bacterial Strains, Cell Lines and Reagents

*M. marinum* M expressing dsRed was used as a WT. Remaining strains were generated as described in chapter 5 and maintained in 7H9 or 7H10 Middlebrook agar, supplemented with ADC, Tween 80 and kanamycin at 50 μg/ml. ΔrpfA::rpfa was additionally supplemented with 50 μg/ml hygromycin.

J774 A.1 murine macrophages were maintained in DMEM supplemented with 10 % FCS and 2 mM L-glutamine at 37 °C and 5 % CO₂. Further detail can be found in 2.5.1.

Macrophage infections were performed as described in 2.5.2. The lipophilic tracer, DilC₁₈(5)-DS (Invitrogen Molecular Probes), was used to visualise intracellular membranes during macrophage infection and AlexaFluor 488 or 633 Phalloidin (Life Technologies) was used to stain macrophage F-actin to visualise ejectosomes and actin tails.

Confocal microscopy was performed using a Leica True Confocal Scanner SP5 Microscope (Leica Microsystems) and images analysed by Leica Application Suite Advanced Fluorescence (LAS AF; Leica Microsystems) and Imaris 3D analysis software (Bitplane).
6.2.1 Flow Cytometry to Monitor Rpf Expression During In Vitro Growth

1 x 10^7 mid-log phase mycobacteria (OD_{600}= 0.7) grown in 7H9 were washed in sterile PBST and incubated with anti-RpfB or anti-Rpf antibody (diluted 1:100 in PBS) for 30 min, shaking at room temperature. Bacteria were washed in PBST and incubated for 30 min in secondary antibody diluted 1:100 (Anti-Rabbit IgG or Anti-Sheep IgG conjugated with AlexaFluor 488) with shaking, in the dark. Bacteria were washed in PBST and resuspended in 100 μl PBST for analysis using a BD Accuri C6 (BD Biosciences) flow cytometer. Flow cytometry analysis was performed using the FL-1 filter (for fluorescence excitation at 488 nm for AlexaFluor 488), the FL-3 filter (for fluorescence excitation at 640 nm for dsRed), the FSC (relative cell size) and side scatter (cell granularity/complexity) detectors. A minimum 20000 events were detected for each sample and data was analysed using C6 Flow Plus software (BD Biosciences).

6.2.2 Rpf Staining of In Vitro Grown M. marinum

Mid –log phase WT M. marinum expressing dsRed was stained as previously described in 6.2.1, 5 μl of stained cells were dried onto glass coverslips and mounted onto slides using ProLong Gold Antifade Mountant with DAPI (Life Technologies). Slides were stored at 4 °C until imaging on a Leica SP5 Confocal Microscope.

6.2.3 Preparation of Coverslips and Staining for Microscopy

M. marinum infected macrophages adhered to coverslips were prepared for imaging 48 hours post infection. Spent DMEM was removed, and the coverslips washed three times in sterile PBS. 4 % w/v paraformaldehyde (PFA) dissolved in PBS was used to fix the cells by incubating the coverslips in 1 ml PFA, for 10 min at room temperature. PFA was removed and the coverslips washed in PBS three times. Macrophages were permeabilised to facilitate the entry of stains and antibodies by incubating at 4 °C for 5
in cold 0.2 % triton. Coverslips were washed three times using PBS, blocked for 1 hour at room temperature in 5 % sterile bovine serum albumin (Sigma) and washed again in PBS to prepare the cells for staining.

Anti-RpfB and/ or anti-Rpf antibodies were incubated in 1 ml 1 % albumin in PBS, overnight at 4 °C at a dilution of 1: 1000 resulting in a final amount of 2.5 μg/ml of antibody. Any unbound primary antibody was removed by washing the coverslips three times in sterile PBS and the secondary antibody added to coverslips at a 1:1000 dilution in 1 ml albumin in PBS. AlexaFluor 488/555 goat anti-rabbit or AlexaFluor 488/555 donkey anti-sheep secondary antibodies (Invitrogen) were used, respectively. Samples were incubated at room temperature for an hour, excess antibody removed by washing three times with PBS and the coverslips allowed to air dry in the dark. Coverslips were mounted onto slides using ProLong Gold Antifade Mountant with DAPI (Life Technologies) and stored in the dark at 4 °C.

### 6.2.4 Confocal Microscopy

A Leica True Confocal Scanning SP5 Microscope (Leica Microsystems) was used to image slides at 63x magnification ( >63x/1.3 Glyc 21 °C) under oil immersion (Leica). All slides were warmed to room temperature prior to imaging. Details of lasers used to image each fluorophore can be found in [Error! Reference source not found.].

<table>
<thead>
<tr>
<th>Fluorescence</th>
<th>Excitation/Emission (nm)</th>
<th>Laser</th>
</tr>
</thead>
<tbody>
<tr>
<td>DsRed2</td>
<td>563/582</td>
<td>10 mW DPSS 561 nm</td>
</tr>
<tr>
<td>GFP</td>
<td>488/509</td>
<td>100 mW Argon Laser 488 nm</td>
</tr>
<tr>
<td>AlexaFluor 488</td>
<td>499/519</td>
<td>100 mW Argon Laser 488 nm</td>
</tr>
<tr>
<td>AlexaFluor 633</td>
<td>632/648</td>
<td>10 mW HeNe 633 nm</td>
</tr>
<tr>
<td>AlexaFluor 555</td>
<td>553/568</td>
<td>10 mW HeNe 561 nm</td>
</tr>
<tr>
<td>DAPI</td>
<td>345/455</td>
<td>50 mW diode 405 nm</td>
</tr>
</tbody>
</table>

*Table 4: Details of Fluorophores and Imaging Conditions.* Summary of the fluorophores and conjugates used in this study, the excitation and emission wavelengths and the laser used to image each one.
Videos were prepared in Imaris Scientific 3D Image Processing Suite (Bitplane) and are best viewed using Apple QuickTime (https://www.apple.com/quicktime/download/) or Windows Media Player (http://windows.microsoft.com/en-us/windows/download-windows-media-player) as described.

