Title
Requirements for the secretion of virulence factors by the Mycobacterium ESX-1 secretion pathway

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Publication Date
2007-10-25

Peer reviewed|Thesis/dissertation

Requirements for the secretion of virulence factors by the *Mycobacterium* ESX-1 secretion pathway

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biochemistry and Molecular Biology

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

[redacted]

UMI Number: 3289303

UMI Microform 3289303

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ACKNOWLEDGEMENTS

I first thank Rick for his mentorship and friendship over the years. Rick cleverly chose to pursue, a decade ago, the *Mycobacterium marinum* model as a tool to understanding the pathogenesis of *M. tuberculosis* infections and I am grateful that I could be part his lab and contribute to the development of this model system. I have always appreciated the confidence that Rick has bestowed on me as well as the time he taken to train me in designing experiments, interpreting data, and presenting results.

I also owe tremendous thanks to the other members of my thesis committee: Joanne Engel and Jeff Cox. Joanne thoroughly and thoughtfully helped me formulate my thesis project early in my graduate work and has remained supportive of me through the completion of my thesis work. Additionally, Joanne has devoted a great deal of her time to the UCSF Microbial Pathogenesis program and thereby has created a supportive community of postdocs and students, for which I am very grateful. Jeff is a brilliant scientist and his students and postdocs are of equally high caliber. I am grateful for the advice that Jeff has given me over the years and I am equally appreciative of the friendly collaborative interactions I enjoy with members of the Cox lab. Another member of the UCSF Microbial Pathogenesis program that I would to thank is Anita Sil, who also devotes a great deal of her time to make the UCSF Microbial Pathogenesis program a collaborative and supportive community.

I owe thanks to three undergraduate students- Janet Chon, Terri Cheng, and David Lin- and one high school student –Sarah Kajihara- that I had the privilege to mentor, work with, and befriend in the course of my research. In particular, Janet devoted over a

year of her time working alongside me enabling the accomplishment of significantly more science than I otherwise would have achieved. Janet, Terri, David, and Sarah made the lab a more fun enthusiastic place and raised the level of learning through their inquisitive questions.

I thank the Von Zastrow, the Bourne, and the Mostov labs for their friendship, especially Gloria Pedro for feeding me fruit nearly every day. I thank Belgica Caro for her kindness and helping me so frequently with the ordering of reagents. I am thankful to my classmate and friend Muluye Liku. I am very grateful for the companionship of my roommates during all six years of graduate school –Matt Cameron and Clement Cheung.

Over the years I have had a great deal of fun sharing stories, laughter, and friendship with my baymates: Mette Johansen, Elsa Dulac, Kim Kajihara and our honorary baymate Todd Nakagawa. I have also appreciated the companionship of my other labmates, in particular Ingrid Koo, Ping Wu, Wouter Hazenbous, and Chen Wang.

I owe the deepest of thanks to my parents, brothers, sister, and grandparents whose unwavering value on education and love has afford me the most fortunate of opportunities. Finally, I thank Dr. Kit Wong, for taking care of me, for joining me on fun adventures, for good advice, for cooking so often for me and for loving me.

The text of Chapter 2 is largely a reprint of the material as it appears in "A *Mycobacterium ESX-1 secreted virulence factor with unique requirements for export*" McLaughlin B, Chon JS, MacGurn JA, Carlsson F, Cheng T, Cox JS, Brown EJ, A . PLoS Pathogens, August 3, 2007, vol. 3, issue 8.

ABSTRACT

Requirements for the secretion of virulence factors

by the *Mycobacterium* ESX-1 secretion pathway

Bryant McLaughlin

Mycobacterium tuberculosis is the causative agent of tuberculosis, a primarily pulmonary disease that is estimated to infect approximately one third of the world population yet the mechanisms by which this bacterium causes disease are poorly understood. A key feature of *M. tuberculosis* pathogenesis is the parasitism of host macrophages following phagocytic entry, which is facilitated by the bacteria's manipulation of the host cell environment. The secretion of virulence proteins is a common mechanism used by pathogenic bacteria for altering the host environment. One such pathway, present in *Mycobacterium* and other Gram-positive genera, is ESX-1 (early secretory antigenic target 6 system 1). Although ESX-1 is required for multiple phenotypes related to the pathogenesis of infection, only three substrates of the secretion machine have been identified to date, and the mechanism by which these substrates are exported is not understood. In this work I identify a novel ESX-1 substrate and find that its delivery to the ESX-1 machine requires different protein interactions than previously identified substrates. I probe the role of this novel substrate in virulence and present data that the various ESX-1 substrates contribute additively to virulence. These results expand our understanding of a major virulence mechanism of *Mycobacteria* as well as mechanisms for the secretion of proteins through thick impermeable bacterial cell walls.

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Chapter 1

Introduction

M. marinum, a robust model organism

Pathogenic species of the genus *Mycobacterium* pose a major threat to public health. In particular, *Mycobacterium tuberculosis* infects at least one-third of the world's population and causes nearly 2 million deaths each year. The burden is most severe on the developing world, where poverty, inadequate health care, and other diseases – especially HIV/AIDS-, are contributing factors. Although some antibiotics are effective for treating tuberculosis infections, the requirement for long courses of therapy, the emergence of multi-drug resistant strains, and the increase in co-infection with HIV has created the need to develop new drugs, new vaccines as well as improved diagnostics. Delineating the molecular mechanisms of pathogenesis promises to inspire such necessary novel strategies for the treatment and prevention of mycobacterial diseases.

Although there is a dire urgency for more progress in *M. tuberculosis* research, the safety issues, the slow rate of growth of the bacilli, and infection models with hosts for which *M. tuberculosis* is not a natural pathogen present high costs and limitations in such research efforts. In this thesis work a closely related, yet safe and faster growing species, *Mycobacterium marinum,* was employed to model the pathogenesis of *M. tuberculosis*. *M. marinum*, is a natural pathogen of fish and amphibians that causes systemic tuberculosis-like diseases. The course of infection in these ectothermic hosts, and the pathology of granuloma formation are indistinguishable from that of *M. tuberculosis* ([1]). Multiple *M. marinum* virulence mutants have similar phenotypes to the orthologous *M. tuberculosis* mutants and can be complemented by the *M. tuberculosis* orthologous genes ([2-6]). Of particular relevance to this thesis, disruptions of genes encoding the ESX-1 secretion system in either species renders each less virulent, and

many of those attenuated *M. marinum* mutants have been complemented with the *M. tuberculosis* orthologs. Thus, *M. marinum* is a pragmatic and effective model to study the pathogenesis of tuberculosis in genetically tractable model organisms such as zebrafish ([7]) and Drosophila ([8]), in cultured mammalian monocyte derived cells, as well as for in vitro biochemical characterization of mycobacterial virulence systems.

M. marinum also is believed to be the progenitor of *M. ulcerans*, the causative agent of the chronic necrotising human skin disease Buruli ulcer and the third most common mycobacterial disease after tuberculosis and leprosy. *M. ulcerans* evolved through lateral gene transfer and reductive evolution with the key driver of its speciation being the virulence plasmid, pMUM001, required for production of the tissue damaging polyketide, mycolactone.

Alternative bacterial secretion systems

Many animal and plant bacterial pathogens use sec-independent, alternative protein secretion systems to alter their host environment to a replication permissive niche. Frequently, these protein secretion systems are encoded within pathogenicity islands - clusters of genes acquired during evolution via horizontal genetic transfer. Thus, distantly related pathogens can harbor closely related virulence genes. Of these secretion systems, the Type III secretion system of gram-negative bacteria is particularly remarkable for the diversity of effector proteins and the assembly of a syringe-like apparatus for direct injection across host membranes. Thus, *Yersinia* and *Erwinia*, which both express a conserved the type III secretion apparatus but secrete entirely distinct effector proteins ,

can give rise to a multitude of diseases that range from bubonic plague in humans to fire blight in fruit trees [9].

