ABSTRACT

Title of Document: THE CHARACTERIZATION OF SECRETION AND CYTOLYTIC FUNCTIONALITY OF MYCOBACTERIUM VIRULENCE FACTORS ESAT-6 AND CFP-10

Joanna Maya Manoranjan,
Master of Science, 2006

Directed By: Dr. Lian-Yong Gao,
Cell Biology and Molecular Genetics
University of Maryland

Mycobacterium tuberculosis, the causative agent for the Tuberculosis disease, contains a genetic virulence locus named Region of Difference 1 (RD1). RD1 encodes for a novel secretion system that exports two small, immunogenic proteins named ESAT-6 and CFP-10. Although these proteins are involved in *M.tuberculosis* pathogenesis, their function remains largely unknown. This work employs the use of *M.marinum*, a species genetically closely related to *M.tuberculosis*, to study the homologous RD1 region with a focus on the function of ESAT-6 and CFP-10. This also involves the characterization of individual RD1 genes in the secretion and stability of ESAT-6 and CFP-10. The RD1 locus has been implicated in playing a role in bacterial spreading through host cell lysis and necrotic granuloma formation- a hallmark of pathogenic mycobacterium infections. Consequently, this study establishes the putative role of ESAT-6 and CFP-10 in pathogenesis via an investigation into their cytolytic abilities against host macrophages and the bacteria's subsequent ability to spread during infection.

THE CHARACTERIZATION OF SECRETION AND CYTOLYTIC FUNCTIONALITY OF MYCOBACTERIUM VIRULENCE FACTORS ESAT-6 AND CFP-10

By

Joanna Maya Manoranjan

Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Master of Science
2006

Advisory Committee:
Dr. Lian-Yong Gao, Chair
Dr. Louisa Wu
Dr. David Straney

Dedication

For my Family.
Your constant love and support mean more to me than I can say.
I am so blessed to have you in my life.

Acknowledgements

First I would like to thank Dr. Lian-Yong Gao for his continuous support and enthusiasm for my project. His help and advice played a tremendous role in my work and have provided me with lessons I shall always carry with me.

I would especially like to thank Dr. Seema Madhavan, who was a great help and mentor while working in the lab. Her generous spirit, friendship, and laughter have meant so much to me.

Many thanks to Ruby Kish, who was my constant companion over the years in the lab and at UMD. What would I have done without you?!

I would like to thank everyone in the Gao lab- all those currently in the lab, and everybody who has come and gone. You each played a part in making coming to lab a great experience. I'm particularly grateful to Mark Masciocchi and Kathleen Shannon. Mark contributed greatly to obtaining the microscopy images for protein-host cell association while Kathleen's creation of the GFP tagged strains helped me immensely with all the fluorescent microscopy work.

Lastly I would like to extend a special thank you to Amro Bohsali. I would not have been able to do any work without your creation of the *esat-6* mutant strains! Your help and friendship made my time at UMD unforgettable.

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Chapter 1: Introduction

Mycobacterium tuberculosis is the causative agent for the tuberculosis disease. Tuberculosis is considered one of the three deadliest infectious diseases along with malaria and HIV/AIDS (*www.globalhealthreporting.org*). According to the World Health Organization, more than eight million people develop active TB annually and approximately two million die each year. The current rate of infection is estimated to be one person per second. At present, preventative treatment against tuberculosis remains relatively ineffective and as such, there is a great interest into the mechanisms of pathogenesis by this bacterium in order to better understand how to combat the disease.

Current preventative treatment against tuberculosis involves the use of the live attenuated BCG (Bacillus Calmette-Guerin) vaccine derived from the pathogenic *Mycobacterium bovis*, a strain of mycobacterium closely related to *M. tuberculosis* (Frothingham et al, 1994). The original BCG Pasteur strain was generated after 230 serial passages of *M. bovis* in liquid culture, resulting in the loss of large portions of genetic material. Subtractive genomic hybridization used to compare the genomes of both BCG and *M. bovis* demonstrated that several regions of difference, or RDs, resulted in the attenuated phenotype (Mahairas et al 1995). Three regions were initially discovered using this method and subsequent studies comparing BCG to *M. tuberculosis* showed 16 large deletions (Behr et al, 1999; Gordon et al, 2001; Phillip et al, 1996). The primary reason for loss of virulence was attributed to a 9.5-kb DNA segment termed RD1 (Lewis et al, 2003; Pym et al 2002). Deletion of RD1 from the *M. tuberculosis* strain H37Rv resulted in BCG-like virulence attenuation in both

macrophage cells and mice, specifically the human macrophage-like cell line THP-1, human peripheral blood monocyte-derived macrophages, and C57BL/6 mice. The attenuation presented itself as a reduction in colony forming units (CFUs) in both cell lines as well as organs such as the lungs and spleen, a much improved survival rate for infected mice, and a general reduction in bacterial growth and spread of infection (Lewis et al, 2003; Guinn et al, 2004). Reintroduction of RD1 into BCG restored virulence and produced a protein expression profile almost identical to that of *M. tuberculosis* (Mahairas et al, 1995; Pym et al, 2002, 2003). Upon these discoveries, the function of RD1 genes and their role in pathogenesis has been a source of intense study and interest.

The RD1 locus, covering nine open reading frames, has recently been shown to encode for a novel secretion system. Many Gram positive bacteria secrete bacterial virulence proteins or exotoxins into the extracellular milieu via a classical Sec pathway (Economou, 1999, Burts et al, 2005); however no specialized secretion systems for virulence in *M. tuberculosis* were previously known (Stanley et al, 2003). Proteins secreted in this manner also lacked the N-terminal sequence that would normally target them to the Sec-dependent pathway (Sonnenberg et al, 1997). The secretion system has been termed the Snm pathway (for secretion in mycobacterium) (Stanley et al, 2003; Converse et al, 2005) and the entire locus required for secretion has been referred to as the ESX-1 locus (Brodin et al, 2004). The Snm/ESX-1 locus is widely conserved in other mycobacterium species and in several Gram positive bacteria including, *Corynebacterium diptheriae, Streptomyces coelicolor, Staphylococcus aureus, Bacillus subtilus, Bacillus anthracis, Clostridium*

acetobutylicum, Listeria monocytogenes, and *Salinispora tropica* (Gey van Pittius, 2001; Pallen, 2002; Burts et al, 2005). It has been shown experimentally that apart from the nine genes within RD1, flanking genes in the region are essential for Snm secretion (Tekaia et al, 1999; Gey Van Pittius et al, 2001; Pallen et al 2002). Full secretion required a total of 11 *M. tuberculosis* genes including the nine RD1 genes (Pym et al, 2003). In the case of another pathogenic strain of mycobacterium, *Mycobacterium marinum,* a total of 13 homologous open reading frames were required for complete secretion, and this region, which includes RD1, has been termed extended RD1 (extRD1). A comparison of the extRD1 genes can be seen in Figure 1 (Gao et al, 2004).

Analysis of the highly conserved Snm pathway has lead to the predicted functions of several of the RD1 genes. These include the following: a PE (proline/ glutamic acid), PPE gene couplet, several unknown membrane associated proteins (including Rv3869), an ATP-dependent chaperone of the AAA family (Rv3868), a membrane bound ATPase from the FtsK-SpoIIIE family (existing as either a single protein or as two proteins; Rv 3870, Rv3871), a transmembrane protein with eleven α-helices (Rv3877), and serine proteases (mycosins) with a hydrophobic anchor at the C-terminal end and a cleavable N-terminal signal sequence. It is also predicted that the PPE acts as a gating protein within the secretion apparatus to regulate protein export. Additionally, the ATP-dependent chaperone may indicate that secreted products would require chaperone activity, perhaps to stop premature protein-protein interactions as in Type III secretion (Pym et al 2003; Brodin et al, 2004; Converse et

al, 2005). These genes within the ESX-1 cluster were grouped into four transcriptional units (Pym et al 2003).

The proteins actually secreted via the Snm system were found to be two small proteins referred to as Early Secreted Antigenic Target- 6 kDa (ESAT-6) and Culture Filtrate Protein- 10 kDa (CFP-10), encoded by Rv3874 and Rv3875 respectively (Sorensen et al, 1995; Harboe et al, 1996; Berthet et al, 1998; Skjøt et al, 2000; Brodin et al 2002). The encoding genes are designated as *esxB* and *esxA* and are located centrally within the RD1 locus. ESAT-6 and CFP-10 are found abundantly in the extracellular milieu as secreted proteins, however, ESAT-6 has also been shown to associate with the cell wall (Pym et al, 2002), suggesting a possible role in the actual secretion apparatus of the Snm pathway and that the protein presence in cell supernatants is the result of protein "sloughing" (Pallen et al, 2002). ESAT-6 and CFP-10 are coordinately regulated and have been shown to form a tight 1:1 dimer in vitro (Berthet et al, 1998; Renshaw et al 2002) (Figure 2). Both proteins form helix-turn-helix hairpin structures and bind antiparallel to each other. The site of contact between the proteins is hydrophobic (Renshaw et al, 2005). ESAT-6 and CFP-10 are highly immunogenic. ESAT-6 is a dominant T cell antigen and the protein complex has been shown to induce a strong Th-1 type immune response (Brodin et al, 2005).

The function of both proteins has been alluded to but has yet to be entirely determined. Attenuation due to loss of ESAT-6 and CFP-10 has been established experimentally. *In vivo* studies in guinea pigs, mice and zebrafish (with *M. marinum* infections) show, through gross pathology and histopathology, that there is a drastic reduction in virulence by lack of tissue invasiveness, and spread of infection (Wards

et al 2000; Hsu et al, 2003; Stanley et al 2003; Lewis et al, 2003; Gao et al, 2004). Additionally, it has been shown that a deletion mutant of both ESAT-6 and CFP-10 in *M. marinum* results in a reduction of cytolytic activity against macrophages (Gao et al, 2004).

Pathogenic mycobacteria are efficiently phagocytosed by macrophages and are able to persist within these cells. The cytotoxicity and lysis of macrophages via membrane disruption, allows for the dispersal of the bacterium to adjacent cells and for deep tissue infection (McDonough et al, 1995). Consequently the loss of cytolysis reduces bacterial spreading (Gao et al, 2004). Supporting evidence for ESAT-6/ CFP-10 in a role as cytolysins includes the fact that either the lack of the RD1 locus or the disruption of the *esxB* gene (encoding for CFP-10) reduced the ability of *M. tuberculosis* to lyse lung epithelial cells, THP-1 cells, and also to disrupt artificial lipid bilayers (Dobos et al, 2000; Guinn et al, 2004; Hsu et al, 2004).

On a similar note, the RD1 locus and its secreted proteins have been shown to be involved in the formation of granulomas. Granulomas are highly organized structures composed of differentiated macrophages and other immune cells which are recruited into this tightly aggregated complex. The formation of tissue granuloma, specifically caseous (literally "turning to cheese") granuloma, is a hallmark of virulent mycobacterium infections. A central region of necrotic or lysed cells characterizes caseous granuloma. So, it is due to the loss of RD1 and its secreted proteins that mycobacterium are attenuated via the lack of caseous granuloma formation (Cosma et al, 2004, 2006; Volkman et al, 2004; Pym et al, 2002; Davis et al, 2002).

It is clear that the RD1 locus and its secreted products ESAT-6 and CFP-10 play a complex and central role in pathogenesis. Drawing from previous work demonstrating a dual role of ESAT-6 and CFP-10 as immunogenic targets as well as virulence factors, this project establishes a clearer role for these proteins in cytolysis and suggests a role in the formation of necrotic granuloma and subsequent bacterial spreading. This study shows that the loss of ESAT-6 results in a significant reduction of haemolytic and cytolytic activities. Data also shows that loss of secreted ESAT-6 and CFP-10 results in a dramatic inability of the bacteria to spread during macrophage cell infection. Immunofluorescence studies suggest that both proteins bind to macrophage cell surfaces, further establishing that ESAT-6 and CFP-10 interact with cell membranes in order to function as cytolytic proteins.

Additionally, this study further details the role of individual ext RD1 genes in the secretion and stability of ESAT-6 and CFP-10. Western blot analysis of culture filtrates and cell lysates from *M.marinum* strains with mutations in different extRD1 genes, elucidates the roles they play in the Snm pathway. The use of pathogenic *M. marinum*, which is genetically closely related to *M.tuberculosis*, takes advantage of the bacterium's relatively fast replication time and variety of natural hosts. *M. marinum* has previously been used to study *M. tuberculosis* pathogenesis (Gao et al, 2003; Pozos et al, 2004; Ramakrishna et al, 1997). Consequently this work further establishes the use of *M. marinum* as an efficient model system.