### 6.2.5 Staining For F-Actin or Membranes in Macrophages Infected With *M. marinum*

To visualise intracellular membrane bound compartments, uninfected or infected J774 murine macrophages were allowed to uptake the lipophilic membrane marker 1,1’-Dioctadecyl-3,3,3′,3′-Tetramethylindodicarbocyanine-5,5′-Disulfonic Acid (DilC₁₈(5)-DS; Life Technologies) for 1 hour at 32.5 °C. The working concentration of the marker was 2 μM. Following incorporation, macrophages were washed with sterile PBS to remove excess dye and to prepare coverslips for fixation and processing as described in (6.2.3).

Host cell actin was visualised by probing specifically for F-actin using Phalloidin conjugated with AlexaFluor 633 (Invitrogen Molecular Probes). Macrophages that had been fixed, permeabilised and blocked with BSA were incubated at room temperature with a 0.165 μM final concentration of phalloidin in 200 μl of 1 % BSA in PBS for 20 minutes. Coverslips were washed with PBS three times and staining using anti-Rpf antibodies continued as described in (6.2.3).

### 6.2.6 Semi-Quantification of Rpf Expression by Confocal Microscopy

Macrophages were infected and coverslips prepared for imaging using the anti-RpfB and anti-Rpf antibodies. Up to 15 representative fields of view were imaged as z-stacks from two independent biological replicates (Figure 30). Individual macrophages were scored by eye according to infection level: Low (1-5 mycobacteria), Medium (6-10 mycobacteria) or High (11 or more mycobacteria). Rpf expression was scored as present or not. Uninfected macrophages stained with either antibody were also included in this analysis as a measure of background fluorescence.
An average of 1600 macrophages were counted per strain, from 30 different fields of view collected from two independent macrophage infections.

**Figure 31: Representative Field of View for Semi-Quantification.** Macrophages stained with (A) anti-RpfB antibody or (B) anti-Rpf antibody. Quantification was carried out using Leica LAS-AF software. Macrophage nuclei was stained using DAPI (blue), dsRed expressing *M. marinum* was the infectious organism (red) and Rpf expression was seen after staining with the secondary antibody AlexaFluor 488 (green).

### 6.3 Results and Discussion

#### 6.3.1 Rpf Expression During *In Vitro* Growth Can be Detected by Flow Cytometry

Anti-RpfB and anti-Rpf antibodies were used to investigate the Rpf expressing population during growth of *M. marinum* in 7H9 media. Analysis of mid log phase bacteria identified a highly heterogeneous mixture of particles of varying sizes and complexity in the samples tested, likely a composition of mycobacterial aggregates, single cells and non-bacterial particles (Figure 31).

Control experiments using only anti-RpfB (Figure 31a), anti-Rpf (Figure 31c) antibodies or the anti-Rpf secondary antibody (Figure 31d) conjugated with AlexaFluor 488 yielded the same distribution of events as unstained *M. marinum* with little evidence of non-specific binding of the antibodies (Figure 31a), however, a non-specific
background was observed when this culture was treated with only the anti-RpfB secondary antibody (2.6 % in UR; 0.8 % in LR; Figure 31b).

The majority of the gated population analyzed (P1) were dsRed expressing cells (upper left quadrant or UL); this value varied from 74 % to 86 % (Figure 31e). Treatment with the anti-Rpf antibody (Figure 31g) identified a clear subset of the population in the upper right quadrant that was positive for both high intensity dsRed and high intensity AlexaFluor 488 fluorescence (15.4 %). Comparatively, 5 % less of the population was consistently double positive for dsRed and AlexaFluor 488 fluorescence when ΔrpfAE *M. marinum* was analysed with the same antibody (Figure 31h). Treatment of WT *M. marinum* with the anti-RpfB (Figure 31f) antibody identified a subset (6.8 %) of the population that was positive for high intensity AlexaFluor 488 fluorescence and low intensity dsRed fluorescence.