Expression of Type III secretion systems is coordinately regulated in response to host environmental stimuli. Structurally conserved chaperones which specifically bind to individual secreted proteins play an important role in Type III protein secretion, apparently by preventing premature interactions of the secreted factors with other proteins. The inner membrane proteins of the Type III secretion apparatus are homologous to components of the flagellar biosynthesis apparatus of both gram-negative and gram-positive bacteria, and while the effectors are secreted independent of the sec system, assembly of the secretion apparatus probably requires the sec pathway, since several components possess sec signal sequences.

One alternative secretion system in *Mycobacterium* is the ESX-1 secretion system, which, though structurally unrelated, is analogous in some ways to the Type III system. ESX-1 is a multi-protein complex; with at least six effectors –ESAT-6, CFP-10, EspA, EspB, EspC, and EspR [10-15]-; and is largely encoded by the RD1 locus. None of the known effectors contain sec signals and the only signal sequence leading to ESX-1 mediated secretion identified thus far is that of CFP-10, which lies at the carboxyterminus of the protein[16].

The ESX-1 secretion system is essential for the virulence of at least *M. marinum*, *M. bovis*, and *M. tuberculosis*. During the multiple in vitro serial passages that caused attenuation of *M. bovis* to the live vaccine strain BCG, an approximately 10kb deletion occurred, termed RD1. Within this locus are 9 genes, several of which have been shown subsequently to encode components of the ESX-1 system. If BCG is complemented with

a cosmid that spans the RD1 region (and adjacent DNA on either side of the region to achieve complete expression of all the genes in the RD1 locus), then the recombinant strain exhibits increased growth of bacteria in the lungs and spleen of mice and markedly increased virulence in SCID mice. Deletion of RD1 from virulent *M. tuberculosis* H37Rv results in attenuation of the ability to grow intracellularly in human macrophages, to cause macrophage cytotoxicity, and to disseminate from the lungs to the spleen of immunocompetent mice after aerosol infection. Similarly, deletion of RD1 from *M. marinum* renders this species less virulent in mouse macrophage infections, as well as defective in macrophage aggregation and granuloma formation during zebrafish embryo infections.

In the evolution of *M. marinum* to *M. ulcerans* 1.2 Mb of the genomic DNA was lost, including the deletion of ESX-1[17]. The *M. ulcerans*, ESX-1 was disrupted by two deletions, which enabled the core ESX-1 operon *Mh3876-Mh3877* and *Mh3878* to be retained, but caused the remainder of the *extRD1* genes from *Mh3864-Mh3883c* to be deleted. Interestingly, *espA* and *espC*, genes encoding two ESX-1 substrates that are located at a non-RD1 locus, are also deleted in *M. ulcerans*. Loss of ESX-1, which triggers granuloma formation by *M. marinum*, may contribute to the predominantly extracellular location of *M. ulcerans* in infected human tissue.

As mentioned above, the RD1 deletion disrupts the expression of nine genes, including core components of ESX-1. Genes adjacent to RD1 or in the distant locus, Rv3616c-Rv3614c, also encode proteins essential for ESX-1. The ESX-1 substrates, ESAT-6 and CFP-10, encoded within RD1 are among the most abundant proteins in the culture filtrate of *M. marinum* and *M. tuberculosis*, during growth in Sauton's media,

while the other known substrates are of much less abundance. Additional ESX-1 effectors and ESX-1 mediated host pathogen interactions continue to be identified, and the prominent questions that have arisen include: how does the ESX-1 system function to transport proteins across both the cytosolic membrane and the thick highly hydrophobic and impermeable mycobacterial cell wall; how is ESX-1 regulated; what are the host targets of the ESX-1 effectors; and do some effectors have a more significant role in virulence than other effectors?

Introduction to thesis work

At the time that I began my thesis work, our lab had just completed screening 900 *M. marinum* transposon insertion mutants for the ability to lyse sheep red blood cells and grow within mouse macrophages. Of the 900 mutants 24 failed to grow within mouse macrophages, and of those 24 mutants, nine were disrupted for genes in an extended RD1 (*extRD1*) locus, *Mh3866-Mh3881c* [3]. Two signature tagged mutagenesis screens of infected mice, one using *M. bovis* and the other *M. tuberculosis*, also suggested that the extRD1 locus was significant in virulence [15,18]. Of our nine mutants, *$espB_M$::tn (Mh3881c::tn)* was the most attenuated for virulence to zebrafish, growth in macrophages, and cytotoxicity to J774 cells. Given the obvious significance of the extRD1 locus suggested by these screens, we decided to investigate in detail the function of these genes and in particular the *$espB_M$::tn* mutant.

In the first part of this thesis I show that $EspB_T$ (Rv3881c) and its *M. marinum* homolog, $EspB_M$, are in fact substrates of the ESX-1 secretion system. Although a substrate of ESX-1, we find that the specific genes required for secretion of EspB differ

from those required for the secretion of at least one other ESX-1 substrate, CFP-10. Through multiple biochemical assays I convincingly demonstrate that EspB forms a complex with Rv3879c and then demonstrate that Rv3879c interacts with Rv3871, the same component of ESX-1 that interacts with the ESAT-6/CFP-10 complex during its secretion.

In the second part of this work, I find that the carboxyterminus of EspB, while dispensable for interaction with Rv3879 and EspB secretion, is required for *M. marinum* growth in macrophages and for hemolytic activity. We report that the *espB*$_M$*::tn* mutant displays wild-type sensitivity to acidic conditions and reactive nitrogen intermediates in in-vitro assays, but has a subtle defect in cording in addition to its smooth colony morphology. Finally, we show that the *espB*$_M$*::tn* mutant is not altered for the transcription of extRD1 genes other than *espB* and *Mh3880c* and that the survival profile of an *espB*$_M$*::tn* mutant in macrophages diverges from that of wild-type after 24 hours of infection.

These data support a model that different substrates are delivered to the ESX-1 machine by molecularly distinguishable pathways and that each of these pathways for ESX-1–mediated secretion contributes to mycobacterial virulence.

Chapter 2

A *Mycobacterium* ESX-1–Secreted Virulence Factor with Unique Requirements for Export

ABSTRACT

Specialized secretion systems of pathogenic bacteria commonly transport multiple effectors that act in concert to control and exploit the host cell as a replication-permissive niche. Both the *Mycobacterium marinum* and the *Mycobacterium tuberculosis* genomes contain an extended region of difference 1 (extRD1) locus that encodes one such pathway, the early secretory antigenic target 6 (ESAT-6) system 1 (ESX-1) secretion apparatus. ESX-1 is required for virulence and for secretion of the proteins ESAT-6, culture filtrate protein 10 (CFP-10), and EspA. Here, we show that both Rv3881c and its *M. marinum* homolog, Mh3881c, are secreted proteins, and disruption of RD1 in either organism blocks secretion. We have renamed the *Rv3881c/Mh3881c* gene *espB* for ESX-1 substrate protein B. Secretion of *M. marinum* EspB ($EspB_M$) requires both the *Mh3879c* and *Mh3871* genes within RD1, while CFP-10 secretion is not affected by disruption of *Mh3879c*. In contrast, disruption of *Mh3866* or *Mh3867* within the extRD1 locus prevents CFP-10 secretion without effect on $EspB_M$. Mutants that fail to secrete only $EspB_M$ or only CFP-10 are less attenuated in macrophages than mutants failing to secrete both substrates. $EspB_M$ physically interacts with Mh3879c; the *M. tuberculosis* homolog, $EspB_T$, physically interacts with Rv3879c; and mutants of $EspB_M$ that fail to bind Mh3879c fail to be secreted. We also found interaction between Rv3879c and Rv3871, a component of the ESX-1 machine, suggesting a mechanism for the secretion of EspB. The results establish EspB as a substrate of ESX-1 that is required for virulence and growth in macrophages and suggests that the contribution of ESX-1 to virulence may arise from the secretion of multiple independent substrates.