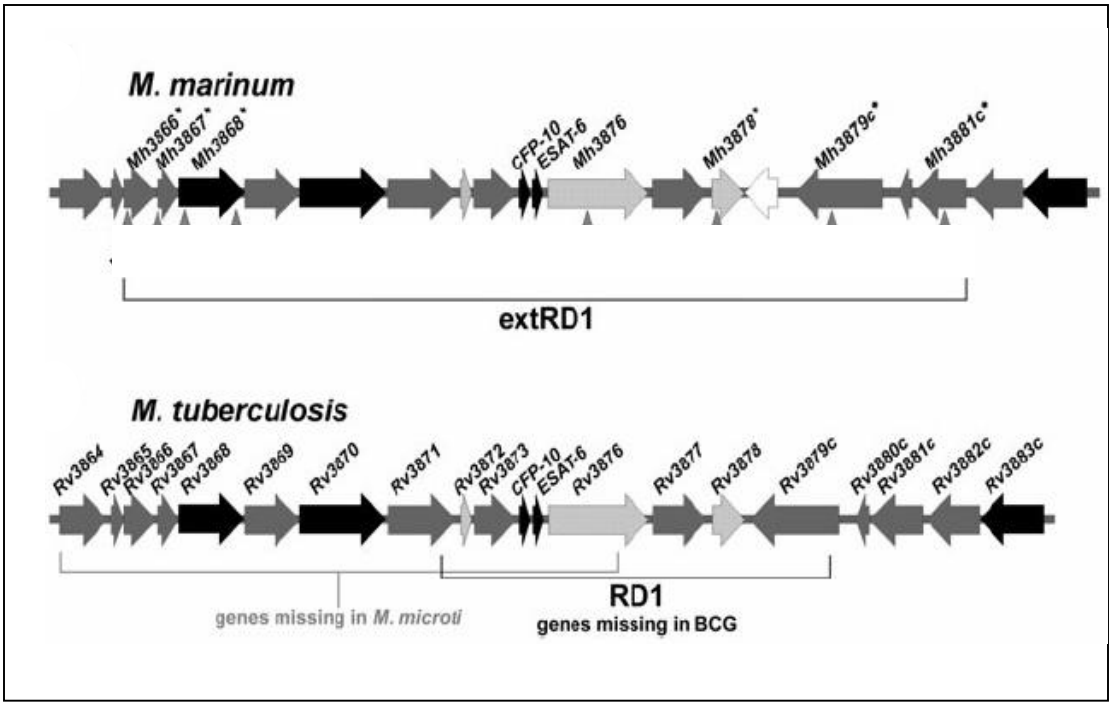

Fig 1. *A schematic representation of the RD1 and extended RD1 genes in Mycobacterium tuberculosis and marinum.* The direction of transcription is denoted and the extent of homology is indicated as follows: black,>90%; dark grey, 70–89%; light grey, 55–69%; and open, < 54%. RD1 genes covering *Rv3871-79c* are absent in the BCG vaccine strain. The *M. marinum* extended RD1 genes cover Mh3866-81c. Asterisk * indicates the location of mutation for the *M.marinum* strains used in this study (Gao et al, 2003).

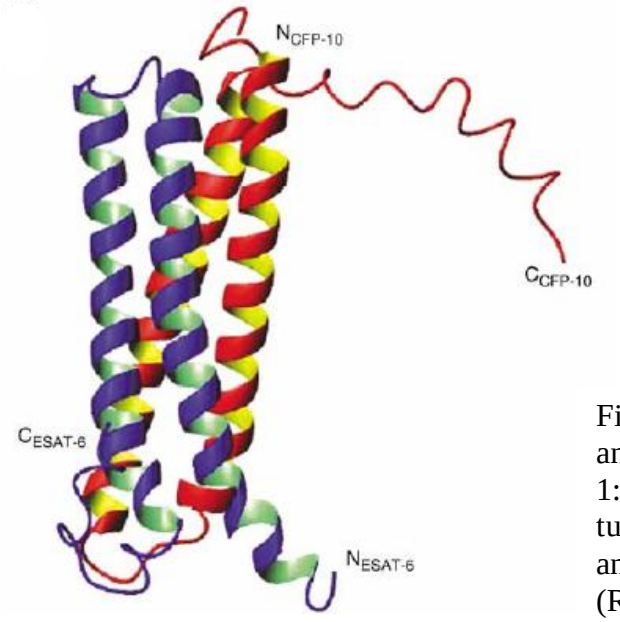

Fig. 2. A ribbon model of ESAT-6 and CFP-10 demonstrates the tight 1:1 dimer formation and the helix-turn-helix hairpin structures that bind antiparallel to each other.
(Renshaw et al, 2002)

Chapter 2

Results Part I: Analysis of Individual extRD1 genes in the secretion and stability of proteins ESAT-6 and CFP-10

In order to determine whether a particular extRD1 gene plays a role in the secretion, stability, or synthesis of ESAT-6 and/ or CFP-10, it was necessary to examine the protein levels produced by the bacterium that have a disruption or mutation of the particular gene of interest. The mutant strains used in this study were previously generated and briefly characterized for their ability to secrete ESAT-6 and CFP-10 at 5-6 days of culture growth (Gao et al, 2004). Additional mutants were recently created in the Gao lab including the first *M.marinum esat*-6 mutant. This study provides in depth analysis of all these mutants in terms of secretion, stability, and synthesis. Furthermore, observing the strains at 2 and 6 days of growth generated protein secretion profiles over time.

To analyze secretion, it was first necessary to evaluate ESAT-6 and CFP-10 in crude cell lysates of wild type and the various extRD1 strains by western blot analysis (Figure 3). The controls included in the western blot of cell lysates include a Δ*secA2* mutant that demonstrates the SecA2 secretion pathway is not involved in the stability and/ or synthesis of ESAT-6 or CFP-10. Also included is GroEL which is a protein that served as a loading control, demonstrating that equivalent amounts of cell lysate protein were loaded for each sample. As seen in Figure 3, no ESAT-6 or CFP-10 protein was detected in the lysates of either the *Δesat-6* mutant or the *Δcfp-10/esat-6* double knockout strain. It is of note that the mutation of the *esat*-6 gene resulted in

undetectable CFP-10, suggesting that although *esat-6* is downstream of *cfp-10*, ESAT-6 may be involved in CFP-10 biosynthesis or stability.

In the case of the extRD1 mutant strains, ESAT-6 and CFP-10 was detected in all the lysates. The protein production levels for most of the mutant strains were in general reduced, but did not differ significantly to the wild type amounts. However, several strains appeared to have more significantly reduced quantities of protein. In terms of ESAT-6, several mutants including *Mh3867::kan*, *Mh3868a::kan*, and *Mh3879::kan* had less detectable protein. In terms of CFP-10, all of the mutants had lower levels of protein, but the strains *Mh3867::kan*, *Mh3878::kan*, and *Mh3879::kan* appeared to have more pronounced reduction of protein. Quantitative analysis of these results can be seen in Figure 4. This analysis consistently indicates that although several of the mutants do exhibit reduced levels of protein, only *Mh3867::kan* shows a conspicuously lower amount of CFP-10. Therefore it appears that *Mh3867* may be a gene that is in fact involved in CFP-10 biosynthesis or intracellular stability. These results also indicate that in general, mutations within the extRD1 region produce slightly reduced amounts of ESAT-6 and CFP-10 when compared to the wild type strain. However, there are only certain genes that play a more significant role in the synthesis or stability of the proteins.

After examining the intracellular levels of ESAT-6 and CFP-10 from bacterial cell lysate, the culture filtrate was analyzed by western blot in order to establish whether the various extRD1 genes were involved the proteins' secretion. Although the cell lysate analysis demonstrated that the extRD1 mutants in general had reduced ESAT-6 and CFP-10, it is evident that the disruption of several genes resulted in a

dramatic decline in secreted protein, as seen in Figure 5. In terms of the mutant strain that appeared defective in either biosynthesis/ stability, *Mh3867::kan* shows almost no detectable protein which can be explained by the highly reduced intracellular protein levels of CFP-10. Strains that appear to be defective in secretion include *Mh3866::kan, Mh3868a::kan, Mh3868b::kan, Mh3868c::kan, Mh3876::kan,* and *Mh3881::kan.* It is of note that *Mh3868b::kan* and *Mh3876::kan* are more defective in either ESAT-6 or CFP-10 but not both. *Mh3868b*::*kan* shows a dramatic reduction in CFP-10, not ESAT-6, while *Mh3876::kan* has extremely lowered amounts of ESAT-6 and relatively high levels of CFP-10. These results imply that several of the extRD1 genes play a role in secretion of ESAT-6 and CFP-10 while others are involved in intracellular synthesis or stability. Certain genes, including *Mh3868b* and *Mh3876* are more involved in either one or the other protein secretion, and mutation of the genes results in discordantly regulated secretion of ESAT-6 and CFP-10. The ΔSecA2 was included again as a control to show that mutation of the SecA2 secretion pathway does not disrupt/affect ESAT-6 or CFP-10 secretion and that these proteins are secreted independently of this pathway. Additionally, Ag85 was used as a control to show equivalent protein loading and also to show that since this protein is secreted in a SecA1-dependent manner, general SecA1 secretion is not disrupted and ESAT-6/CFP-10 are exported independently of the SecA pathway. Analysis and graphical representation of these results can be seen in Figure 6.

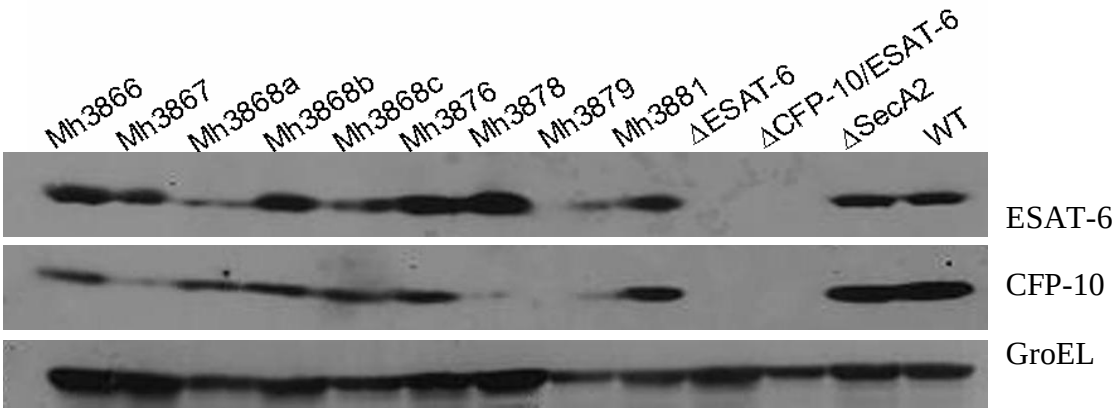

Fig. 3. *extRD1 mutants levels of intracellular ESAT-6 and CFP-10.* Western blot analysis of cell lysates from extRD1 mutants shows intracellular ESAT-6 and CFP-10 protein levels. Strains are labeled by the gene in which they are mutated. Cell lysate samples were harvested after 6 days of bacterial growth. With the exception of a few strains, protein levels are, in general, reduced for the mutant strains in comparison to the wild type. A SecA2 mutant serves as a control to show that the SecA2 secretion pathway is not involved in stability/synthesis of ESAT-6 or CFP-10. GroEL serves as an internal control to demonstrate equal protein loading. All three membranes were run on the same gel and the CFP-10 blot was obtained after stripping the membrane clean of ESAT-6 bound antibodies.