The data obtained indicates that *in vitro* expression of Rpf can be detected using the antibodies characterised in chapter 4. The profile observed with the anti-Rpf antibody, and lack of background from the associated secondary antibody, suggests that 15 % of WT *M. marinum* appears to express cell associated Rpf during exponential *in vitro* growth and that deletion of *rpfA* and *rpfE* reduces Rpf expression by 5 % under these conditions. The association of high intensity AlexaFluor 488 fluorescence with low intensity dsRed events could suggest that RpfS are being detected on a small subset of individual mycobacteria (as judged by low dsRed fluorescence), however the background that is seen with the secondary anti-rabbit antibody implies that part of this population may also be a result of non-specific binding of the secondary antibody to bacterial or non-bacterial particles. Additionally, this antibody has been documented to detect diffuse Rpf more often than cell attached (Figure 33). This preliminary data thus requires further investigation using a quadruple *rpf* deletion mutant to confirm how specific this binding is to RpfS.
Figure 32: Quantification of Rpf Expression in *In Vitro* Grown *M. marinum* by Flow Cytometry. All samples have been analysed for relative size (FSC-A) and complexity/granularity (SSC-A), or dsRed (FL3-A) and Alexafluor 488 (FL1-A) expression. Representative dot plots of controls: WT *M. marinum* stained (A) with only the anti-RpfB antibody, (B) with only the anti-rabbit conjugated with AlexaFluor 488 secondary antibody, (C) with only the anti-Rpf antibody or (D) with the anti-sheep conjugated with AlexaFluor 488. The percentage of the population in each quadrant is highlighted in red.
Figure 33 cont.: Quantification of Rpf Expression in In Vitro Grown M. marinum by Flow Cytometry. All samples have been analysed for relative size (FSC-A) and complexity/granularity (SSC-A), or dsRed (FL3-A) and Alexafluor 488 (FL1-A) expression. Representative dot plots of (E) WT M. marinum not stained for Rpf, (F) WT M. marinum stained using the anti-RpfB antibody or (G) anti-Rpf antibody. (H) Analysis of ΔrpfAE M. marinum with anti-Rpf antibody. The percentage of the population in each quadrant is highlighted in red.
6.3.2 *In Vitro* Grown Bacteria Express Detectable Levels of Rpf

Exponential phase WT *M. marinum* grown in 7H9 was incubated with anti-Rpf or anti-RpfB antibody and imaged by confocal microscopy. Both antibodies could detect surface associated fluorescence likely to be Rpf attached to the bacterial envelope on a subset of the population observed. Fluorescence was seen as discrete flecks or beads covering part of the mycobacterial surface (Figure 32; Video 1). Cultures that had been incubated with anti-RpfB antibody also showed fluorescence discretely localised to the bacterial pole (Figure 32; yellow arrow; Video 2) supporting previous microscopy based observations describing the close *in vitro* interaction of *Mtb* RpfB with RipA, a protein that localises at the bacterial pole, and interacts with peptidoglycan synthesizing enzymes like PBP-1 (Hett et al. 2007; Hett et al. 2008).

Figure 34: *In Vitro* Expression of Rpf. Confocal microscope images of mid-log phase WT *M. marinum* (red) stained with anti-Rpf (top panels) or anti-RpfB antibody (bottom panel), and secondary antibody conjugated to AlexaFluor 488 (green). Yellow arrow highlights polar localised Rpf. All scale bars are 5 μm.
6.3.3 Rpfs are Found in the Cytoplasm and Localised to the BacterialEnvelope

Having obtained two Rpf-specific antibodies with differing titres to Rpfs, and generated a panel of Rpf deletion mutants, it was now possible to address the localisation and expression patterns of Rpfs during infection. J774 macrophages were infected as described in 2.5.2, stained with one of two anti-Rpf antibodies and imaged by confocal microscopy. Two different patterns of Rpf localisation were observed.

When staining with the anti-Rpf antibody, presumably, multiple Rpf homologues were found to closely associate with the mycobacterial surface- either as a consistent smooth envelope surrounding the whole cell (Figure 33b,d; Video 3, Video 4), or as irregular flecks (Figure 33b, c; Video 5). Staining with the anti-RpfB antibody revealed patches of bright, irregularly shaped fluorescence in the cytoplasm of the macrophage; in most cases this lacked any obvious association with the bacterial membrane (Figure 33f, g, h; Video 6, Video 7) indicating that Rpfs were secreted into the cytoplasm of the cell. Non-specific background fluorescence can be seen in the uninfected controls and is significantly less intense than Rpf-specific signals (Figure 33a, e). The localisation patterns of Rpfs that are described above remain unaffected in ΔrpfA, ΔrpfE and ΔrpfAE M. marinum (data not shown).