INTRODUCTION

The cell surface–associated and secreted proteins of pathogenic bacteria promote the uptake of nutrients; facilitate attachment to specific surfaces, cells, or proteins; function in cell wall maintenance and cell division; and offer protection from harsh environmental conditions, including the host immune system. In *Mycobacteria,* there are at least four pathways to secrete proteins—Sec, SecA2, twin-arginine translocase, and the early secretory antigenic target 6 (ESAT-6) system 1 (ESX-1). Much attention has been focused on the ESX-1 pathway because it is required for virulence and for the secretion of ESAT-6 and culture filtrate protein 10 (CFP-10), two major targets of the immune response in infected individuals.

M. tuberculosis ESX-1 is required for virulence in mice, growth in macrophages, and the suppression of macrophage inflammatory and immune responses, including the arrest of phagosome maturation and the reduced expression of IL-12 and TNF-α [15,19-23]. The homologous *M. marinum* ESX-1 is required for virulence in zebrafish, growth in macrophages, cytolysis and cytoxicity, and cell-to-cell spread, in addition to ESAT-6 and CFP-10 secretion [3,5]. In zebrafish embryo infections, *M. marinum* ESX-1 is required for macrophage aggregation and granuloma formation [6]. In *M. smegmatis,* ESX-1, in addition to being required for secretion of ESAT-6 and CFP-10, modulates conjugal DNA transfer [24,25]. In contrast, most strains of *M. ulcerans,* which is closely related genetically to *M. marinum* and *M. tuberculosis,* but persists in extracellular locations during mammalian infection, lack most of the ESX-1 components as well as orthologs of the genes extending from *Rv3879c* thru *Rv3883c* [17,26]. Although the ESX-1 secretion machinery (Rv3870, Rv3871, and Rv3877) is required for the arrest of phagosome

maturation by *M. tuberculosis* during an infection of macrophages, the known ESX-1 substrates are dispensable [23]. The multiple phenotypes and host responses dictated by the ESX-1 secretory apparatus suggest that there may be additional substrates, components, and regulatory molecules yet to be identified.

Recently, a third ESX-1 substrate, EspA (Rv3616c), was identified [10]. Unlike ESAT-6 and CFP-10, EspA is encoded at a locus distant from the ESX-1 machine, yet this substrate is codependent with both ESAT-6 and CFP-10 for secretion. The mechanism for this interdependence has not been determined, but the interaction between ESAT-6 and CFP-10 in the bacterial cytosol appears to be required for secretion of the heterodimer [16,27-29]. Presumably, the stable heterodimer is also required for the secretion of EspA.

The *M. tuberculosis* region of difference 1 (RD1) locus *(Rv3871-Rv3879c)* and the neighboring genes encode the ESX-1 substrates ESAT-6 and CFP-10, as well as core components of the secretion machine [15,19,20]. These core components include at least two putative SpoIIIE/FtsK ATPase family members (Rv3870 and Rv3871), a proline-rich predicted chromosome-partitioning ATPase (Rv3876), and a putative transporter protein with 12 transmembrane domains (Rv3877). The non-RD1 gene cluster *Rv3616c-Rv3614c* also is required for secretion of the known substrates [10,12]. Additional proteins are likely to be necessary for the assembly of the ESX-1 machinery, because in *M. smegmatis,* genes extending from homologs of *Rv3866* through *Rv3883c* have been shown to be required for ESX-1–mediated secretion [25]; an *M. bovis* mutant disrupted for the expression of the genes homologous to *Rv3867* through *Rv3869* fails to secrete ESAT-6 and CFP-10 [18]; and in *M. marinum,* the locus required for ESX-1–mediated secretion

extends at least from the homolog of *Rv3866 (Mh3866)* to the homolog of *Rv3881c (Mh3881c),* which in this work we rename *espB* (see below) [3].

Although these studies have identified multiple genes required for ESX-1 function, the biochemical interactions necessary for assembly of the secretion machine and for transport of substrates are still not understood. A model for CFP-10 secretion is that the carboxyterminus of the CFP-10 substrate is recognized by Rv3871, which in turn interacts with the integral membrane protein Rv3870 to direct CFP-10 through the secretion pore [16]. The interaction of CFP-10 with Rv3871 is also required for secretion of ESAT-6, suggesting that this is a requisite step in secretion of the ESAT-6/CFP-10 heterodimer by the ESX-1 machine.

Here, we show that Rv3881c and its *M. marinum* homolog, Mh3881c, are substrates for secretion by ESX-1. For this reason, we have named the gene product of this locus ESX-1 substrate protein B (EspB). In both species, *espB* encodes a gylcine-rich protein with a predicted molecular weight of ~47 kDa, without any region of apparent similarity to the secretion signal of CFP-10 or other known secretion signals. Although a substrate of ESX-1, we find that the specific genes required for secretion of EspB differ from those required for the secretion of CFP-10. Biochemical investigation demonstrates that EspB forms a complex with Rv3879c and that Rv3879c interacts with Rv3871, the same component of ESX-1 that interacts with the ESAT-6/CFP-10 complex during its secretion. These data support a model that different substrates are delivered to the ESX-1 machine by molecularly distinguishable pathways. Moreover, each of these pathways for ESX-1–mediated secretion contributes to mycobacterial virulence.

RESULTS

Both M. tuberculosis and M. marinum EspB Restore Intracellular Growth to the M. marinum Mutant $espB_M$::tn

A previous genetic screen for *M. marinum* mutants that fail to cause hemolysis led to the isolation of eight mutants in the extended RD1 (extRD1) locus, *Mh3866-Mh3881c* [3]. Of the eight mutants, *$espB_M$::tn (Mh3881c::tn)* was the most attenuated for virulence to zebrafish, growth in macrophages, and cytotoxicity to J774 cells. Thus, we decided to investigate the *$espB_M$*–encoded protein ($EspB_M$) and its *M. tuberculosis* homolog ($EspB_T$) in detail. The gene, *$espB_M$*, is the first in a two-gene operon. Using quantitative RT-PCR, we found that the mutation disrupts the expression of both genes in the operon (unpublished data). We then sought to determine the genetic requirements for restoration of intracellular growth to the mutant. Introduction of a non-integrating plasmid, expressing either $EspB_M$ from the *$espB_M$* promoter or $EspB_T$ from its native promoter, was sufficient to appreciably restore growth in macrophages to *$espB_M$::tn* (Figure 2.1A). The non-integrating plasmids expressing both $EspB_M$ and Mh3880c or both $EspB_T$ and Rv3880c were not superior in restoration of intracellular growth. Thus, EspB is necessary and sufficient to appreciably complement *$espB_M$::tn,* and the *M. tuberculosis* homolog functions equally well in *M. marinum,* demonstrating conservation of function.

While the expression of $EspB_M$ from a non-integrating plasmid appreciably restored growth in macrophages to *$espB_M$::tn,* the complementation was not complete. Among possible explanations are that the transposon insertion exerted a polar effect on the operon upstream, *Rv3883c-Rv3882c*, which also might have a role in intracellular

growth, or that a proper stoichiometry between EspB and ESX-1 is required for complete complementation. Therefore, integrating plasmids encoding either *espB$_M$* along with the *espB$_M$* promoter, *espB$_M$-Mh3880c* along with the *espB$_M$* promoter, or the entire locus *Mh3883c-Mh3880c* along with the *Mh3883c* promoter, were introduced into *espB$_M$::tn.* The locus *Mh3883c-Mh3880c,* along with the *Mh3883c* promoter, was also introduced into *espB$_M$::tn* on a non-integrating plasmid. Of these constructs, only the integrating plasmids encoding *espB$_M$-Mh3880c* or *Mh3883c-Mh3880c* fully complemented the growth defect of *espB$_M$::tn* (Figure 2.1B). Similarly, *espB$_M$* alone appreciably restored a rough colony morphology to the *espB$_M$::tn* mutant, but *espB$_M$-Mh3880c* or *Mh3883c-Mh3880c* fully restored the rough colony morphology to *espB$_M$::tn* (Figure 2.8). These results suggest that *Mh3880c* can contribute to *M. marinum* growth in macrophages when it is expressed along with *espB$_M$* from the bacterial chromosome. In contrast, *espB$_M$* contributes equally well to bacterial virulence whether expressed episomally or on the chromosome, suggesting that its contribution is more independent of its stoichiometry with respect to other virulence components.