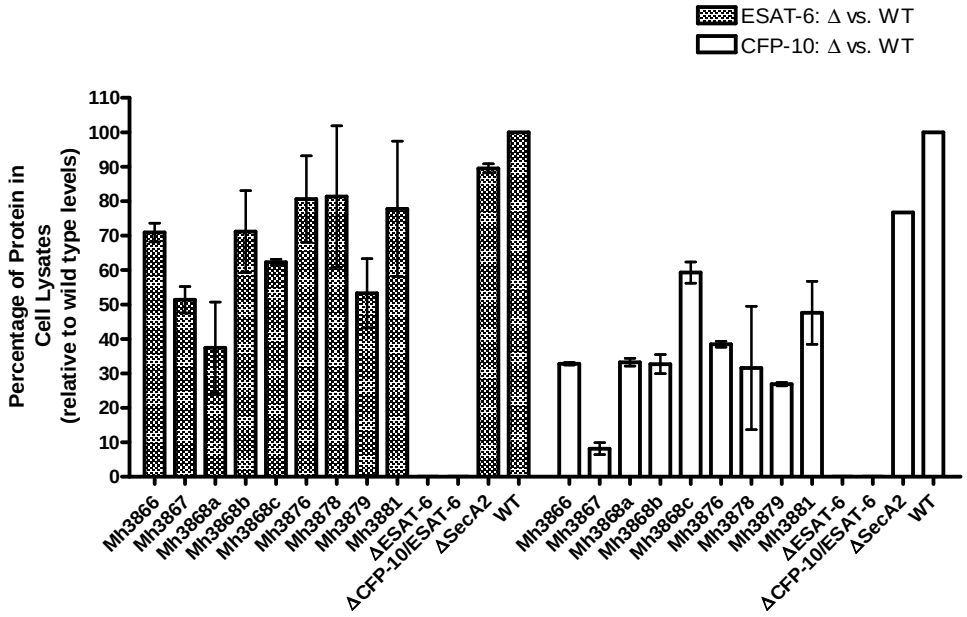

Fig. 4. *Analysis of ESAT-6 and CFP-10 protein levels in cell lysates from various extRD1 mutants relative to wild type.* Strains are labeled by the gene in which they are mutated. ImageJ software was used to analyze the bands/ protein amounts from westerns blots of cell lysates from various extRD1. Protein levels were adjusted according to the amounts of GroEL for each sample and were then calculated as a percentage in relation to the wild type. Although there are reduced levels of protein for all the ΔextRD1 strains, the mutant Mh3867 strain shows significant loss of CFP-10. The error bars indicate ± standard error derived from western blots analyzed in duplicate

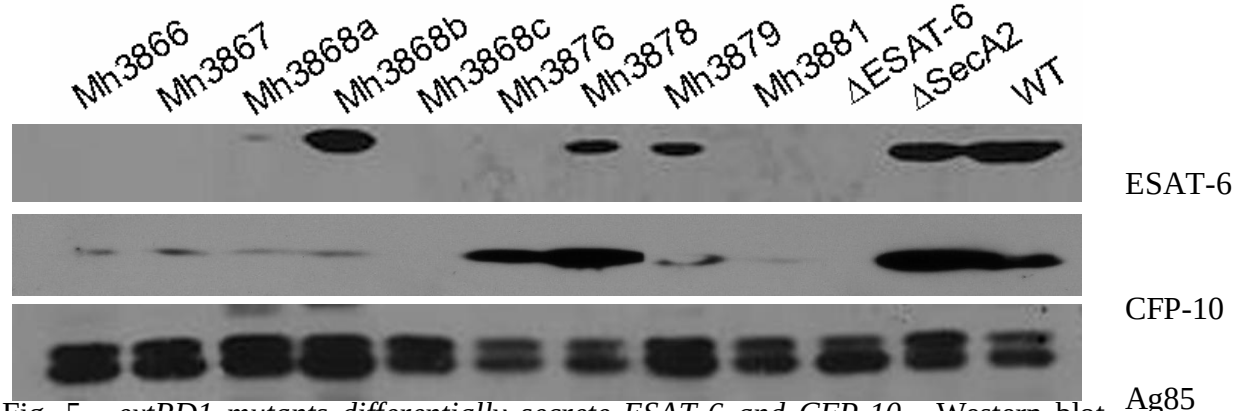

Fig. 5. *extRD1 mutants differentially secrete ESAT-6 and CFP-10.* Western blot analysis of culture filtrates from extRD1 mutants shows individual genes play varying degrees of importance in ESAT-6 and CFP-10 export. Strains are labeled by the gene in which they are mutated. The culture filtrate samples were harvested after 6 days of bacterial growth. SecA1-dependent Ag85 shows even secretion, while the SecA2 mutant demonstrates wild type level export of both ESAT-6 and CFP-10. These controls confirm that general SecA1-dependent secretion is not disrupted, and that ESAT-6 and CFP-10 are both secreted in a Sec-independent manner.

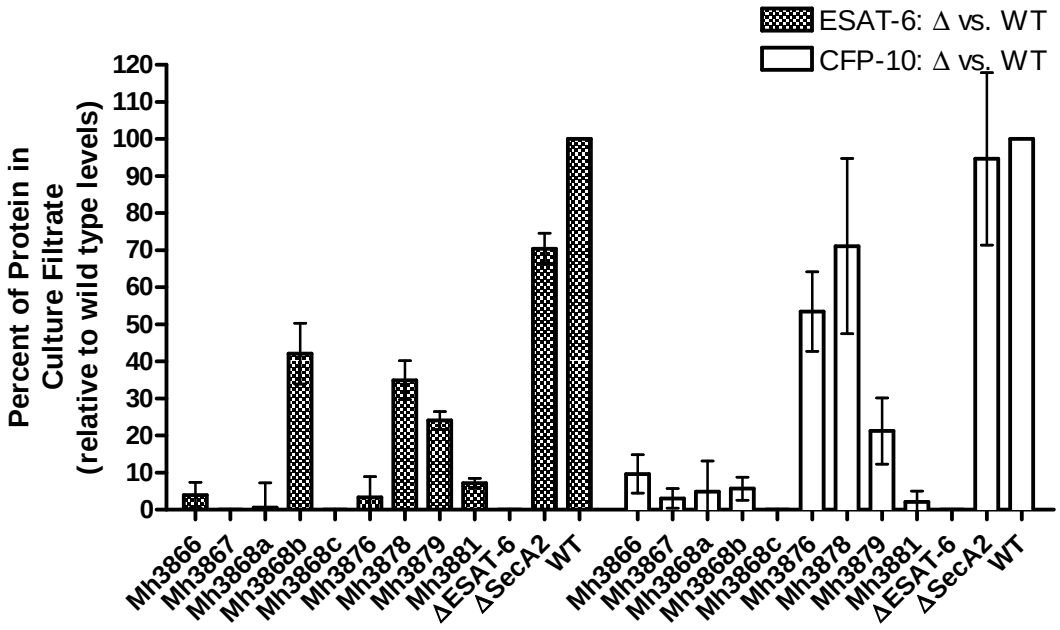

Fig. 6. *Analysis of ESAT-6 and CFP-10 protein levels in culture filtrates from various extRD1 mutants relative to wild type.* Strains are labeled by the gene in which they are mutated. ImageJ software was used to analyze the bands/ protein amounts from westerns blots of culture filtrates from various extRD1. Protein levels were adjusted according to the levels of Ag85 and were then calculated as a percentage in relation to the wild type. The error bars indicate ± standard error derived from western blots analyzed in duplicate.

In order to determine whether the reduced levels of ESAT-6 and CFP-10 in the culture filtrate were due to an actual deficiency in secretion or due to a lack of protein stability, culture filtrates harvested at two days were compared to the previously described six day harvests. The western blot analysis can be seen in Figure 7 and quantification of results can be seen in Figure 8 and 9. Sample loading was based on normalization to the weight of the bacterial pellet. Therefore the sample loaded is relative to the same weight or approximate number of bacteria. Results from Figure 7 show the mutant strains *Mh3868a::kan* and *Mh3868c::kan* appear to be clearly defective in secretion and not stability. This can be inferred by the fact that regardless of whether samples were collected at 2 days or 6 days, there was no detectable protein. It is of note that *Mh3868b::kan* is able to secrete higher levels of ESAT-6 and CFP-10 by 2 days but has lowered amounts at 6 days. It implies a change in secretion over time that is perhaps a result of the lack of stability of the proteins. The three mutant strains listed as *Mh3868a, Mh3868b,* and *Mh3868c* all have mutations in the same gene but at varying locations on the gene as given in table 1. It appears that depending on where the mutation is, it affects the ESAT-6 and CFP-10 levels differently. It seems that in the case of *Mh3868b* the mutation at the stop codon affects the intracellular levels of protein, whereas partial loss of other regions results in a loss only of secretion. This implies the gene is involved in both intracellular stability and secretion of both ESAT-6 and CFP-10.

The strain *Mh3867::kan* was previously shown to be defective in intracellular levels of CFP-10 and accordingly has very little detectable protein in both 2 and 6 days culture filtrates. Since there is a defect in intracellular levels of CFP-10 that

may result from either a defect in biosynthesis or intracellular stability, it is difficult to determine whether this gene is involved in secretion or solely involved in synthesis or stability of CFP-10 within the bacterial cell. In terms of ESAT-6 it does seem to be defective in secretion since the intracellular levels were not dramatically reduced. This loss of ESAT-6 could be linked to the reduction in CFP-10 synthesis as well.

Based on the comparison of 2 and 6 day culture filtrates shown in Figure 9, it does appear that the mutation of the following genes results in ESAT-6 protein instability: *Mh3866*, *Mh3876*, *Mh3878*, *Mh3879*, and *Mh3881*. These mutants are also defective in CFP-10 secretion, with the exception of *Mh3866* and *Mh3878*. The reason they appear to be defective in stability is that at 2 days there is relatively high amounts of protein that is no longer detectable by 6 days. This implies a lack of protein stability or a change in regulation over time. Taken together, these results clarify the role played by the individual extRD1 genes in biosynthesis, stability, and secretion of ESAT-6 and CFP-10. A summary of these findings can be seen in Table 1.

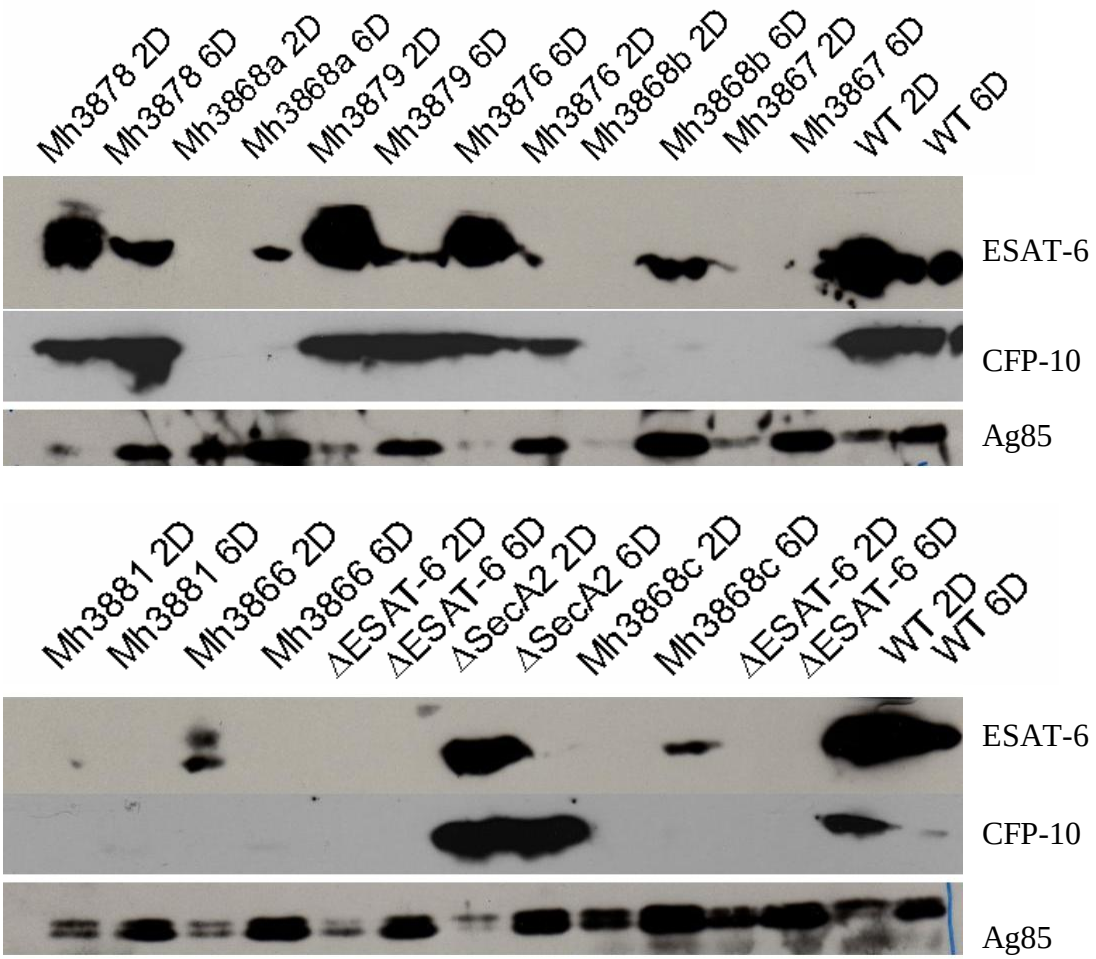

Fig. 7. *Analysis of ESAT-6 and CFP-10 protein levels in 2 and 6 day harvested culture filtrates from wild type and various extRD1 mutants.* Western blot analysis of 2 and 6 day harvested culture filtrates from extRD1 mutants shows that individual genes play varying roles in ESAT-6 and CFP-10 stability and/or secretion. Strains are labeled by the gene in which they are mutated. 2D and 6D indicate 2 or 6 day harvested samples. Two *esat-6* mutant clone strains (both having a mutation in the *esat-6* gene) are included. With comparison of 2 day and 6 day levels, it can be determined if the reduction in protein levels is due to an actual defect in secretion or if the 2 day proteins are possibly unstable and are degraded over time. Sample loading was adjusted by normalizing to bacterial pellet weight to ensure that protein from relatively equal number of bacteria was loaded. The SecA1-dependent Ag85 shows relative secretion independent of ESAT-6/CFP-10 while the SecA2 mutant demonstrates high levels of export of both ESAT-6 and CFP-10 at 2 days. These controls confirm that general SecA-dependent secretion is not disrupted.