A control experiment was performed using ΔrpfB M. marinum infected macrophages that had been incubated with anti-RpfB antibodies (Figure 34). WT M. marinum infected macrophages showed the same pattern of fluorescence as previously described (Figure 33). The discrete patches of intense fluorescence were not observed in macrophages infected with the RpfB deletion strain, although, the same diffuse background staining was present in uninfected and infected cells (Figure 34). No difference in Rpf localisation, compared to the WT strain, was observed when ΔrpfB M. marinum infected macrophages were incubated with anti-Rpf antibody (data not shown).
**Figure 35: Cellular Localisation of *M. marinum* Rpfs.** WT *M. marinum* and the uninfected control stained with anti-Rpf antibody (A-D) or the anti-RpfB antibody (E-H). Constitutively dsRed expressing *M. marinum* is red, the DAPI stained nucleus of the macrophage is blue and Rpf signals are in green due to the use of a AlexaFluor 488 as a secondary antibody. Some background fluorescence is visible as diffuse green. All scale bars are 5 μm.
Figure 36: $\Delta rpfB$ M. marinum Infected Macrophages Treated With Anti-RpfB Antibodies. Top panel shows the intensely fluorescent patches characteristic of Rpf expression when treated with anti-RpfB antibody observed in macrophages infected with WT M. marinum. Middle and lower panels show $\Delta rpfB$ M. marinum infected macrophages also stained with this antibody. Notice loss of intense fluorescence. All scale bars are 5 μm.
Figure 37: Polar Localisation of RpfS During Macrophage Infection. Staining of RpfS with the (A-C) general anti-Rpf antibody or the (D-F) specific anti-RpfB antibody shows localisation to the apical ends of bacteria. This was seen to occur in WT *M. marinum* (A, D), ΔrpfA (B, C, E) and ΔrpfE (F). Red is *M. marinum*; blue is the nucleus of macrophage; green is Rpf-specific signals. Polar localised Rpf is highlighted by a yellow arrow. All scale bars are 5 μm.
Rpfs have already been recognized to be secreted into culture supernatant (Mukamolova et al. 1999), shown to localise to the bacterial cell wall when grown in vitro (de Souza et al. 2011; Mukamolova et al. 2002), appear to be diffuse in the vicinity of bacteria in human tissues infected with Mtb (Davies et al. 2008) and have been detected in the tissues of infected guinea pigs (Kruh et al. 2010). As such, it is not surprising to find that during macrophage infection, all of these forms of localisation can also be observed suggesting Rpfs function in a dynamic manner. The biological significance of associating closely with the mycobacterial cell wall is most likely to allow the Rpfs to exert their muralytic function and cleave the peptidoglycan. The presence of diffuse Rpf that is not co-localised to bacteria supports that these proteins can be secreted into the surrounding environment and may represent a mechanism of regulation of Rpf function. The correct regulation of Rpfs and peptidoglycan hydrolases is key for normal bacterial growth and cell division as these enzymes can be lethal for bacterial cells if their activity is not controlled. Alternatively, this subset of protein may be performing, or responding to, a currently unknown task or cue.

Both antibodies identified Rpf that appeared to be localised to the polar ends of bacteria in WT M. marinum and a number of the deletion mutants (Figure 35; Video 2, Video 8, Video 9). Mycobacteria elongate apically, incorporating nascent peptidoglycan at the distal ends of the cell (Hett & Rubin 2008; Joyce et al. 2012); most likely, the polar localisation of Rpfs occurs to create the ‘space’ required in the existing cell to facilitate the incorporation of peptidoglycan monomers. Both RpfB and RpfE from Mtb have been demonstrated to function synergistically with RipA primarily at the ends of dividing cells (Hett et al. 2007; Hett et al. 2008) supporting the mounting evidence that Rpfs are involved bacterial extension and growth, and not only resuscitation from dormancy.

It is of interest to note that although the anti-Rpf and anti-RpfB antibodies predominantly show differing patterns of Rpf localisation, there still appears to be significant overlap when using them for immunofluorescence. The anti-Rpf antibody identifies both cell associated Rpf and the discrete ‘blobs’ of Rpf characteristic of the anti-RpfB antibody, and vice versa (Figure 36) suggesting that to some extent, the latter may recognise other Rpf homologues.
Survival within the phagosome by arresting maturation is a key feature of mycobacterial infection and a number of groups studying mycobacterial gene expression have detected the upregulation of certain Rpf s during phagosome-like conditions. In vitro exposure of Mtb to low pH for 24 hours resulted in significant upregulation of rpfE and to a lesser degree, rpfD (Gupta et al. 2010), whilst upregulation of Mtb rpfD and rpfE in response to increases in Cl− and acidification of the surrounding environment has also been corroborated by Tan et al. (2013).
Therefore, to investigate Rpf expression and whether it is associated with mycobacteria residing in the phagosome, J774 macrophages were infected with WT *M. marinum* or the Rpf deletion strains. Prior to fixation and processing of slides for confocal microscopy, infected cells were allowed to uptake the amphiphilic tracer DilC$_{18}$(5)-D. This tracer is highly fluorescent and photostable once incorporated into membranes and facilitates the visualisation of membrane bound compartments. However, the lipophilic nature of the dye means that it does not discriminate between cell membranes and is capable of staining phagosomes, vesicles and organelles equally. Phagosomes were thus defined as the rod-shaped membranes found associated to the outside of dsRed expressing mycobacteria (highlighted with a white arrow in Figure 37 and Figure 38).

No obvious correlation was observed between Rpf localisation and phagosomal bacteria when using either the anti-Rpf (Figure 37) or the anti-RpfB antibody (Figure 38). Whilst WT, ΔrpfA and ΔrpfE *M. marinum* could readily be found in phagosomes, Rpf expression seemed to be indiscriminate and could be detected in the cytoplasm, localised to free and phagosomal bacteria. Such an observation does not, necessarily contradict the results of Gupta et al. (2010) or Tan et al. (2013). It would be of importance to assess a quadruple Rpf deletion mutant, or a strain retaining a single Rpf, for the ability to reside within the phagosome as single mutants clearly show no obvious phenotype, likely due to the redundancy within this family of protein. The antibodies used, whilst specific to Rpf$s$ do not allow sensitive enough differentiation between the different homologues and it could be possible that one Rpf is more often found localised to phagosomal bacteria than the others.
Figure 39: Use of Anti-Rpf Antibody to Observe Rpf Expression and Phagosomal Mycobacteria. The general anti-Rpf antibody was used to monitor if there was any correlation between *M. marinum* residing in the phagosome (visualised using DilC$_{18}$(5)-DS (middle panel; white arrow)) and Rpf expression using the secondary antibody AlexaFluor 488 (left panel; yellow arrow). Overlays of Rpf expression (green), macrophage nuclei (DAPI; blue), *M. marinum* (red) and membrane compartments (grey) are shown on the right. All scale bars at 7 μm.
Figure 40: Use of Anti-RpfB Antibody to Monitor Rpf Localisation in Relation to Phagosomal Mycobacteria.