EspB Is a Secreted Protein That Undergoes Carboxyterminal Processing

As a first step toward understanding the role of EspB in virulence and growth in macrophages, we determined its localization in *Mycobacteria* grown in broth culture. The cell lysate and culture filtrate fractions of *M. tuberculosis* H37Rv, wild-type *M. marinum,* and *M. marinum espB$_M$::tn* were probed with a mouse polyclonal antibody raised against a 100 amino acid fragment of EspB$_T$ extending from amino acid 234 to 333 (Figure 2.2A). EspB was detected in both the cell lysate and the culture filtrate fractions of *M.*

tuberculosis, as well as in both the cell lysate and the culture filtrate fractions of wild-type *M. marinum*. EspB was not detected in either fraction of the *espB$_M$::tn* culture, verifying the specificity of the antibody. GroEL, a non-secreted bacterial cytoplasmic protein, was found exclusively in the cell lysate, demonstrating that EspB did not appear in the culture filtrate as a result of cell lysis.

The EspB in the cell lysate had an M_r of 55 kDa on SDS-PAGE, while the EspB in the culture filtrate of both species ran at a slightly lower molecular weight. A lower molecular weight of EspB in the culture filtrate was also observed in a prior proteomic analysis of *M. tuberculosis* H37Rv proteins [30], in which EspB in the cell lysate was observed on a 2-D gel as a single spot with an apparent molecular weight of 55.6 kDa, while the EspB in the culture filtrate was observed as two spots with apparent molecular weights of 49.7 kDa and 48.4 kDa. Therefore, EspB might be cleaved either during or after secretion. To test this possibility, a V5 epitope tag was fused to the N-terminus of EspB$_M$ and a His6x epitope tag was fused to the C-terminus. The resulting construct, V5-EspB$_M$-His6x, was expressed in the *espB$_M$::tn* mutant. Like the native protein, V5-tagged EspB was detected in the cell lysate as a single band and as a doublet in the culture filtrate (Figure 2.2B). In contrast, His-tagged protein was only detected in the cell lysate fraction, suggesting that EspB$_M$ in the culture filtrate is C-terminally truncated.

EspB Secretion Requires a Distinct Set of extRD1-Encoded Genes for Secretion

To assess which ESX-1 genes are required for EspB secretion, its compartmentalization between cell lysate and culture filtrate was determined for several *M. marinum* ESX-1 mutants (Figure 2.2C). Although EspB$_M$ was found in both the cell

lysate and culture filtrate fractions of most mutants, $EspB_M$ was not detected in the culture filtrates of *MmΔRD1, Mh3868::tn, Mh3879c::tn,* or *Mh3871::tn*.

The *Mh3868::tn* mutants failed to accumulate protein in the pellet, suggesting that the Mh3868 protein could be involved in EspB synthesis or stability. Thus, of the ESX-1 genes tested, only *Mh3879* and *Mh3871* were clearly involved in $EspB_M$ secretion. In contrast, none of the mutants secreted ESAT-6 [3], and only *Mh3879c::tn* and *Mh3878c::tn* secreted CFP-10 normally. This difference in secretion requirements for ESAT-6 and CFP-10 in *M. marinum* has been noted previously [3]. Complementation of *Mh3879::tn* and *espB::tn* restored $EspB_M$ secretion. GroEL was absent from culture filtrates of all strains, and secretion of the fibronectin attachment protein (FAP), a protein secreted in a Sec-dependent manner [31], was not disturbed in any of the extRD1 mutants. Thus, the product of the $espB_M$ gene is a secreted protein that requires Mh3871, a core component of the ESX-1 secretion machine, for export; we have therefore named it ESX-1 substrate protein B (EspB). However, EspB, ESAT-6, and CFP-10 differ with respect to the extRD1 genes required for their secretion. $EspB_M$ secretion depends on Mh3879c, but is independent of Mh3866 and Mh3867, while CFP-10 shows the inverse pattern.

To demonstrate the importance of the ESX-1 machine in EspB secretion in another strain of *M. marinum,* we examined the 1218R strain and an isogenic mutant in which the *Mh3871* gene had been disrupted. The M strain, used for the previous experiments, is a human isolate, whereas 1218R was originally isolated from an infected fish. Wild-type 1218R secreted EspB, but the *Mh3871* mutant did not (Figure 9), confirming the importance of ESX-1 in the secretion of this protein by *M. marinum*. Complementation of the mutant with either the *M. marinum* or *M. tuberculosis* homolog

of *Mh3871* restored secretion of $EspB_M$ to this mutant, suggesting parallel functions for the genes in the two species.

To test directly whether ESX-1 was required for EspB secretion by *M. tuberculosis,* we examined culture filtrates from *M. tuberculosis* Erdman and the isogenic mutants *Rv3870::tn, Rv3871::tn,*and *ΔCFP-10* (Figure 2.2D). Secretion of $EspB_T$ by wild-type *M. tuberculosis* was abrogated in the *Rv3870* and *Rv3871* mutants, but not in the *ΔCFP-10* mutant. Thus, EspB is a secreted protein in both *M. marinum* and *M. tuberculosis,* and its secretion requires core ESX-1 components in both species of *Mycobacteria*. Importantly, EspB is the first ESX-1 substrate in *M. tuberculosis* whose secretion is not disrupted in the *ΔCFP-10* mutant.

MmΔRD1, $espB_M$::tn, and Mh3871::tn Are More Attenuated for Growth in Macrophages than the Other M. marinum extRD1 Mutants

Of the ten *M. marinum* extRD1 mutants we examined, *MmΔRD1, $espB_M$::tn,* and *Mh3871::tn* were disrupted for the secretion of all three substrates: ESAT-6, CFP-10, and $EspB_M$. In contrast, the *Mh3879::tn* mutant was disrupted only for the secretion of ESAT-6 and $EspB_M$, while the *Mh3866::tn* and *Mh3867::tn* mutants were disrupted only for ESAT-6 and CFP-10 secretion. To assess the importance of the multiple ESX-1 substrates for growth in macrophages, we infected murine bone marrow–derived macrophages (BMDMs) with wild-type *M. marinum,* with strains lacking one secreted effector, or with strains lacking secretion of all the known ESX-1 substrates. As shown in Figure 2.3, *MmΔRD1, $espB_M$::tn,* and *Mh3871::tn,* which fail to secrete all substrates, are

more attenuated for growth in macrophages than *Mh3866::tn,* which still secretes $EspB_M$, or *Mh3879c::tn,* which still secretes CFP-10. Therefore, we conclude that the various substrates of ESX-1 each contribute to virulence.

EspB Physically Interacts with the Rv3879c

To learn more about the involvement of ESX-1 in EspB secretion, we tested whether EspB would interact with other ESX-1 genes by bacterial two-hybrid analysis (Figure 2.4). An advantage of the bacterial two-hybrid system is that it can allow detection of interactions of membrane-bound proteins [32]. In this assay, potential protein–protein interactions are assessed by determining the ratio of colonies that grow on selective medium to the number grown on non-selective medium. For each of the bait plasmids, co-transformation with an empty target resulted in a ratio of colonies on selective to non-selective medium of less than 0.1%, as did co-transformation of the $EspB_T$ target with an empty bait. In contrast, the Rv3879c bait and $EspB_T$ target resulted in a ratio of 7.6%, an increase of more than 75-fold. An Rv3876 bait also showed interaction above background with $EspB_T$, but since the *M. marinum Mh3876::tn* mutant showed significant $EspB_M$ secretion (Figure 2.2C), any interaction between Rv3876 and $EspB_T$ is not likely to be required for EspB secretion and thus was not pursued.