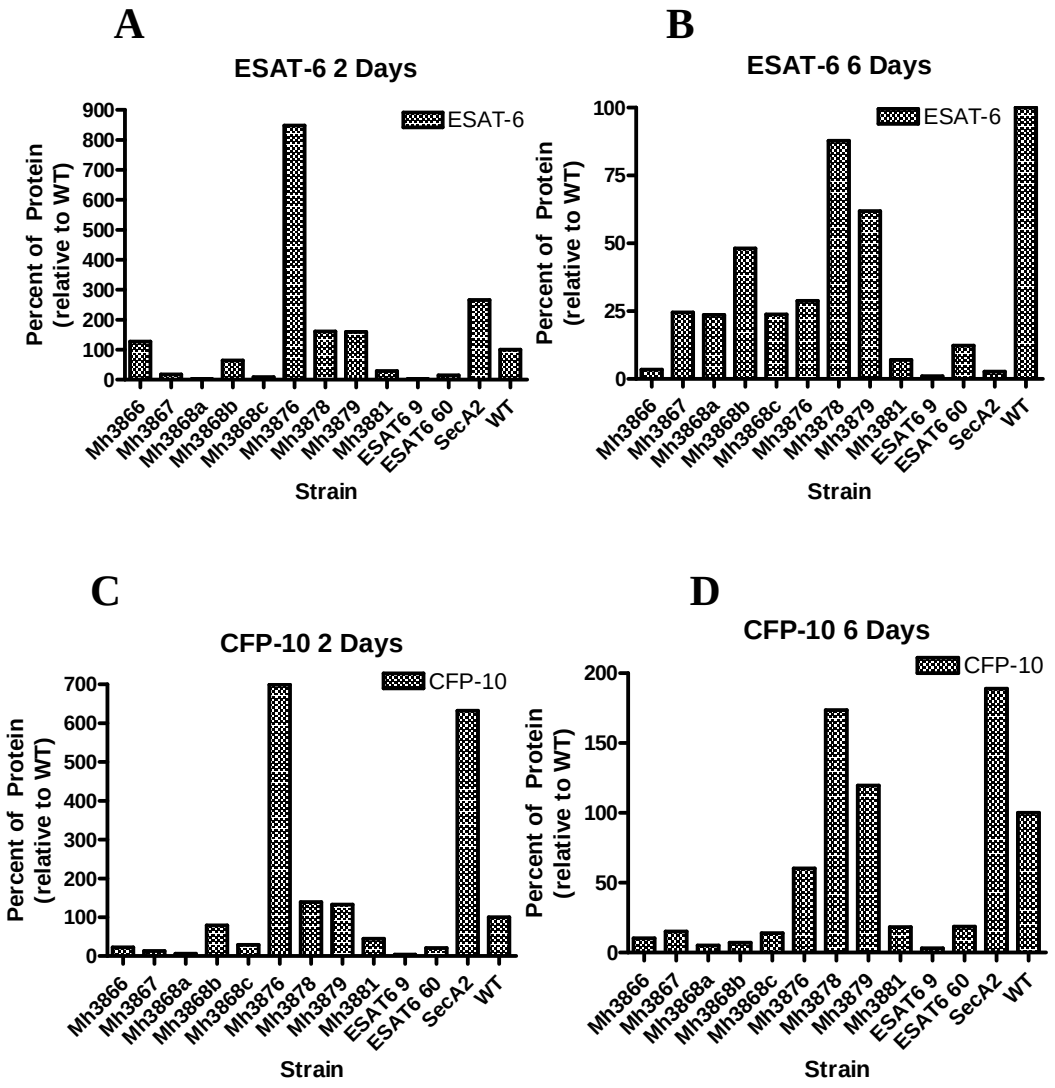

Fig. 8. *Quantification of ESAT-6 and CFP-10 protein levels in 2 and 6 day culture filtrates from wild type and extRD1 mutant strains.* Strains are labeled by the gene in which they are mutated. Panels A and B demonstrate culture filtrate protein levels of ESAT-6 at 2 and 6 days of bacterial growth respectively. Panels C and D demonstrate culture filtrate protein levels of CFP-10 at 2 and 6 days of bacterial growth respectively. All protein percentages are shown relative to wild type amounts for either 2 or 6 days and are normalized to Ag85. Analysis is derived from western blots shown in Figure 7 and data are summarized in table 1 taking into account the western analysis seen in Figure 4 & 6.

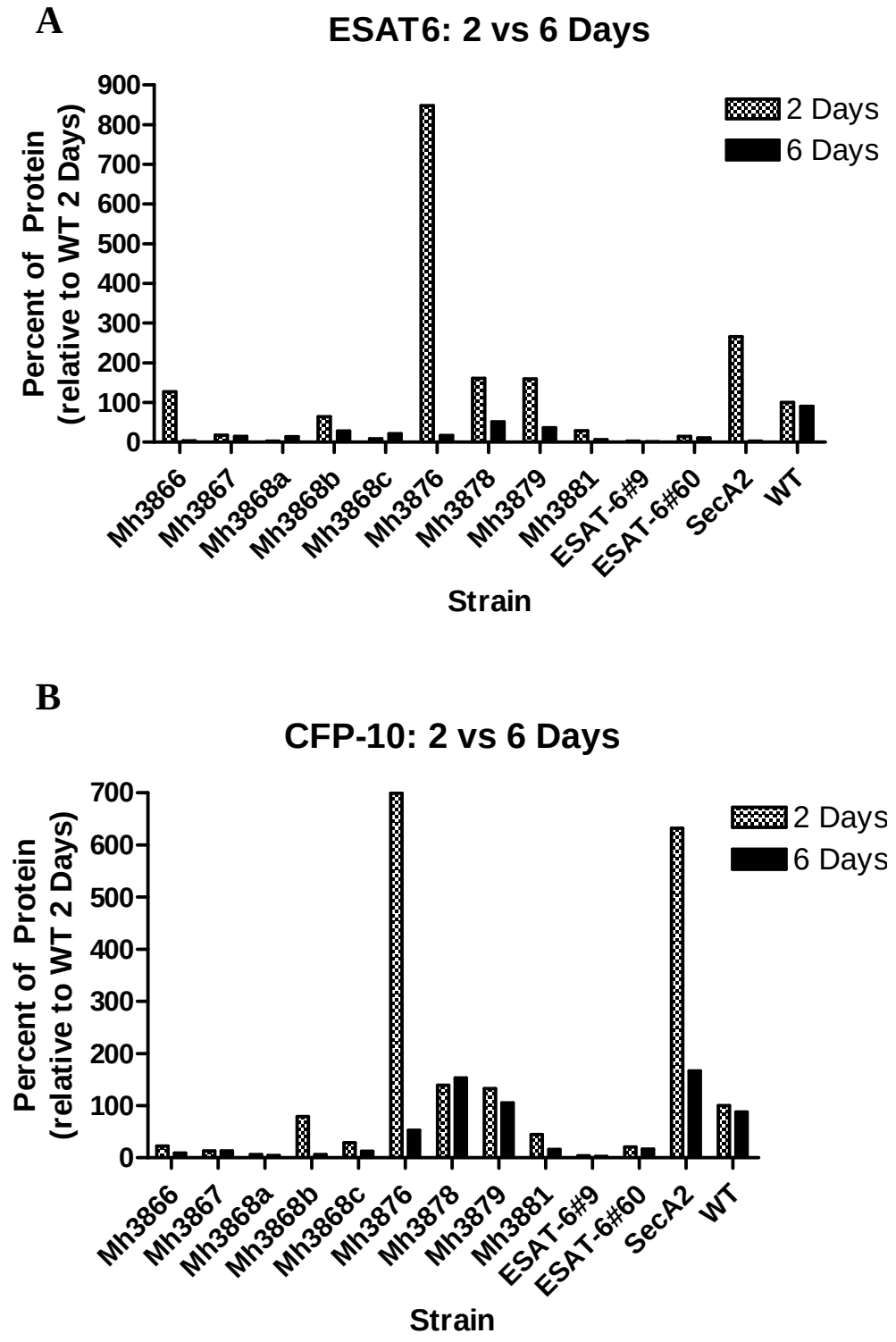

Fig. 9. *Comparative Analysis of 2 and 6 day culture filtrates of ESAT-6 (A) and CFP-10 (B) levels in wild type and extRD1 mutant strains.* Strains are labeled by the gene in which they are mutated. All protein percentages are shown relative to wild type amounts for 2 days and are normalized to Ag85. Analysis is derived from data shown in Figure 8 and is summarized in table 1 taking into account the western analysis seen in Figure 4 & 6.

Gene/ Location of mutation	Function	Involved in ESAT-6 synthesis or intracell-ular stability?	Involved in CFP-10 synthesis or intracell-ular stability?	Involved in ESAT-6 stability?	Involved in CFP-10 stability?	Involved in ESAT-6 secretion?	Involved in CFP-10 secretion?
Mh3866*	Hypothetical protein	No	No	**Yes**	No	--/No	**Yes**
Mh3867*	Hypothetical protein	No	**Yes**	No	--/No	**Yes**	**--/No**
Mh3868a* (Δ at middle of gene; partial loss of gene)	ATP-dependent chaperone of AAA family with ATP binding site	No	No	No	No	**Yes**	**Yes**
Mh3868b* (Δ at stop codon; partial loss of gene)	ATP-dependent chaperone of AAA family with ATP binding site	No	No	**Yes**	**Yes**	**--/No**	**--/No**
Mh3868c* (Δ at N-terminus; loss of entire gene)	ATP-dependent chaperone of AAA family with ATP binding site	No	No	No	No	**Yes**	**Yes**
Mh3876	ATPase	No	No	**Yes**	**Yes**	--/No	--./No
Mh3878	Hypothetical alanine rich protein	No	No	**Yes**	No	--/No	No
Mh3879	Hypothetical protein	No	No/ partial	**Yes**	**Yes**	--/No	No
Mh3881*	Hypothetical protein	No	No	**Yes**	**Yes**	--/No	--/No

Table 1. *The involvement of individual extRD1 genes in the biosynthesis/ stability and, or secretion of ESAT-6 and CFP-10.* *Asterisk indicates extRD1 genes outside of RD1. Genes can be categorized as involved in biosynthesis, stability and/or secretion of both ESAT-6 and CFP-10. Mutations of genes that resulted in significantly reduced levels of ESAT-6/CFP-10 and were categorized as playing a role in biosynthesis. Mutation of genes that resulted in reduced protein levels in six day culture filtrates but had wild type level secretion at 2 days were categorized as involved in extracellular protein stability. Mutations of other genes resulted only in significantly reduced levels at both 2 and 6 days and were therefore categorized as being involved in secretion. As certain genes appeared to play a role in synthesis/stability it can not be determined if they are involved in secretion and are therefore indicated as -- in the table. Data is a summary of the western blot analysis seen in Figures 4, 6, and 8.