The anti-RpfB antibody that was raised in this project was used to visualize Rpf expression using the secondary antibody AlexaFluor 488 (left panel; yellow arrow) and its correlation with phagosomal *M. marinum* (membranes were stained with DilC18(5)-DS; middle panel; white arrow). Overlays of Rpf expression (green), macrophage nuclei (DAPI; blue), *M. marinum* (red) and membrane compartments (grey) are shown on the right. All scale bars at 5 μm.
6.3.5 Deletion of RpfA Does Not Affect Actin Polymerisation

The short, highly cross-linked actin tails formed by \textit{M. marinum} have been well documented by Stamm et al. 2003, however the \textit{M. marinum} genes and proteins responsible for this have not been defined. We hypothesised that RpfA could be involved in actin tail formation due to the similarities of this protein to ActA, a critical mediator of actin polymerisation in \textit{L. monocytogenes} (section 1.5.2).

Another important actin based event that occurs during infection is the formation of ejectosomes. These F-actin based cellular organelles facilitate the non-lytic intercellular movement of \textit{M. marinum} and other intracellular pathogens. Beyond the requirement of an intact ESX-1 locus, the deletion of which abolishes the formation of ejectosomes entirely, the mechanism involved in their formation is unknown and actin tails were rarely observed as the propelling force that allow mycobacteria to move through the ejectosome and into neighbouring cells (Hagedorn et al. 2009). Clearly, it can be extrapolated that a secreted mycobacterial protein is involved in this process, and due to the previously identified connection of RpfA to a well documented actin-assembly protein, we also investigated a possible role for RpfA in this actin based event.

\textit{Δ}rpfA \textit{M. marinum} was generated as previously described in chapter 5 and infected macrophages were treated with AlexaFluor Phalloidin 633 to specifically stain F-actin. Phalloidin is extensively used in the study of actin filaments; \textit{M. marinum} actin tails have been demonstrated to be short, curved and more often found at the pole than along the length of the bacteria (Stamm et al. 2003; Stamm et al. 2005; Figure 39b, c, e); ejectosomes are typically observed as bright, F-actin dense, barrel shaped structures spanning the macrophage membrane with bacteria protruding directly through the structure (Figure 40c). Bacteria can also be seen to be partially surrounded by ejectosomes (Hagedorn et al. 2009; Figure 40b,e).
Figure 41: F-actin Polymerisation in WT and ΔrpfA M. marinum. Overlay of images where the macrophage nucleus is stained with DAPI (blue), F-actin is stained with AlexaFluor Phalloidin 633 (cyan) and dsRed expressing bacteria can be seen in red. The DAPI stain has been removed from some overlays for clearer viewing of tails that are highlighted in yellow. All scale bars are 5 μm.
Figure 42: Confocal Microscopy of Ejectosomes in WT and ΔrpfA M. marinum. The nucleus of macrophages has been stained with DAPI (blue), bacteria are constitutively expressing dsRed (red) and actin is stained with Alexafluor Phalloidin 633 (cyan). An uninfected macrophage shows no actin polymerisation. Ejectosomes are highlighted by white arrows. All scale bars are 10 μm.
Actin tails were not visible in uninfected J774 macrophages; however, both WT and \( \Delta rpfA \) M. marinum showed evidence of actin polymerisation around bacterial poles (Figure 39; yellow arrow; Video 10, Video 11, Video 12, Video 13) that was similar to previous reports of actin tails. Whereas, it was somewhat difficult to discern the tails due to the dense infection of cells and background from the phalloidin stain; ejectosomes were unmistakably visible in macrophages infected with WT or \( \Delta rpfA \) M. marinum (Figure 40; Video 14, Video 15). Additionally, Rpf localisation, as observed by using the anti-Rpf or anti-RpfB antibodies, demonstrated no pattern of association (or consistent lack of) with actin-associated bacteria (data not shown). When taken together, this data suggests that RpfA does not appear to be a critical factor for mediating host cell actin polymerisation under the conditions tested.

As such, RpfA may have a specific role beyond its general capacity to digest bacterial peptidoglycan and resuscitate dormant bacteria that has not yet been elucidated. Due to the close relationship between L. monocytogenes ActA and M. marinum RpfA, it may be possible to hypothesise that RpfA is involved in functions that ActA has been implicated in including cell adhesion, attachment and ultimately biofilm formation. Alternatively, the presence of a ydaO riboswitch in the 5’ UTR region of rpfA that has been implicated in osmotic shock responses (Barrick et al. 2004) may suggest that RpfA is required for osmotolerance or recovery from osmotic shock.

Prior to this work, M. marinum ejectosomes had only been demonstrated during the infection of the model organism Dictyostelium. Evidence of their formation during infection of murine macrophages highlights the importance of this conserved mechanism of cell-to-cell spread and provides another tool by which to study them.