To test for an analogous interaction between $EspB_M$ and Mh3879c and to confirm the potential interaction between $EspB_T$ and Rv3879c suggested by the two-hybrid assay, we performed in vitro pull-down assays. All of the proteins used were expressed in *Escherichia coli* as GST- or V5-epitope tagged fusions. Controls for nonspecific interactions included GST alone, as well as GST-syntaxin2, and GST-Shp1. As shown in

Figure 2.5A, GST-tagged $EspB_M$, but none of the GST controls, bound specifically to V5-tagged Mh3879c. In the reciprocal experiment, GST-tagged Mh3879c bound specifically to V5-$EspB_M$. Similarly, as shown in Figure 2.5B, GST-tagged $EspB_T$ bound specifically to V5-tagged Rv3879c, and GST-tagged Rv3879c bound specifically to V5-tagged $EspB_T$. These data demonstrate that recombinant $EspB_T$ and Rv3879c, as well as their *M. marinum* homologs, interact in vitro.

Since *Rv3871* mutants in both *M. tuberculosis* and *M. marinum* fail to secrete EspB, we used GST pulldowns to test whether Rv3871 interacts with either $EspB_T$ or Rv3879c. GST-tagged Rv3879c bound to V5-tagged Rv3871, whereas the GST controls and GST-$EspB_T$ did not bind to Rv3871. This suggests that Rv3879c may facilitate $EspB_T$ secretion through an interaction with Rv3871.

The Carboxyterminus of EspB Is Dispensable for Interaction with Mh3879c and for Secretion

To identify whether EspB, like CFP-10, requires its carboxyterminus for secretion, we constructed a series of $EspB_M$ deletion mutants with N-terminal V5 tags and expressed them in the *$espB_M$::tn* mutant strain using the *$espB_M$* promoter. As shown in Figure 2.6A, V5-tagged full-length $EspB_M$ was secreted. This N-terminally tagged protein, like native $EspB_M$, underwent C-terminal truncation either during or after secretion. $EspB_M$ deletion mutant constructs Δ(2-31), Δ(264-271), and Δ(400-454) were stably expressed in *M. marinum,* but only $EspB_M$ Δ(400-454) accumulated in the culture filtrate. The secreted $EspB_M$ Δ(400-454) had a higher apparent molecular weight than the

secreted full-length $EspB_M$, presumably because deletion of the C-terminal 55 amino acids inhibits some of the carboxyterminal proteolytic processing. This result demonstrates that the C-terminus of $EspB_M$ is dispensable for secretion, but N-terminal and internal amino acids are required. Next, we tested how these $EspB_M$ mutants interacted with Mh3879c. Lysates of *E. coli* expressing V5-tagged $EspB_M$ mutants were incubated with GST-Mh3879c. While full-length $EspB_M$ and $EspB_M$ Δ(400-454) bound to GST-Mh3879c, the stably expressed but non-secreted $EspB_M$ Δ(2-31) and $EspB_M$ Δ(264-271) constructs did not bind to GST-Mh3879c (Figure 2.6B). These data support a model in which EspB interacts with Rv3879c, which in turn interacts with Rv3871, to facilitate the secretion of EspB.

Mh3879c Is Not Secreted

Because CFP-10 and ESAT-6 are secreted as a heterodimer, we assessed whether Mh3879c and EspB might be secreted similarly. The fusion constructs V5-Mh3879c, Mh3879c-His6x, and V5-Mh3879c-His6x were expressed from the endogenous *Mh3879c* promoter on non-integrating plasmids in both wild-type *M. marinum* and in the *Mh3879c::tn* mutant. Introduction of V5-Mh3879c fully complemented the $EspB_M$ secretion defect of the *Mh3879c::tn* mutant, but Mh3879c-His6x and V5-Mh3879c-His6x failed to complement the secretion defect (Figure 2.10A). In wild-type *M. marinum,* V5-Mh3879c and V5-$EspB_M$ were expressed at nearly identical levels in the cell lysate, but only V5-$EspB_M$ was detected in the culture filtrate (Figure 2.10A). To determine whether failure of secretion reflected inefficient competition of V5-tagged protein with native protein, the V5-$EspB_M$ secretion was also analyzed in the *Mh3879::tn* mutant. In this

strain as well, V5-Mh3879c was found only in the cell lysate. Thus, V5-tagged Mh3879c, while fully competent to mediate EspB secretion, was not itself secreted, suggesting that Mh3879c and EspB are not secreted as a heterodimer. The C-terminally His6x-tagged Mh3879c, which did not restore EspB secretion to the *Mh3879::tn* mutant, also was detected only in the cell lysate. Since Mh3879c-His6x failed to complement the $EspB_M$ secretion defect of the *Mh3879c::tn* mutant, we hypothesized that the carboxyterminus of Rv3879c might be required for interaction with EspB. To test this hypothesis, lysates of *E. coli* expressing V5-tagged Rv3879 mutants were incubated with GST alone, GST-Rv3871, or GST-$EspB_T$. While full-length Rv3879 and Rv3879 Δ(1-166) bound to GST-$EspB_T$, Rv3879 Δ(564-729) failed to bind to GST-$EspB_T$ (Figure 2.10B). None of the constructs bound to GST alone, and all three constructs bound to GST-Rv3871. Thus, the carboxyterminal 166 amino acids of Rv3879 are required for EspB secretion, but not for interaction with the ESX-1 machine.

DISCUSSION

In this study, we identified EspB as a novel substrate of the ESX-1 secretion system and demonstrated a requirement for the *Mh3879c* and *Mh3871* genes in the secretion of $EspB_M$. Further, we showed protein complex formation between $EspB_M$ and Mh3879c, as well as identical behavior of their *M. tuberculosis* homologs. Two mutants of $EspB_M$ that were stable after synthesis but failed to bind Mh3879c were not secreted, while a large carboxyl-terminal deletion did not interfere with either Mh3879c binding or secretion. Additionally, the carboxy-terminus of Rv3879c/Mh3879c is required for

interaction with and secretion of EspB. These results suggest that the EspB/Mh3879c protein complex is required for $EspB_M$ secretion. While complex formation between ESAT-6 and CFP-10 is required for their secretion as a heterodimer by *M. tuberculosis,* Mh3879c appears not to be secreted. Our data, though, do not exclude the possibility that the aminoterminus of Mh3879c is quantitatively removed during or immediately after secretion, since we do not have and could not probe with antibodies to the native protein. We hypothesize that Mh3879c acts as a cytosolic chaperone to deliver $EspB_M$ to the secretion machine. We showed that Rv3879c interacts directly with Rv3871 and that Rv3871, in addition to being required for the secretion of ESAT-6/CFP-10, is required for the secretion of EspB. Although our work does not reveal precisely how EspB is delivered to the ESX-1 machine, our data demonstrate that Rv3879c can interact with Rv3871 as well as with $EspB_T$, suggesting that EspB may be targeted to Rv3871 in this way. We propose that the mechanisms of EspB and CFP-10 secretion intersect at binding to Rv3871 (Figure 2.7).

We also found that disruption of *Mh3868* leads to loss of accumulation of EspB in the bacterial cytosol. We previously observed that disruption of *Mh3868* prevents bacterial accumulation of ESAT-6 and CFP-10 [3]. *Mh3868* and its *M. tuberculosis* homolog *Rv3868* are predicted to be AAA ATPases, which suggests that they may function as chaperones for the translocation of ESX-1 substrates, but little is known about this key protein. We have found that *CFP-10* and $espB_M$ mRNAs are expressed in the *Mh3868::tn* mutants (B. McLaughlin and E. Brown, unpublished data), suggesting that the *Mh3868* gene product affects either the translation or stability of the ESX-1

substrates. Characterizing the function of Mh3868 will certainly be important to better understand ESX-1–mediated secretion.