Chapter 3

Results Part II: *Mycobacterium marinum* extRD1 secreted virulence factors, ESAT-6 and CFP-10, are required for cytolysis

The esat-6 gene plays an essential role in haemolysis

In order to study the cytolytic activity of Δ*esat-6* in comparison to the wild type, a contact dependent haemolysis assay was used. Cytolysis is a characteristic of pathogenic mycobacterium (Rudnicka et al, 1999), and haemolysis has been shown to be relevant to cytolysis of nucleated cells (Gao et al, 2004; Maslow et al, 1999). This assay allowed for measurement of red blood cell lysis by optical density (OD) to assess the release of hemoglobin. It is evident that the incubation of Δ*esat-6* resulted in a dramatic reduction in haemolysis compared to the wild type (Figure 10). The mutant produced a negligible level of lysis comparable to the levels induced by the PBS control. Wild type bacteria produced high levels of haemolysis, with an OD value of approximately 7 times greater than the mutant. These results clearly demonstrate that the *esat-6* gene is essential for haemolytic activity.

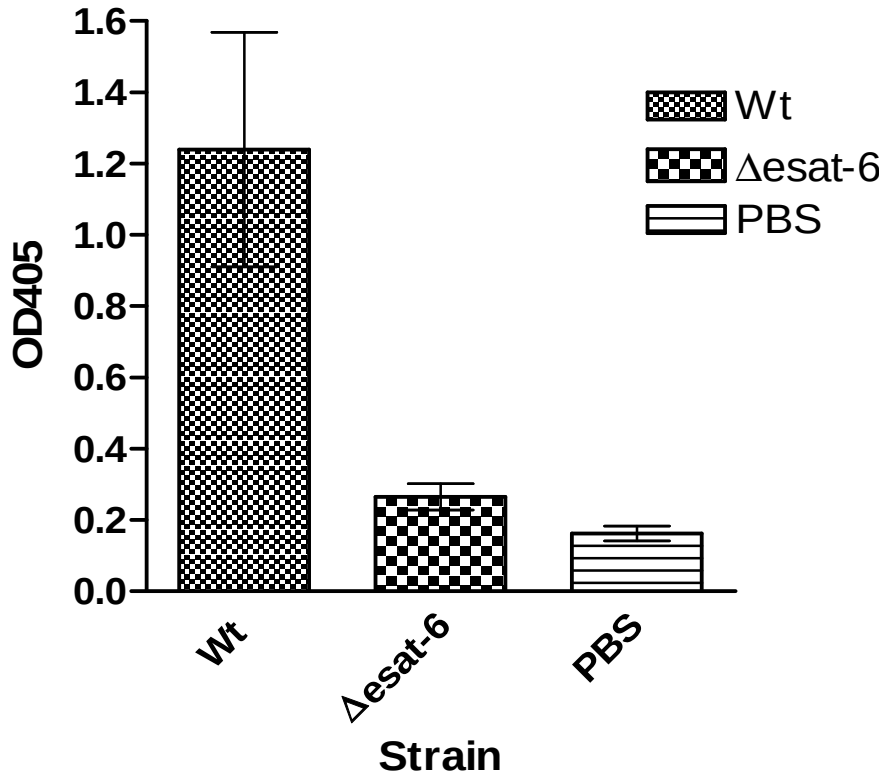

Fig. 10. The *esat-6 gene is involved in haemolytic activity.* Red blood cells (RBCs) were incubated for two hours with either wild type or Δ*esat-6* strains of bacteria at an MOI of 20. The assay screened for contact-dependent haemolysis since bacteria and RBCs were centrifuged together at a speed of 8,000 RPM and incubated at 32 degrees for 2 hours. Haemolysis was measured by taking the optical density of the bacteria/RBC supernatant at a wavelength of 405 nm. The resulting OD reading reflected the release of hemoglobin during haemolysis. Error bars indicate ± standard error derived from three experiments.

The esat-6 gene is involved in macrophage necrosis

In order to observe the role of ESAT-6 in the necrosis or lysis of macrophages, J774 cells were incubated with wild type or Δ*esat-6* green fluorescent protein (GFP) tagged bacterial strains at a multiplicity of infection (MOI) of 50 bacteria to one cell. After two hours, the cells were stained with Calcein acetomethoxy (calcein AM) and Ethidium homodimer-1 (Eth-1) and observed by fluorescent microscopy. Calcein AM is transported into the cells through the cell membrane and will be retained only in viable or live cells to make them fluoresce green. The Eth-1 will only penetrate necrotic cells with compromised or disintegrating cell membranes and stain DNA or RNA, making cells fluoresce red. As seen in Figure 11A, wild type bacteria induced much higher levels of necrosis in macrophages when compared to the Δ*esat-6* (shown in Figure 11B). Quantification of these results can be seen in Figure 11C and shows that the wild type causes approximately seventy percent necrosis, while the mutant induces roughly ten percent. The data strongly indicate that the *esat-6* gene plays a critical role the necrotic activity of *Mycobacterium*.

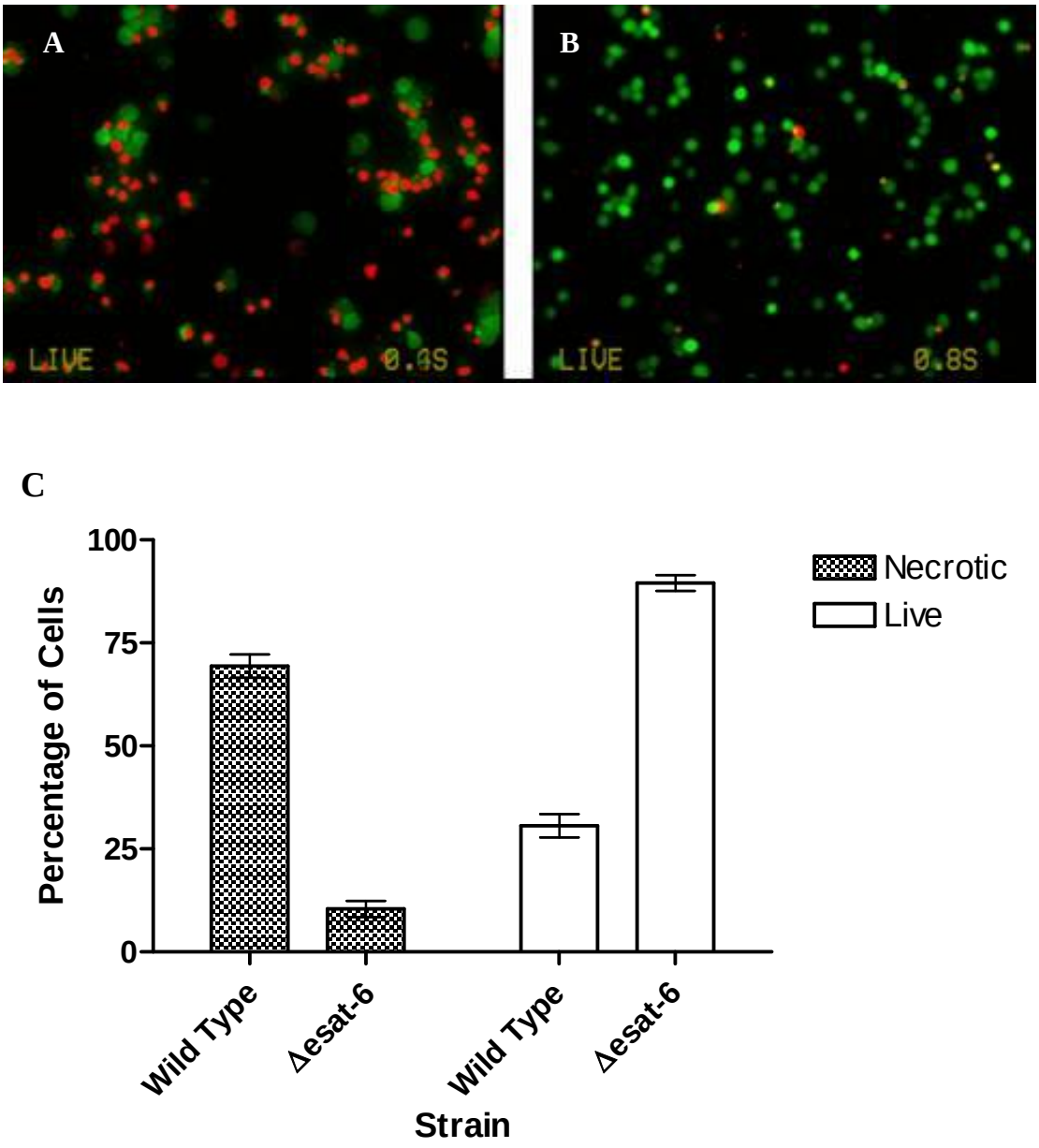

Fig. 11. *Loss of ESAT-6 results in diminished rapid necrosis of J774 macrophages.* J774 cells were infected by wild type (A) and Δ*esat*-6 (B) strains at a multiplicity of infection of 50 bacteria to 1 cell. Images are representative of cells observed by fluorescent microscopy after two hours and stained with Calcein AM and Ethidium homodimer-1 (Eth-1). Necrotic or lysed cells appear red due to the Eth-1 and live cells appear green via the fluorescent Calcein staining. (Images courtesy of Amro Bohsali and Seema Madhavan). C. *Analysis of rapid necrosis assay.* The total necrotic and live cells were counted within three different microscopic fields of vision and the percentage of each was calculated. Error bars are ± standard error obtained by observing the three different fields of vision for the rapid necrosis assay.

The esat-6 gene is involved in mediating bacterial spreading during macrophage infection

Loss of cytolysis would greatly diminish the bacteria's ability to spread to adjacent cells during infection. To observe if the loss of ESAT-6 would affect the bacteria's capacity to spread, macrophages were infected with wild type and Δ*esat*-6 strains expressing GFP. The infection was done at a MOI of 0.4 and cells were observed by fluorescent microscopy every 24 hours over the course of three days. In order to determine the degree of bacterial spreading by both strains, the percent of cells infected over time was calculated. This assay resulted in the observation that the percentage of cells infected by the wild type appeared to increase over time while the number of Δ*esat*-6 infected cells remained static (Figure 12). The time zero count of infected cells (taken after 2 hours of bacterial incubation with the cells) shows there was no initial difference between the wild type and mutant strains in their ability to infect cells. In other words there is no defect in invasion by the ΔESAT-6 strain compared to the wild type. Similarly there was no drop in the number of infected cells, indicating that there was no obvious lack of intracellular survival as a result of the disruption of the *esat*-6 gene. Microscopic observation also showed that *Δesat-6* was in fact able to grow within infected cells over time (data not shown). The data shown here imply that the *esat-6* gene is involved in the ability of *M.marinum* to spread during macrophage infection.