### 6.3.6 Semi-Quantification of Rpf Expression During Macrophage Infection

To investigate the expression levels of RpfS at the single cell level, the previously described panel of mutants was used to infect murine macrophages, stained with the aforementioned antibodies and macrophages quantified according to infection load (uninfected, low, medium or high) and for Rpf expression (present/not present) by
microscopy. Initial attempts to use fully- or semi-automated systems including the Java-based image processing software ImageJ, Scan^R microscope for high-throughput screening (Olympus) and the 3D image analysis programs Imaris (Bitplane) and Volocity (Perkin Elmer) were unsuccessful due to a number of reasons. These included the inability of the software to discriminate between multinucleate macrophages and numerous macrophages that were infected with the same bacteria and due to these systems being configured to recognise circular objects resulting in the expression profile observed with the anti-Rpf antibody remaining virtually unrecognisable. Automated scanning of slides resulted in images that lacked resolution due to poor autofocus and there was a huge loss of expression data due to the inability to image z-stacks. To overcome these obstacles, the imaging and quantification was performed manually.

The results indicate that all strains were capable of infecting macrophages with comparable levels (40 %–55 % macrophages infected) seen 48 hours post infection. The exception to this pattern is the rpfA complemented strain (ΔrpfA::rpfA) which appears to have infected approximately 28 % of macrophages in the same time period (Error! Reference source not found.5). Whilst this strain shows no growth defects in in vitro growth, or in its ability to replicate intracellularly (Figure 27 and Figure 29), this lower than expected infection level may reflect the inherent variability of this protocol where macrophages are infected with live cultures that are prone to clumping and slow growth.

Generally, when using either antibody, as the number of bacteria infecting each macrophage increases, so does the level of Rpf expression that can be observed. In all cases, more Rpf expression can be observed with the general anti-Rpf antibody; this is most probably due to the known ability of this antibody to recognise multiple Rpf homologues. Additionally, the functional redundancy of this protein family has been demonstrated by gene expression profiling whereby the remaining Rpfs in a Rpf deletion mutant have been upregulated (Gupta et al. 2010). This is demonstrated in the results obtained here (Error! Reference source not found.) where Rpf expression is higher in ΔrpfE M. marinum (35 %) in comparison to WT M. marinum (17 %) when stained with the anti-Rpf antibody.
### Table 5: Semi-Quantification of Rpf Expression During Macrophage Infection.

Analysis of Rpf expression when staining cells with **(A)** the specific anti-RpfB antibody raised in this project or **(B)** the more general anti-Rpf antibody. Low infection was scored at 1-5 mycobacteria per macrophage, medium infection as 6-10 mycobacteria and high infection as more than 10 mycobacteria per macrophage. Percentage total expression was corrected for background ‘expression’ obtained from scoring uninfected macrophages for areas of bright fluorescence similar to Rpf expression (0.51% from staining with the anti-RpfB antibody; 0.55% from the anti-Rpf antibody). Background associated with the secondary antibody AlexaFluor 555, used alongside the GFP expressing pMV_GFP_rpfA, was 0.64% for the anti-RpfB antibody, and 0.87% when using the anti-Rpf antibody. Data shown is the average of two biological replicates.

**A.**

<table>
<thead>
<tr>
<th>Anti-RpfB</th>
<th>% total infection</th>
<th>% total expression</th>
<th>% low infection</th>
<th>% expression</th>
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**B.**

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Strains in which \textit{rpfA} has been deleted seem to show the least amount of detectable antibody when using the anti-RpfB antibodies (8 % for \textit{ΔrpfA} and 9 % for \textit{ΔrpfAE}) in comparison to WT (17 % total expression) suggesting a particular importance to this protein and a role that cannot be easily compensated for by the remaining RpfB.

Manual semi-quantification and flow cytometry analysis of Rpf expression identified comparable levels of Rpf in WT \textit{M. marinum} when using the anti-Rpf antibody (17 % by microscopy, Error! Reference source not found.5; and 15 % by flow cytometry, Figure 31). Comparative analysis of Rpf expression in the \textit{ΔrpfAE} strain using the anti-Rpf antibody identified 5 % less Rpf expression when detected by flow cytometry (Figure 31) whereas it remained close to WT Rpf expression in the microscopy based semi-quantification (Error! Reference source not found.). This difference may be due to the different gene expression profiles that exist between \textit{in vitro} grown mycobacteria, and those in a more biologically relevant system such as a macrophage (Butcher 2004; Schnappinger et al. 2003). It is difficult to draw conclusions between the patterns of expression observed with the anti-RpfB antibody using these two techniques due to non-specific staining of low fluorescence events by the anti-rabbit secondary antibody during flow cytometry (Figure 31b).

Although much of this data supports published information on Rpf expression and the effects of Rpf deletion, it highlights an important feature that has not been previously addressed- that only a subset of bacteria express Rpf at any given time during infection. Previous investigation into the expression levels of this protein family suggested that they are expressed, to differing degrees, at all stages of the life cycle (Gupta et al. 2010; Downing et al. 2004; Tufariello et al. 2004), however, there is increasing evidence for the stochastic nature of gene expression in \textit{E. coli} (Li & Xie 2011), \textit{B. subtilis} (Maamar et al. 2007; Locke et al. 2011) and \textit{M. smegmatis} (Wakamoto et al. 2013) that results in population heterogeneity and is overlooked when measuring gene expression at the population level. This phenomenon is intricately linked to antibacterial tolerance and persistence (Zahrt 2003; Maisonneuve & Gerdes 2014; Balaban et al. 2004) and explains how some bacteria can remain viable during antimicrobial treatment- it may also be the mechanism by which Rpf expression is governed. Constant expression of RpfB (Gupta et al. 2010; Downing et al. 2004)
would result in a constant signal for growth and resuscitation, and would presumably impair formation of NRP. Similarly, the constant presence of a muralytic enzyme may result in a weaker bacterial cell wall due to constant cleavage of the peptidoglycan. Dynamic bursts of Rpf expression would explain the presence of Rpf in only a subset of bacteria which is likely to be related to the growth state (replicating or persisting), or stage (resuscitating or scouting) of that particular bacterium.