Like ESAT-6 and CFP-10, EspA is secreted by the ESX-1 machine. Whether any of the *M. marinum* genes with sequence similarity to *espA* are functional orthologs has not yet been determined. Loss of either EspA or EspB inhibits secretion of ESAT-6 and CFP-10, but the reason for their requirement is unknown. It may be that as substrates reach the final common pathway for secretion, they interact in a manner that leads to cooperative secretion. Clearly, though, the secretion of EspB is quite distinct from that of EspA. While EspA requires CFP-10 for its secretion, EspB secretion is independent of CFP-10. EspB secretion is not disrupted in the *M. marinum* mutants *Mh3866::tn* and *Mh3867::tn,* neither of which secrete CFP-10, nor is EspB secretion disrupted in the *M. tuberculosis ΔCFP-10* mutant. These data are consistent with the model that EspB, unlike either ESAT-6 or EspA, is targeted to the ESX-1 machine independently of CFP-10.

These studies beg the question of whether it is possible to determine which ESX-1 substrates are most important for virulence. This has been a difficult task because of the apparent codependence of the various substrates on each other for secretion. However, our results allowed a somewhat different approach. We used a set of extRD1 mutants in which some *(Mh3866::tn* and *Mh3867::tn)* failed to secrete CFP-10, but did secrete EspB; while another mutant *(Mh3879::tn)* secreted CFP-10 but failed to secrete EspB; while mutants that disrupted the core secretion machinery *(MmΔRD1* and *Mh3871::tn)* and $espB_M$*::tn* itself failed to secrete all substrates. We found that mutants lacking secretion of both substrates had a more marked growth defect in macrophages than the mutants lacking secretion of only one substrate. This suggests that the different substrates

make distinct, and potentially additive, contributions to virulence. Although we cannot say that the defects in intracellular growth of the various mutants are caused by the substrates we have identified, our work does support the hypothesis that ESX-1 secretes more than one substrate that contributes to the virulence of *Mycobacteria* and that different substrates may have independent contributions to bacterial pathogenesis.

In summary, this work has identified a novel substrate for ESX-1–dependent secretion and has demonstrated interactions of this substrate with a protein encoded within RD1, expanding our understanding of how genes within this locus contribute to this novel secretion pathway. Furthermore, we have demonstrated that secretion of distinct ESX-1 substrates follows variable pathways to interaction with the core secretion machinery, and that the different substrates may contribute independently to intracellular survival and growth of the bacteria. These data extend the understanding of a major virulence mechanism of *Mycobacteria*.

MATERIALS AND METHODS

Bacterial strains and plasmids.

All strains and plasmids used in this study are listed in Table 2.1. *M. marinum* strains were grown as previously described [2]. The designations assigned by the Sanger Institute in the annotation of the *M. marinum* genome and the corresponding DNA sequences are available at http://www.sanger.ac.uk/Projects/M_marinum/.

TABLE 2.1

Strain/Plasmid	Name	Relevant gene product	Genotype using "Marinum homolog" of H37Rv nomenclature	Genotype using Sanger Institute nomenclature
M. marinum M	WT			
	ΔRD1		*Δ(Mh3871 - Mh3879)*	*Δ(MM5446 - MM5455)*
	M1		*Mh3866::tn*	*MM5441::tn*
	M2		*Mh3867::tn*	*MM5442::tn*
	M3		*Mh3868::tn-1*	*MM5443::tn*
	M4		*Mh3868::tn-2*	*MM5443::tn*
	M5		*Mh3876::tn*	*MM5451::tn*
	M6		*Mh3878::tn*	*MM5454::tn*
	M7		*Mh3879::tn*	*MM5455::tn*
	M8 (*$espB_M$::tn*)		*Mh3881::tn*	*MM5457::tn*
	M9		*Mh3871::tn*	*MM5446::tn*
M. marinum 1218	WT			
	MRS1459		*Mh3871::tn*	*MM5446:::tn*
	MRS1459 +p*Rv3871*		*Mh3871::tn + Rv3871*	*MM5446:::tn + Rv3871*
	MRS1459 +p*Mh3871*		*Mh3871::tn + Mh3871*	*MM5446:::tn + Mm5446*
M. marinum M	WT *attB::hyg^r*			
	$espB_M$::tn; attB::hyg^r			
	$espB_M$::tn; attB:: $espB_M$ hyg^r			
	$espB_M$::tn; attB:: $espB_M$ –Mh3880c hyg^r			
	$espB_M$::tn; attB:: Mh3883c-Mh3880c hyg^r			
M. tuberculosis Erdman	WT			
	Rv3870::tn			
	Rv3871::tn			
	ΔCFP-10			
E. coli protein expression plasmids	pBM856	GST-Mh3879c		
	pBM553	GST-Rv3879c		
	pBM550	GST-$EspB_M$		
	pBM551	GST-$EspB_T$		
	pBM843	V5-Mh3879c		
	pBM540	V5-Rv3879c		
	pBM504	V5-$EspB_M$		
	pBM810	V5- $EspB_T$		
	pBM841	V5- Rv3871		
	p*GST-Shp1*	GST-Shp1		
	p*GST-syntaxin2*	GST-syntaxin2		
	pBM589	V5- $EspB_M$ Δ(1-30)		
	pBM398e	V5- $EspB_M$ Δ(264-271)		
	pBM400ve	V5- $EspB_M$ Δ(400-454)		
	pBM1010	V5-Rv3879c Δ(1-166)		
	pBM1013	V5-Rv3879c Δ(564-729)		
Mycobacterium plasmids	pBM332	V5- $EspB_M$		
	pBM336	V5- $EspB_M$-His6x		
	pBM367	V5- $EspB_M$ Δ(1-30)		
	pBM398	V5- $EspB_M$ Δ(264-271)		
	pBM400v	V5- $EspB_M$ Δ(400-454)		
	pBM869	V5-Mh3879c		
	pBM870	Mh3879c-His6x		
	pBM871	V5-Mh3879c-His6x		
	p*Mh3879c*	Mh3879c		
	pMV306.hyg`	empty integrating plasmid		
	pL.yg206	empty non-integrating plasmid		
	p(*$espB_M$)*	$EspB_M$		
	p(*$espB_M$-Mh3880c*)	$EspB_M$ and Mh3880c		
	p(*Mh3883c-Mh3880c*)	Mh3883c thru Mh3880c		
	p(*$espB_T$)*	$EspB_T$		
	p(*$espB_T$-Rv3880c*)	$EspB_T$ and Rv3880c		

The transposon insertion in the mutant *espB$_M$::tn* lies between the 175th and 176th base pairs of the *espB$_M$* gene, and the kanamycin gene within the transposon is transcribed opposite to the direction of transcription of the *espB$_M$* gene. The strains *M. marinum* M *attB::hyg^r* and *espB$_M$::tn attB::hyg^r* were constructed by transforming the strains *M. marinum* M WT and *espB$_M$::tn* with the plasmid pMV306.hyg. The strains *espB$_M$::tn attB:: espB$_M$ hyg^r* and *espB$_M$::tn attB:: espB$_M$-Mh3880c hyg^r* were constructed by ligating 250 bp upstream of *espB$_M$* along with *espB$_M$* or *espB$_M$-Mh3880c* into pMV306.hyg and then transforming the resulting plasmids, pBM264 and pBM262, into *espB$_M$::tn.* The strain *espB$_M$::tn attB:: Mh3883c-Mh3880c hyg^r* was constructed by ligating 345 bp upstream of *Mh3883c* along with *Mh3883c-Mh3880c* into pMV306.hyg and then transforming the resulting plasmid, pBM263, into *espB$_M$::tn.* To construct the plasmids pBM841, pBM540, and pBM810, the genes *Rv3871, Rv3879c,* and *Rv3881c* were PCR amplified from the cosmid RD1-2F9 [33] and ligated into pBM510, a derivative of pET22b+ in which the N-terminal His tag was replaced with the V5 epitope tag. To construct the plasmids pBM843 and pBM504, the genes *Mh3879c* and *Mh3881c* were PCR amplified from *M. marinum* M genomic DNA and ligated into pBM510. To construct the plasmids pBM332 and pBM336, a series of fragments were ligated into pLYG206 to achieve the following sequence ligated into the NotI and XbaI sites: 250 bp upstream of *espB$_M$*, then the V5 epitope, then the *espB$_M$* gene, and finally, in the case of the pBM336 plasmid, the His6x epitope. The plasmids pBM869, pBM870, and pBM871 were made in a manner synonymous to that of pBM332 and pBM336, where the *Mh3879* promoter and gene were used. The plasmids pBM367 and pBM400v were constructed by PCR from pBM332 and re-ligation of the truncated gene fragments back into pBM332,