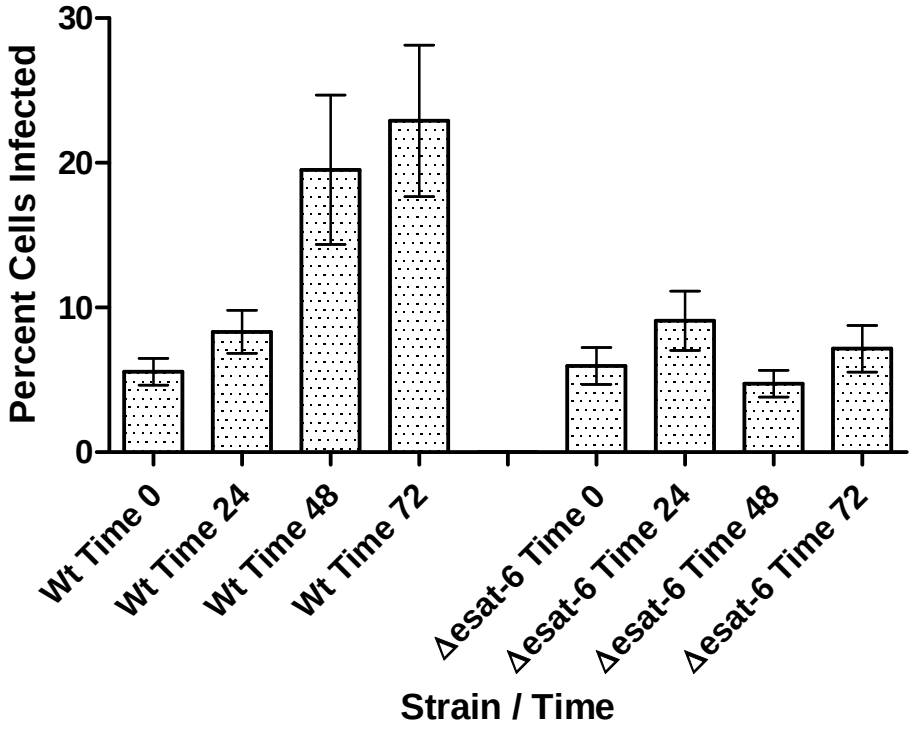

Fig. 12. *Loss of ESAT-6 results in the inability of M. marinum to spread during macrophage infection.* Bone marrow derived macrophages were infected by GFP-tagged wild type and Δ*esat*-6 strains at an MOI of 0.4. The percentage of cells infected was observed by fluorescent microscopy over the course of 72 hours. The graph is representative of duplicate experiments and error bars indicate ± standard error derived from using ten replicate microscopic fields of vision to count cells at each time point (each microscopic field showed approximately 20-23 total cells).

The secreted ESAT-6 and CFP-10 proteins may associate with the macrophage cell surfaces

To further characterize the involvement in cytolysis, it was important to observe whether ESAT-6 and/or CFP-10 exhibited a level of association with host cells. J774 macrophages were incubated with wild type or Δ*esat*-6 bacteria expressing GFP at an MOI of 50. Cells and bacteria were incubated together for 30, 60, or 90 minutes, fixed, and then probed with antibody to either ESAT-6 or CFP-10. The use of fluorescently labeled secondary antibodies allowed for immunofluorescence analysis via fluorescent microscopy.

The results showed that in the case of the wild type infected cells, ESAT-6 or CFP-10 was localized at certain regions where bacteria and cells were in contact. Figure 11A, D, and G, shows the locations of the GFP-tagged bacteria, while panels B, E, and H show the areas of protein/antibody concentration in red. The overlay images seen in Figure 13, panels C, F, and I, show the colocalization of the antibody with the bacteria on the macrophage cells. The antibody appears to localize in areas on or around the site of contact between bacteria and cells. It is of note that the bacteria within the field of vision that are not in contact with the cells do not show any protein. This also confirms that antibody is not penetrating the cells or the bacteria but is in fact specific to protein found extracellularly. In comparison, cells infected with the Δ*esat*-6 strain yielded no localized red fluorescence to indicate antibody binding (Figure 13K, N) and consequently there was no colocalization of bacteria and protein (Figure 13L, O). Similar results were observed for CFP-10 (Figure 14).

For both wild type and mutant infections there appeared to be low levels of background red staining on all the cells. In the case of the wild type infected cells, although there is a certain degree of background red fluorescence visible on the cells, the highly localized regions of antibody binding remain distinct. Therefore in order to further confirm that the binding of the primary antibody was in fact specific, a control of wild type infection without the subsequent addition of primary antibody was done (Figure 15A-F). This resulted in no visible regions of localized red fluorescence but did also faintly stain the background cells, which may be explained by a low degree of secondary antibody non-specific binding. The control resulted in images similar to those cells infected with Δ*esat*-6.

To quantify the degree of colocalization between bacteria and either ESAT-6 or CFP-10 protein, ImageJ software was used to analyze the above-mentioned fluorescent images. Figure 16A,B shows the levels of colocalization between GFP bacteria and ESAT-6 or CFP-10 respectively. In the case of both proteins, there is a much higher degree of overlay for the wild type than the mutant infected cells. The mutant infected cells exhibit a level of colocalization that is comparable to the control. Since there was a certain amount of background staining due to some degree of non-specificity by the secondary antibody, the image analysis of overlay between the red and green pixels resulted in the appearance of colocalization for the control. The comparison therefore, of the mutant and control, indicates that the measure of colocalization was primarily due to the background secondary antibody fluorescence.

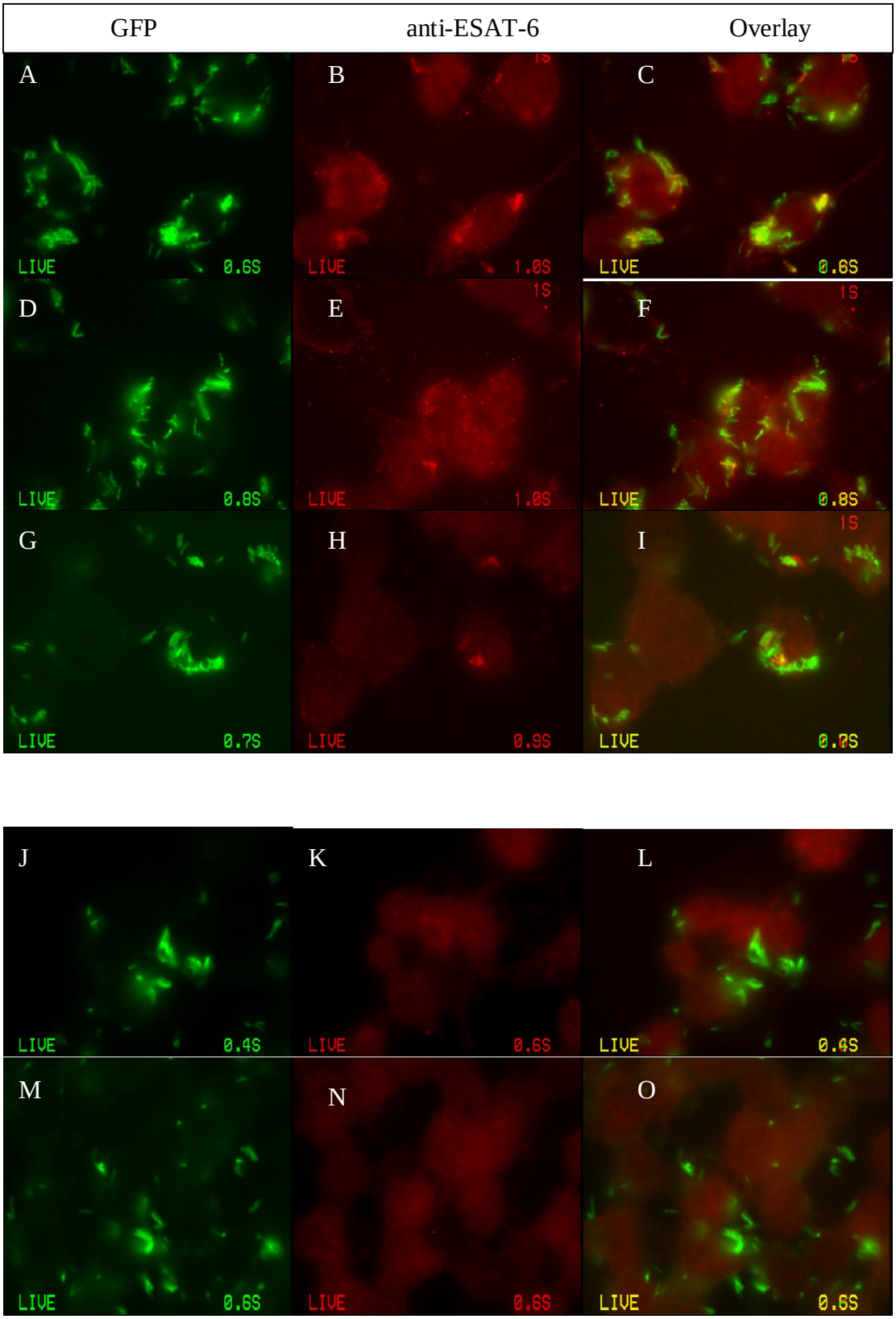
GFP
anti-ESAT-6
Overlay
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
LIVE

Fig. 13. *Immunofluorescence shows wild type secreted ESAT-6 is able to associate with the surface of J774 macrophage cells.* J774 cells were infected by wild type-GFP (A-I) or Δ*esat*-6-GFP (J-O) strains for 90 minutes, fixed, probed for ESAT-6, and observed by fluorescent microscopy. Green (FITC) shows the GFP tagged bacteria, red (TRITC) indicates the binding of the primary antibody anti-ESAT-6, and the overlay shows both images together with colocalization (yellow) of bacteria and antibody.

GFP	anti-CFP-10	Overlay

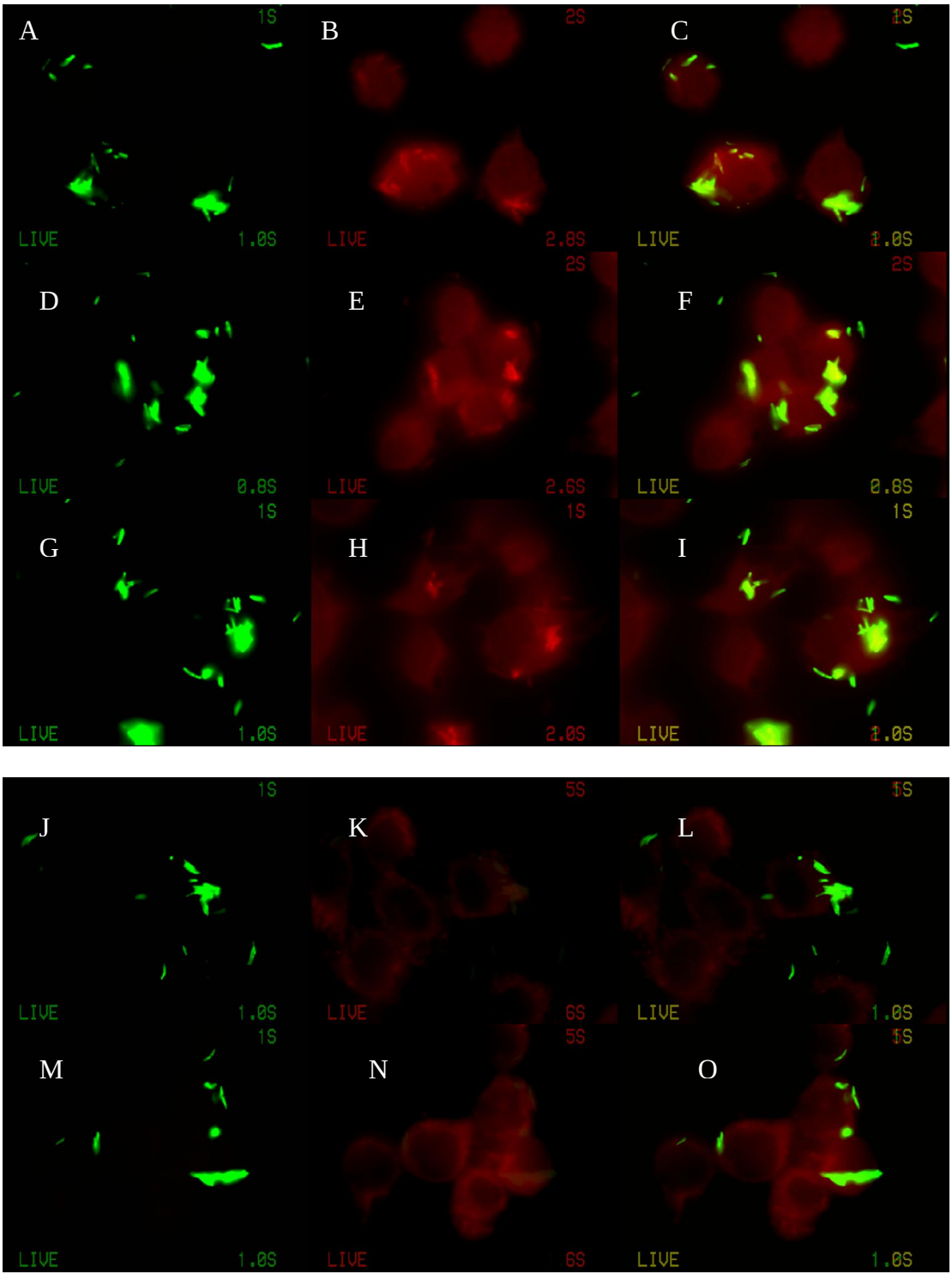

Fig. 14. *Immunofluorescence shows wild type CFP-10 is able to associate with the surface of J774 macrophage cells.* J774 cells were infected by wild type-GFP (A-I) or Δ*esat*-6-GFP (J-O) strains for 60 minutes, fixed, probed for CFP-10, and observed by fluorescent microscopy. Green (FITC) shows the GFP tagged bacteria, red (TRITC) indicates the binding of the primary antibody anti-CFP-10, and the overlay shows both images together with colocalization (yellow) of bacteria and antibody.