6.4 Conclusions

This chapter describes the use of two anti-Rpf antibodies to characterise the localisation and expression patterns of *M. marinum* Rpfs, at the single cell level and in the context of macrophage infection. The anti-Rpf antibody was demonstrated to recognise an Rpf expressing population when using flow cytometry to analyse *in vitro* grown *M. marinum*, however the preliminary results obtained when using the anti-RpfB antibody need further investigation to clarify the extent of non-specific binding by the secondary antibody. Both antibodies readily detected Rpf on the cell surface of *in vitro* grown bacteria when observed by microscopy.

Rpfs were found to be cell wall-associated, free in the cytoplasm and localised to the polar ends of the bacterial cell suggesting a role in apical extension, and possible spatial regulation whereby Rpfs localised to the cell wall are released from this position to stop cleavage of bacterial peptidoglycan. Attempts to discern roles for Rpfs in mycobacteria residing in the phagosome identified no distinct patterns that implicated their involvement at this stage. The observed phenotype of Δ*rpfA* *M. marinum* during macrophage infection showed no difference to WT *M. marinum* suggesting that RpfA does not play a role in actin polymerisation under the conditions tested.

Most interestingly, Rpf expression was associated with only a subset of bacteria, as demonstrated by microscopy and semi-quantification of the presence of Rpfs during macrophage infection, suggesting that Rpfs are expressed in pulses during the mycobacterial life cycle.
7 Final Conclusions and Future Work
7.1 Final Conclusions

*Mtb* remains a major global health threat; part of its success as an infectious organism lies in its ability to persist in a non-replicating state within its host, and reactivate years after the initial infection. With an estimated third of the world’s global population thought to be latently infected, there is an urgent need to understand this stage of the mycobacterial life cycle, and more specifically to elucidate the means by which these dormant organisms are reactivated. The identification and characterisation of Rpfs, bacterial factors implicated in growth stimulation and resuscitation of dormant bacteria by cleavage of the peptidoglycan cell wall (Mukamolova et al. 1998; Mukamolova et al. 2006), has begun to shed light on this phenomenon. However, questions remain regarding the exact mechanisms that allow Rpf activity to result in growth and reactivation of mycobacteria and how this is regulated. The overarching aim of the work described here has been to begin to unravel the individual roles of the Rpf homologues and to gain insight on the mechanisms by which Rpfs exert their function by using an *M. marinum*-macrophage infection model.

These aims were addressed using a multidisciplinary approach including the generation and purification of recombinant *M. marinum* RpfB which was confirmed to be folded and active in muralytic assays (chapter 3); the production of a polyclonal anti-RpfB antibody, which, when used alongside a previously generated antibody that broadly recognises all Rpf homologous, facilitated the study of Rpf expression and localisation during *in vitro* growth and macrophage infection using immunofluorescence (chapter 4); and the generation of a panel of Rpf deletion mutants that would allow any phenotypic changes attributed to loss of Rpfs to be observed (chapter 5).

The outcome of the work described here has multiple implications. Firstly, the generation of a novel anti-RpfB antibody, and the construction of a panel of deletion mutants provides another means by which to study these proteins, and their effects on replication, survival and persistence. The use of *M. marinum* specifically allows for modelling of TB infection when used to infect its natural host, the model organism, zebrafish.
Secondly, the use of these reagents during macrophage infection, and consequent imaging by confocal microscopy has provided insight into the patterns of Rpf localisation. Whilst no noticeable differences were observed between the deletion strains in their ability to grow in vitro (Figure 27) or in murine macrophages (Figure 29), RpfS were seen to localise to the mycobacterial cell wall as a smooth or disrupted envelope, to localise to the distal end of the bacterial cell, or found to be diffuse in the cytoplasm of macrophages. Rpf expression did not seem to be correlated with phagosomal mycobacteria (chapter 6). Collectively, this data provides a foundation to begin forming some mechanistic inferences towards how this protein family may function.

![Diagram](image)

**Figure 41: Proposed Model of Rpf Function Derived from Protein Expression and Localisation Patterns.** Mycobacteria (red) express Rpf (green) localised to the distal end of the cell facilitating the incorporation of nascent peptidoglycan monomers and resulting in bacterial growth and extension. The continuous or ‘flecked’ Rpf envelope occurs due to RpfS localising to the bacterial envelope resulting in cell wall remodelling and resuscitation. The significance of these forms of expression is currently unknown. Diffuse ‘blobs’ of Rpf represent secreted Rpf, or Rpf that has been released from the bacterial envelope after localisation to the cell envelope.