while pBM398 was generated by quick-change mutagenesis (Stratagene, http://www.stratagene.com/). For pBM589, pBM398e, and pBM400ve, the *espB$_M$* gene fragments in the plasmids pBM367, pBM398, and pBM400v were cut by restriction digest and ligated into pBM504. For pBM856, pBM550, pBM553, and pBM551, the genes *Mh3879c, espB$_M$*, *Rv3879c,* and *espB$_T$* were cut by restriction digest from the plasmids pBM843, pBM504, pBM540, and pBM810 and ligated into the GST expression vector pGex-KG. To construct the plasmid p*Mh3879,* 250 bp upstream of the gene *Mh3879c* together with *Mh3879c* was PCR amplified from *M. marinum* genomic DNA and inserted into pLYG206. The plasmids pBM1010 and pBM1013 were constructed by restriction digests of pBM540 to excise portions of *Rv3879c,* and ligation of 5' phosphorylated hybridized oligos that restored the frame and created the *Rv3879* deletions Δ(1-166) and Δ(564-729). To construct the plasmid p(*Mh3883c-Mh3880c*), the 345 bp upstream of *Mh3883c* along with *Mh3883c-Mh3880c* was cut by restriction digest from the plasmid pBM263 and inserted into pLYG206. To construct the plasmid p(*espB$_T$-Rv3880c*), 250 bp upstream of the operon *Rv3881c-Rv3880c* together with the operon were inserted into pLYG206.

Protein preparation and analysis.

M. marinum strains were grown in 40-mL cultures to 0.5 OD_{600} in 7H9 medium. The cultures were centrifuged and washed three times with 15 mL of PBS before re-suspension in 40 mL of Sauton's medium, supplemented with 0.015% Tween-80. When strains containing non-integrating plasmids for complementation were grown in Sauton's medium, the Sauton's medium was supplemented with Zeocin (5 μg/ml; Invitrogen,

http://www.invitrogen.com/). After growth for 36 h at 30 °C, 105 rpm, in Sauton's medium, the cells were harvested by centrifugation. Supernatants were filtered through a 0.22-μm-pore-size filter with a glass pre-filter and concentrated with an Amicon Ultra-15 (5,000-molecular-weight cutoff; Millipore, http://www.millipore.com/) to 200 μL, which was saved as the culture filtrate (CF) fraction.

Pelleted cells were washed and resuspended in 1.5 mL of PBS with a protease inhibitor cocktail and 1 mM PMSF. Pellets were lysed using glass beads and the mini-bead beater (BioSpec Products, http://www.biospec.com/) with three 40-s pulses at maximum speed and incubations on ice in between each pulse, and then centrifuged at 3,000*g* for 2 min at 4 °C to remove unbroken cells. The resulting supernatant was collected and saved as the cell lysate (CL) fraction. *M. tuberculosis* (Erdman) culture filtrate and cell lysate fractions were prepared as previously described [15]. Total protein concentrations were determined by a Bradford assay.

Western immunoblot assay.

Pellet and culture filtrate fractions were separated by SDS/PAGE on 10%–20% gradient polyacrylamide gels for detection of CFP-10; 7.5% polyacrylamide gels for detection of EspB, GroEL, or V5-tagged Mh3879; and 12.5% polyacrylamide gels for detection of FAP. Proteins were visualized by immunoblotting by using antibodies against EspB at a concentration of 1:500 (mouse polyclonal to the 100 amino acid fragment of Rv3881c [234-333aa], Arizona State University CIM Antibody Core), and the blot was developed using ECL reagent West Dura (Pierce, http://www.piercenet.com/). Anti-CFP-10 (rabbit polyclonal; Colorado State University,

http://www.cvmbs.colostate.edu/microbiology/tb/top.htm) was used at a concentration of 1:50000, blots of the culture filtrate fraction were developed using West Pico (Pierce), and blots of the cell lysates were developed using West Dura (Pierce). Anti-GroEL (rabbit polyclonal, SPA-875 / SPS-875; Stressgen, http://assaydesigns.com/) was used at a concentration of 1:10000, and blots were developed using West Pico (Pierce). Anti-FAP [31] for *M. marinum* samples was a rabbit polyclonal, used at a concentration of 1:10000 and developed using West Pico (Pierce). Anti-FAP for *M. tuberculosis* Erdman samples was CS-93 (Colorado State University), mouse monoclonal, used at a concentration of 1:20, and developed using West Pico (Pierce). His6x epitope was detected with a mouse monoclonal (Novagen, http://www.emdbiosciences.com/html/NVG/home.html) at a concentration of 1:1500, and V5 epitope was detected with a mouse monoclonal (R960-25, Invitrogen), at a concentration of 1:5000, and these blots were developed using West Pico (Pierce).

Bacterial two-hybrid system assay.

The genes *Rv3614c, Rv3615c,* and *Rv3616c,* which were PCR amplified from genomic DNA, and each of the genes in the region *Rv3864* through *Rv3883,* which were PCR amplified from the cosmid RD1-2F9 [33], were cloned into the "bait" vector pBT (BacterioMatch II; Stratagene) in frame with cI. *Rv3881c* was cloned into the "target" vector pTRG in frame with the N-terminal subunit of RNA polymerase according to the manufacturer's instructions. The constructs were co-transformed into the *E. coli* two-hybrid system reporter validation strain XL1-Blue MRF *hisB lac* [*F′ laqIq HIS3 aadA Kanr*] and plated onto both the selective (+5 mM 3-AT) and the non-selective screening

medium according to the manufacturer's instructions. The non-selective screening plate is histidine-dropout M9 agar supplemented with 0.5 mM IPTG, 12.5 μg/ml tetracycline, and 25 μg/ml chloramphenicol. The selective screening plate is histidine-dropout M9 agar supplemented with 0.5 mM IPTG, 12.5 μg/ml tetracycline, 25 μg/ml chloramphenicol, and 5 mM 3-amino-1,2,4-triazole.

GST pull-down.

GST fusion proteins, GST alone, and V5-tagged proteins were expressed in the BL21-RP codon plus *E. coli* strain (Stratagene) by addition of 0.2 mM IPTG (3 h at 30 °C). Bacterial cultures were lysed in buffer containing 50 mM HEPES (pH 7.4), 300 mM NaCl, 1% Triton X-100, 0.5 mM EDTA, and protease inhibitor cocktail (Roche). Solubilized proteins were separated by centrifugation at 20,000*g* for 10 min. The GST fusion proteins and GST alone were bound to glutathione agarose beads (Amersham Biosciences, http://www.gelifesciences.com) by incubation overnight at 4 °C. The beads were then extensively washed with PBS containing 0.1% Triton X-100. Bacterial lysates containing solubilized V5-tagged proteins, in lysis buffer, were incubated with the GST protein–loaded agarose beads overnight at 4 °C. After washing three times with PBS containing 1% Triton X-100, bead-bound protein was eluted in Laemmli buffer, seperated by SDS-PAGE, and analyzed by western blot.

Macrophage infections.