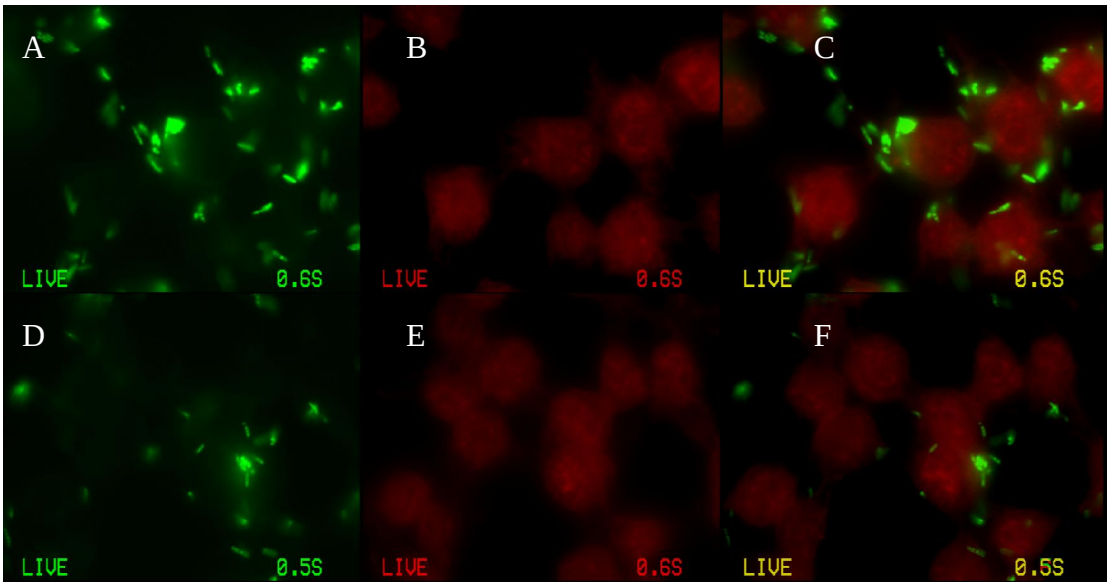

Fig. 15. *Immunofluorescence control, lacking primary antibody, shows no localized regions of red fluorescence on the surface of J774 macrophage cells.* J774 cells were infected at an MOI of 50 by wild type-GFP bacteria for 60 minutes, fixed, probed with only secondary antibody, and observed by fluorescent microscopy. Bacteria are visible in green (FITC). The background level of red fluorescence on all cells indicates the degree of non-specific binding of the secondary antibody. The concurrent lack of primary antibody, localized regions of red fluorescence, and colocalization of red fluorescence with bacteria demonstrates the specificity of the primary antibodies.

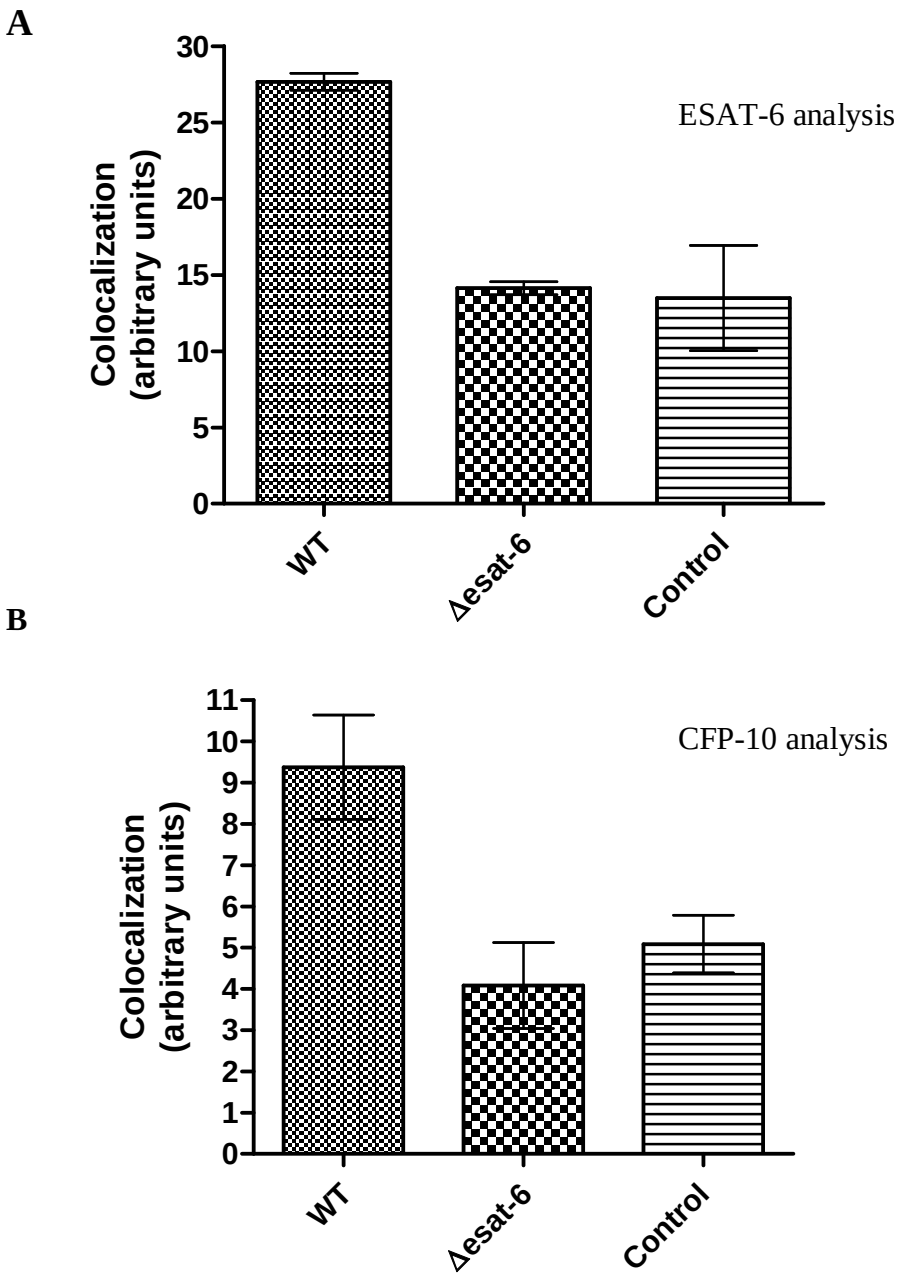

Fig. 16. *Immunofluorescence analysis of colocalization of GFP-tagged bacterial strains and antibody against ESAT-6 (A) or CFP-10 (B) proteins*. Overlay of green and red pixels was analyzed by ImageJ software to quantify colocalization. The graph shown is a representative example of duplicate experiments using four images (examples of which are seen in Figures 11-13) per strain for quantification. The control indicates samples treated with secondary but not primary antibody. The level of colocalization for the control is indicative of the nonspecific background binding of the secondary antibody to cell surfaces. A threshold of 50 (0-255) for both red and green channels was used during analysis in ImageJ. Error bars are ± standard error of the mean (SEM).

Chapter 4: Experimental Procedures

Bacterial Strains and Media

Mycobacterium marinum strains were cultured at 32°C, shaking at 100 rpm, in 7H9 liquid supplemented with Tween 80 and ADC or on solid 7H10 agar as previously described in Gao *et al*., 2003**.** Additionally**,** strains were cultured in Sauton's minimum defined media for culture filtrate and cell lysate western blot analysis. The *esat-6* mutant was generated by Amro Bohsali (Dept. Cell Biology & Molecular Genetics, University of Maryland), while both wild type and ΔESAT-6 GFP tagged strains were created by Kathleen Shannon (University of Maryland).

Cell Culture

J774 cells were cultured at 37°C in DMEM supplemented with 10% Fetal Bovine Serum (FBS) and 1% Glutamine. Bone marrow derived macrophages obtained from C75BL/6 mice (Jackson Laboratories, Bar Harbour, Maine) were cultured at 37°C in DMEM supplemented with 10% FBS, 2% Hepes buffer, 1% glutamine, and 15% L929 cell supernatant.

Culture Filtrate & Cell Lysate Preparation

M.marinum strains were cultured to late-log phase (OD600 ~1.2-1.4) in 7H9 medium. Cultures were washed twice in Sauton's minimum defined media by centrifugation at 3000xg for ten minutes. Bacteria were then inoculated into Sauton's media and grown to OD600 ~1.2-1.4 and passaged for a second time into Sauton's media. Bacteria were cultured for either 2 or 6 additional days to obtain cultures at

varying log phases. Culture filtrate was harvested at these times by centrifugation at 3000xg for 10 minutes to separate the supernatant and bacterial pellets. The supernatant was concentrated to 1 ml using Centriplus columns after adding EDTA for a final concentration of 1mM (pH 7-8) and a cocktail of protease inhibitors (Sigma Protease Inhibitor Cocktail for general use with broad spectrum inhibition of serine, cystein, and metalloproteases; used at recommended concentration per gram of cell extract). Culture filtrates were normalized according to the weight of each bacterial pellet.

For preparation of cell lysates, bacterial pellets were resuspended in 20 mM Tris buffer, 1mM EDTA, containing a cocktail of proteinase inhibitors (pH 7.5). Bacteria underwent bead-beating followed by centrifugation at 3000xg for ten minutes at 4°C. Samples were analyzed at this stage as crude cell lysate or further separated by centrifugation at 16,000xg for 60 minutes. At this stage the samples were separated into cell wall and cytosolic/plasma membrane fractions for continued analysis. Cell lysates were normalized with a BCA™ Protein Assay Kit (Pierce) in order to determine the concentration of protein and adjust samples to get equal total protein loading for western blot analysis.

Western Blot Analysis

Culture filtrates and cell lysates were mixed with SDS buffer with 2-mercaptoethanol. Immunoblot was performed by separating sample proteins in a 4-20% SDS-PAGE and visualizing by chemiluminesence (Biorad). Antibodies used

included the following: Rabbit polyclonal anti-Mtb CFP-10 obtained from Colorado State University (CSU) (titer 1:50,000); mouse Mab HYB76-8, anti-ESAT-6 (titer 1:35) courtesy of Peter Anderson, Statens Serum Institut, Denmark; mouse monoclonal anti-GroEL, obtained from CSU (titer 1:50); rabbit polyclonal anti-Ag85, obtained from Marcus Horowitz's lab UCLA (1:2,000).

Analysis of Cytolytic Activity

Contact Dependent Haemolysis Assay:

Red blood cells (RBCs) were washed by centrifugation at 2,000 rpm for seven minutes and finally resuspended in PBS. Wild type or ΔESAT-6 *M.marinum* strains were processed to a single cell suspension by centrifugation at 3800 rpm for 10 minutes, resuspension in PBS, centrifugation at 6000 rpm for 3 minutes, and finally passage through a 26G needle. RBCs were incubated with bacterial strains at a ratio of 1:20 by centrifuging both together 8000 rpm for five minutes followed by two hours incubation at 32°C / 5%CO2. The RBC/bacterial pellets were resuspended, centrifuged again, and the OD reading at 405nm was observed to measure the hemoglobin release into the supernatant.

Necrosis Assay:

J774 macrophages were conditioned with phenol-red free media at 32°C, 24 hours prior to infection. Cell infection was done at an MOI of 50 with wild type or ΔESAT-6 strains. Upon addition of the bacterial suspensions to cells, both were centrifuged at 2000rpm for 5 minutes in order to pellet the bacteria down to the cells.

Cells were incubated for 1, 2, or 3 hours at 32°C/ 5%CO2. Cells were washed with PBS and then incubated for 45 minutes with J774 culture medium containing EthD-1 (4um) and Calcein (1uM). Observation was by fluorescent microscopy. Analysis and quantification of fluorescent images was done using ImageJ software and plotted with GraphPad Prism 4.0 software.