The results from immunofluorescence studies of Rpf localisation suggest that the growth stimulatory effect of RpfS may occur as a result of polar localisation, where the protein is able to nick the mycobacterial peptidoglycan and allow the incorporation of
nascent peptidoglycan at the polar end, resulting in bacterial elongation (Figure 41). We suggest that the smooth Rpf envelope that entirely surrounds the bacterial cell wall arises from Rpf aggregating irregularly at the cell wall— a step that is observed as flecks of Rpf attached to the cell wall. Alternatively, the disrupted Rpf envelope might also form following smooth envelope expression as the Rpfs respond to a yet unknown cue that removes them from the bacterial cell wall, and releases them into the cytoplasm. Rpf can also be directly secreted by bacteria into the surrounding environment. By microscopy, this is observed as discrete patches of fluorescence in the cytoplasm of macrophages (Figure 41). At this stage, cytoplasmic Rpf may be degraded or may bind to nearby mycobacteria to begin the growth or resuscitation process again.

Semi-quantification of Rpf expression during infection of murine macrophages indicated a positive correlation between the extent of macrophage infection and the presence of Rpf; the results showed that only a subset of mycobacteria express Rpf during infection, supporting the increasing view that bacterial gene expression can occur as transient pulses (Wakamoto et al. 2013; Locke et al. 2011; Li & Xie 2011). This was corroborated when Rpf expression was monitored by flow cytometry using the anti-Rpf antibody. Although this phenomenon requires more direct experimental confirmation, it could be the mechanism that facilitates the spontaneous resuscitation of dormant mycobacteria that are then able to scout the environment and respond accordingly.

Finally, having identified significant homology between M. marinum RpfA and the actin polymerisation protein ActA from L. monocytogenes, we hypothesised that RpfA could be involved in the actin polymerisation events induced by M. marinum in its host and that ActA may have Rpf-like activity. The muralytic activity of recombinant ActA, purified from E. coli, was confirmed in zymograms and by the ability of this protein to hydrolyse the fluorescent peptidoglycan analog, MUF tri-NAG. ActA has previously been implicated in aggregation and biofilm formation of L. monocytogenes (Travier et al. 2013) and it could be suggested that this muralytic activity may be required for regulating bacterial attachment through cleavage of the peptidoglycan cell wall. The generation of an rpfA deletion mutant in M. marinum allowed the ability of this strain
to form actin tails and ejectosomes during macrophage infection to be observed. No differences were observed between WT *M. marinum* and ΔrpfA, suggesting that this protein plays no essential role in the intracellular motility of *M. marinum* under the experimental conditions tested here.

### 7.2 Future Work

The work described here attempts to elucidate the roles of RpfS at the single cell level. The advantages of using Rpf sensitive antibodies and immunofluorescence to study Rpf expression and localisation patterns are compounded by the potential cross reactivity of these antibodies to multiple homologues of RpfS. An elegant solution that would further advance our knowledge on the behaviour of individual RpfS could be achieved by generating mycobacterial strains expressing RpfS fused to red fluorescent protein (Hett et al. 2010) which would allow the localisation patterns of solitary RpfS to be monitored *in vivo* and in real time. A similar method can be employed to investigate the regulation and expression patterns of specific RpfS by replacing the chromosomal genes with fusions to dsRed2 (Wakamoto et al. 2013). Both of these methods have been exploited in mycobacteria, can provide information at the single cell level, and in the context of infection, or at a specific stage of the mycobacterial life cycle. By combining the use of fluorescent reporters of protein or gene expression with fluorescence live cell microscopy, meaningful data will be generated which can be used to address the accuracy of the proposed model of Rpf localisation (Figure), and the potential of Rpf expression being stochastic.

Fluorescence resonance energy transfer (FRET) microscopy is commonly used to study protein protein interactions within living tissues (Meyer & Dworkin 2007; Ellinger & Voigt 2014). This would be a key technique to employ to definitely confirm if the localisation of Rpf to the polar end of the cell is genuine, or just a random distribution of Rpf on the bacterial surface.

Whilst preliminary experiments performed using ΔrpfA *M. marinum* suggest that the deletion of RpfA has no adverse effect on mycobacterial motility and actin
polymerisation, it remains possible that RpfA could be involved in actin polymerisation under very specific growth conditions that have not been tested in this project. Direct evidence of the ability of RpfA to induce actin polymerisation can be obtained using in vitro cell free assays based on lysed extracts of Xenopus oocytes (Clark & Merriam 1978). By coating polystyrene beads in recombinant, purified Rpf and observing for bead motility by fluorescence microscopy during incubation with cell free extract, it would be possible to test individual RpfA for their ability to polymerise actin (Cameron et al. 1999). This would provide a clear demonstration of whether RpfA can be responsible for, or at least involved in the polymerisation of host actin and could also facilitate the discovery of any additional proteins that may be involved in this process.

Determining the reason behind the muralytic activity of ActA would also yield valuable insight into the multi-faceted role this protein plays during the pathogenesis of L. monocytogenes. Given that this organism can also exist in biofilms and in a dormant state (Pinto et al. 2013), and the context of this project, it would be particularly interesting to observe if the muralytic activity of ActA also correlates with resuscitation from dormancy or if this activity is required for another function. Site directed mutagenesis of the ActA catalytic residue would address the essentiality of this residue for muralytic activity, and in turn, provide a basis to begin dissecting the precise sites of cleavage and their relevance in the pathogenesis of L. monocytogenes.
8 References


Sampson, S.L., (2011) “Mycobacterial PE/PPE proteins at the host-pathogen interface.” *Clinical and Developmental Immunology, 2011*(Figure 1), p.497203.