Analysis of ESAT-6/CFP-10 binding to J774 cell surfaces

J774 cells were infected at a multiplicity of infection of 1:50 with wild type-GFP or ΔESAT-6-GFP *M.marinum* strains. J774 cells were conditioned at 32°C in DMEM with 2% FBS for one hour prior to infection. Cells were infected for 30, 60, or 90 minutes after which cells were fixed with 4% paraformaldehyde. After blocking with 5% BSA solution, cells were probed with either anti-CFP-10 (1:300 titer) or anti-ESAT-6 (1:15 titer) antibodies obtained courtesy of Peter Anderson (Statens Serum Institut, Denmark). Secondary antibodies used were Alexafluor goat-anti-rabbit 633 and Alexafluor goat-anti-mouse 594 for anti-CFP-10 and anti-ESAT-6 respectively. Cells were analyzed by fluorescent microscopy. Analysis and quantification of fluorescent images was done using ImageJ software and plotted with GraphPad Prism 4.0 software.

Analysis of Intracellular Growth

Bone marrow derived macrophages were grown on fibronectin treated coverslips and conditioned 24 hours prior to infection by incubating cells in DMEM with 2% FBS, at 32°C. Cells were infected for two hours with wild type-GFP or

ΔESAT-6-GFP strains at an MOI 0.4. After infection, cells were incubated with culture medium containing streptomycin (4ug/ml) and incubated for 0, 24, 48, or 72 hours. Cells were fixed with 4% paraformaldehyde and observed using fluorescent microscopy. Analysis was done by observing cells infected with </= 2, 2-5, 5-10, or >10 bacteria and calculating the total percentage of cells infected at each time point. Data was analyzed via the GraphPad Prism 4.0 program.

Chapter 5: Discussion

This study provides a better understanding of how the Mycobacterial proteins ESAT-6 and CFP-10 are secreted, as well as providing new insight into the stability of both of these proteins. Previous work done by Gao et al in 2004, suggested that several genes including *Mh3876, Mh3878,* and *Mh3881* all were specifically involved in ESAT-6 secretion while the genes *Mh3866, Mh3867,* and *Mh3868* were involved in synthesis or intracellular stability. The mutation of genes *Mh3866, Mh3867, Mh3868,* and *Mh3881* resulted in either abolished or strongly reduced secretion of CFP-10, although it was mentioned that there were reduced levels of intracellular CFP-10 for strains having mutations in *Mh3866, Mh3867,* and *Mh3868*. The genes *Mh3876, Mh3878,* and *Mh3879* were suggested to have no effect on CFP-10 secretion. Several of these findings were confirmed here, however, the quantification done in this study for the first time, modified some of the previously suggested conclusions. It appears that although the mutation of genes *Mh3866, Mh3867,* and *Mh3868* reduced the amount of intracellular ESAT-6 protein, it did not result in such dramatically different levels of ESAT-6 to suggest a clear defect in synthesis or intracellular stability. These differences suggest that initial loading or exposure of western blot analysis did not show sufficient detectable protein although it was in fact present in either the cell lysate or culture filtrates.

The extensive quantitative analysis done here also modified previous findings by introducing strikingly novel data to suggest the genes *Mh3866, Mh3876, Mh3878, Mh3879,* and *Mh3881* may be involved in ESAT-6 stability as well as CFP-10 stability (with the exception of *Mh3866* and *Mh3878)*. It was previously suggested

by Gao et al (2004) that the mutation of the genes *Mh3878* and *Mh3879* resulted in defective secretion, while the disruption of the genes *Mh3866* and *Mh3876* resulted in reduced secretion due to defects in either synthesis or intracellular stability. These conclusions were suggested after observing culture filtrate harvested at approximately five days of bacterial growth. In this study, data resulting from the comparison of early/ two day culture filtrate harvests, to later/six day harvests, shows that this does not appear to be entirely true. No dramatic intracellular defects were seen when compared to the other extRD1 mutants. However, the analysis of culture filtrates at two days of bacterial growth versus six days of growth profiled protein expression to indicate that relatively high levels of protein were secreted earlier and were significantly reduced by six days. This suggests that there is a defect in extracellular stability of ESAT-6 or that it is somehow being degraded over time. It could also be suggested however that there are differences in regulation for the mutant strains. The observations at six days may be the result of differential regulation compared to that at two days, which limits the production of ESAT-6. At six days of growth, the bacteria do appear to enter late log phase and this may also contribute to changes in cellular regulation and general expression of proteins.

It is very interesting to note that the mutant *Mh3876::kan* has extremely high levels of both ESAT-6 and CFP-10 at 2 days of culture, and less that wild type levels at 6 days. This extreme secretion of protein at 2 days is very surprising and implies not only a defect in stability due to the varying amounts over time, but also a defect in regulation of secretion at early time points. This mutation results in over secretion or

super secretion so perhaps it disrupts a gene responsible for controlling or negatively regulating protein secretion at an early time.

In the examination of two and six day culture filtrates, the SecA2 mutant showed rather varying degrees of secretion for both ESAT-6 and CFP-10 in a manner similar to the *Mh3876::kan* strain. It is apparent that at two days, the mutant produces much higher amounts of both ESAT-6 and CFP-10, while at six days it shows approximately wild type levels. Figure eight appears to be flawed in experimental procedure due to the fact that very little ESAT-6 protein is detected for the SecA2 mutant when it is clear from the previously shown westerns in figure five, that wild type levels are seen in the culture filtrate. These results do suggest however that there may be a link between the SecA2 pathway and the RD1 encoded pathway. It seems that a mutation of the general secretion pathway affects the bacterium to somehow secreting more ESAT-6 and CFP-10 at early time points. Further studies should be done to characterize this potentially important relationship between two previously unlinked secretion pathways. It may be suggested that the disruption of one pathway induces early compensatory secretion in the other. On a slightly different note, further analysis must also be done on all the mutant strains to observe mRNA levels comparatively at two and six days on order to confirm these results and determine whether stability, regulation, or both are affecting the levels of ESAT-6.

The genes implicated here in ESAT-6 stability, encode for several hypothetical proteins (*Mh3866, Mh3878, Mh3879, Mh3881*), and an ATPase (*Mh3876*). Although the *M. marinum* gene Mh3866 is not characterized, it was possible to find that the homologue of the gene in *M. tuberculosis, Rv3866,* also

encodes for a hypothetical protein but has a possible DNA binding site (www.ncbi.nlm.nih.gov). This possible DNA binding site may indicate the likelihood that rather than being involved in stability, this gene plays a role in regulation of either expression of the proteins themselves, or, of intermediate proteins that may affect secretion at different growth stages (such as those involved in regulating the actual secretory apparatus). However, the homologous gene found in *M.smegmatis, Sn3866,* is found to have limited homology with ABC ATPases involved in type I secretion; so the possibility remains that it forms a part of the secretory apparatus to translocate protein. In the case of the ATPase (*Mh3876),* these enzymes are known to be involved in secretory apparatuses through the hydrolysis of ATP resulting in translocation of proteins to the periplasmic space. A defect in the activity of the ATPase may result in improper secretion or translocation of the protein which may in turn cause protein instability. In the case of the other genes shown to be involved in stability, very little is known at present as to their hypothetical functions or cellular location.

The idea of differential secretion of ESAT-6 and CFP-10 over time also has a potential impact in the future of vaccine development. Since both ESAT-6 and CFP-10 are known to be highly immunogenic (Brodin et al, 2005) it would be a useful tool to create an attenuated strain of *Mycobacterium* that has the ability to elicit a strong immune response. A mutant strain defective in stability and able to secrete ESAT-6 and CFP-10 only at an early time may have such an effect. It would allow for a strong immune response early on and then would also lack the virulence features

associated with the presence of both proteins at later times, allowing the strain to be cleared from the host.

In terms of functionality of ESAT-6 and CFP-10, the work done in this study shows that both proteins are involved in cytolysis. Disruption of the *esat-6* gene leads to a loss of both ESAT-6 and CFP-10 protein, and bacteria with this mutation are shown here to be defective in both red blood cell and macrophage cell lysis. It remains unknown however, whether the ESAT-6 and CFP-10 proteins are directly or indirectly involved in mycobacterium cytolytic abilities. It is possible that the protein complex of ESAT-6 and CFP-10 acts as a cytolysin, but there also remains the option that they may require an intermediate in order to have cytolysis occur. It has recently been shown that ESAT-6 and CFP-10 may be required for secretion of an additional protein that is chromosomally unlinked to the RD1 locus and is encoded on a gene called *espA*. It appears that the secretion of all three proteins is mutually dependent (Fortune et al, 2005). Based on this finding, it remains a possibility that additional proteins work together with ESAT-6 and CFP-10. Recent work (unpublished Gao lab) has implied that the enzyme Phospholipase C (PLC) secretion may also be regulated by RD1. This data brings to light the possibility that PLC and ESAT/CFP-10 may act in concert for cytolytic activity; the enzymatic activity by PLC could serve as a means of disrupting the phospholipids in cell membranes, while ESAT-6 and CFP-10 may penetrate or further interfere with the membrane. Structural analysis of ESAT-6/ CFP-10 complex shows that there are extensive hydrophobic regions in the sites of contact between both proteins (Renshaw et al, 2005). These

hydrophobic regions could potentially interact with the interior hydrophobic regions of the phospholipid bilayer in cell membranes and coulld eventually disrupt the membranes.

The work done here also provides a more physiologically relevant system to show ESAT-6/CFP-10 involvement in cytolysis. Previous work by Hsu et al (2003) has shown that purified ESAT-6 protein can disrupt artificial lipid bilayers. Studies done here involved the use of whole bacterium, of wild type and Δesat-6 strains, to see whether the presence of ESAT-6/CFP-10 affected cytolytic activity. This method provides a more biologically relevant model to studying cytolysis rather than using purified protein alone. Additionally, this work uses red blood cells and macrophages to observe cytolysis rather than artificially created membranes.

The ability to lyse macrophages is very physiologically relevant in the study of Mycobacterial infections, as macrophages are one of the primary cells infected. In an attempt to clear infections, macrophages will normally phagocytose bacteria, enclosing them in an endosomal compartment with a membrane that is topologically the same as the outer cell membrane. In order for the bacteria to escape the phagosome and spread to other cells, they need to lyse this phagosomal membrane. Since it is shown here that ESAT-6/CFP-10 are involved in macrophage lysis, it is not surprising that the loss of the proteins also results in a defect in bacterial spreading, as seen in Figure 12. The loss of ESAT-6 and CFP-10 may affect the bacteria's ability to lyse the phagosomal membrane, just as it does for the outer cell membrane. It is worth noting however, that although the bacteria may escape into the cytosol by lysis of the phagosomal membrane they still have to overcome the barrier of the cell

membrane to escape into the extracellular milieu. It is unknown whether the topology of the cytosolic side of the cell membrane may differ enough from the exterior side and affect the bacteria's cytolytic abilities. It has been previously shown that *M.marinum* can polymerize cytosolic actin and propel itself to spread to adjacent cells through actin-based motility (Stamm et al, 2003). This suggests that when mycobacterium have the opportunity to polymerize actin, they will exploit this as a mechanism for propulsion and escape through cell membranes. However when there is no actin available (such as in the phagosomal compartment or the extracellular milieu) it may employ other methods of cytolysis to disrupt membranes and spread /escape. It would be interesting for future studies to observe the integrity of phagosomal membranes during Mycobacterial infection by wild type and *Δesat-6* strains. Additional work may also be done to understand the proteins interactions with cell membranes. Similar studies to the immunofluorescence microscopy work done here can be done, and also observed using confocal microscopy. The three dimensional or Z-stack capabilities of this method of microscopy may help determine the exact localization of the secreted proteins and whether they are located between the bacteria and the cell wall.

Based on the cytolysis results shown here, future work may also be directed in the area of necrotic or caseous granuloma formation. Since the necrotic central regions of granulomas are hallmarks of pathogenic infections, and RD1 has been previously shown to be involved in granuloma formation (Cosma et al, 2006), there is a possibility that ESAT-6 and CFP-10 may also be involved in this aspect of Mycobacterial infection. As both proteins are shown here to be involved in cytolysis,

it is possible that this cytolytic or necrotic activity may be pertinent to the lysis of cells in caseous granuloma formation.

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