All macrophages used in these experiments were derived from bone marrow cells of C57BL/6 mice that were differentiated for 6 d in DMEM supplemented with 10%

CMG supernatant [34] and 10% fetal bovine serum (FBS; HyClone, http://www.hyclone.com/). Immediately prior to infection, macrophage monolayers were washed once with FBS-free DMEM. *M. marinum* strains were each grown to OD_{600} of 1.0, prepared for infection, and incubated with macrophages as previously described [3]. All infections were performed at a multiplicity of infection of 1, for 2 h at 32 °C, in a 5% CO2, humidified environment, in 24-well plates. The time at which *M. marinum* was added to the well was designated time zero. At the end of the 2 h incubation period ($T = 2$ h), infected monolayers were washed twice with DMEM and further incubated in DMEM containing 0.1% FBS and 200 µg of amikacin/ml for 2 h to kill extracellular bacteria. At the end of the antibiotic treatment, monolayers were washed twice with DMEM and incubated in DMEM containing 0.1% FBS at 32 °C and 5% CO2. Intracellular bacteria were enumerated by lysing macrophage monolayers and diluting and plating bacteria exactly as described [3]. Statistical analysis was performed by calculating the one-way analysis of variance (ANOVA) with GraphPad Prism 4.0 (GraphPad Software, http://www.graphpad.com).

Figure 2.1 Either $EspB_T$ or $EspB_M$ Is Sufficient to Restore Growth to the *$espB_M$::tn* Mutant in Mouse BMDMs

BMDMs were infected at a multiplicity of infection of 1. Colony-forming units (cfu) were determined by lysing infected monolayers and plating lysates at indicated time points. Data were combined for three experiments, and with growth indicated as fold increase compared to the initial level of infection for each strain.

(A) Wild-type (Wt) and *$espB_M$::tn* each contain the empty non-integrating plasmid pLYG206; p*$espB_M$* and p*$espB_T$* are plasmids for expression of $EspB_M$ and $EspB_T$, respectively; and p(*$espB_M$-Mh3880c*) and p(*$espB_T$-Rv3880c*) encode the second gene of the operon as well as *espB* for each species.

(B) Wild-type (Wt) and *$espB_M$::tn* each were transformed with the empty integrating plasmid and the indicated genes were integrated into the *attB* of the *$espB_M$::tn* mutant. *$espB_M$::tn* + p(*Mh3883c-Mh3880c*) expresses the locus *Mh3883-Mh3880c* on a non-integrating plasmid. Strains differed significantly by one-way ANOVA in (A) after 24 h, 48 h, 72 h, and 96 h and in (B) after 16 h, 43 h, 75 h, and 92 h ($p < 0.001$ for each).

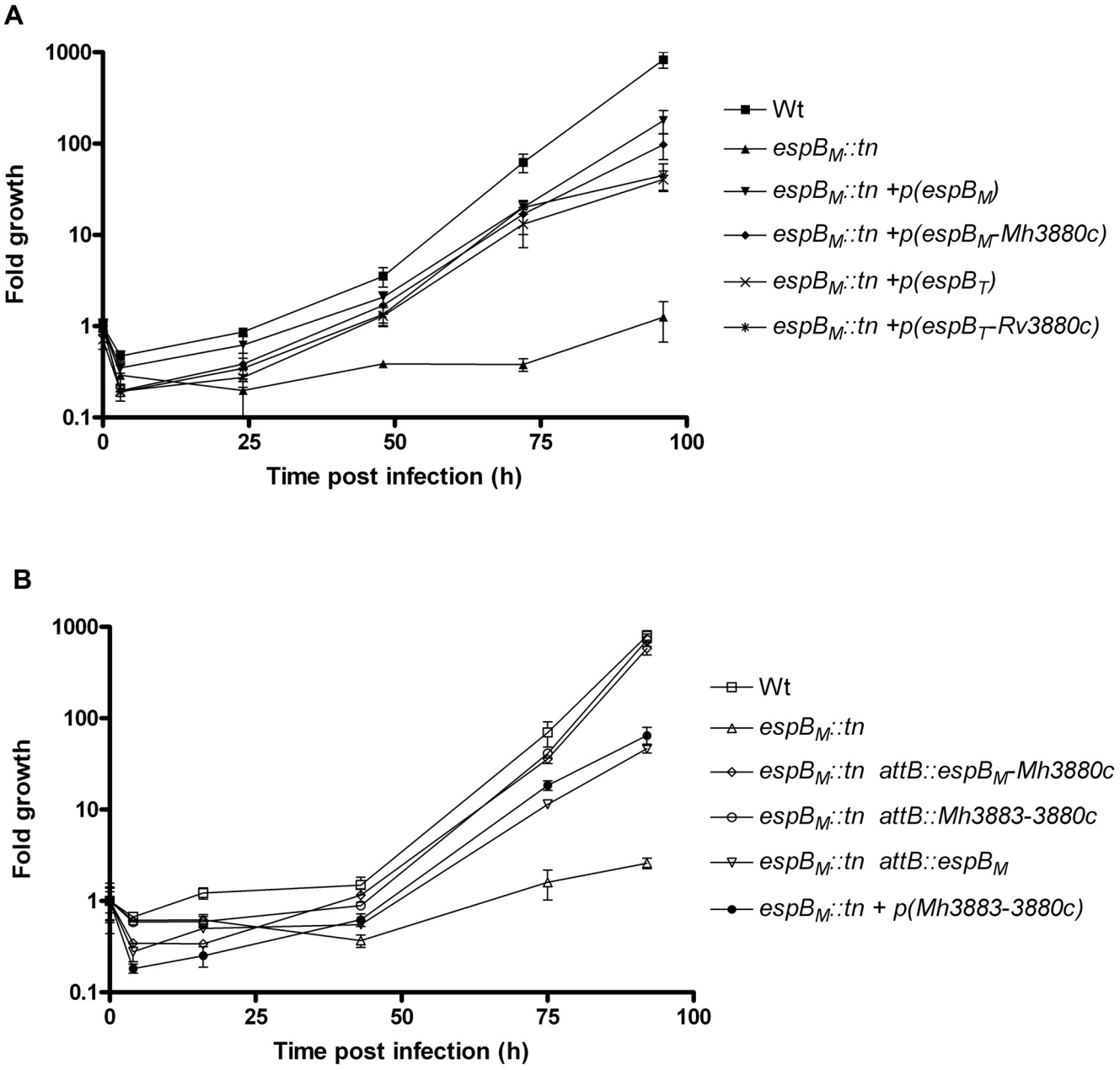

A
1000
100
10
1
0.1
Fold growth
0
25
50
75
100
Time post infection (h)
Wt
espB_M::tn
espB_M::tn +p(espB_M)
espB_M::tn +p(espB_M-Mh3880c)
espB_M::tn +p(espB_T)
espB_M::tn +p(espB_T-Rv3880c)
B
1000
100
10
1
0.1
Fold growth
0
25
50
75
100
Time post infection (h)
Wt
espB_M::tn
espB_M::tn attB::espB_M-Mh3880c
espB_M::tn attB::Mh3883-3880c
espB_M::tn attB::espB_M
espB_M::tn + p(Mh3883-3880c)

Figure 2.2 Requirements for EspB Secretion in *M. marinum* and *M. tuberculosis*

Cell lysates (CL) and culture filtrates (CF) were prepared from the indicated strains as described in Materials and Methods. Proteins were separated by SDS-PAGE, and the indicated proteins were detected by western blot as described in Materials and Methods.

(A) 60 μg of total CL and 30 μg of total CF of *M. tuberculosis* Erdman and each of the *M. marinum* strains were loaded in each well.

(B) 60 μg of total CL and 30 μg of total CF were loaded in each well.

(C) Cultures of each strain were grown in 7H9 to an OD of 0.5 and then inoculated into Sauton's medium at an OD of 0.5 and grown for 36 h. Therefore, the samples of each strain are normalized by OD readings. Of the total CL and CF fractions collected for each strain from one experiment, 3% of the CL was loaded in each lane and 15% of the total CF was loaded into each lane. The results shown are representative of the results obtained in four replications of this experiment.

(D) 30 μg of total CL and 30 μg of total CF of each *M. tuberculosis* Erdman strain were loaded in each well.

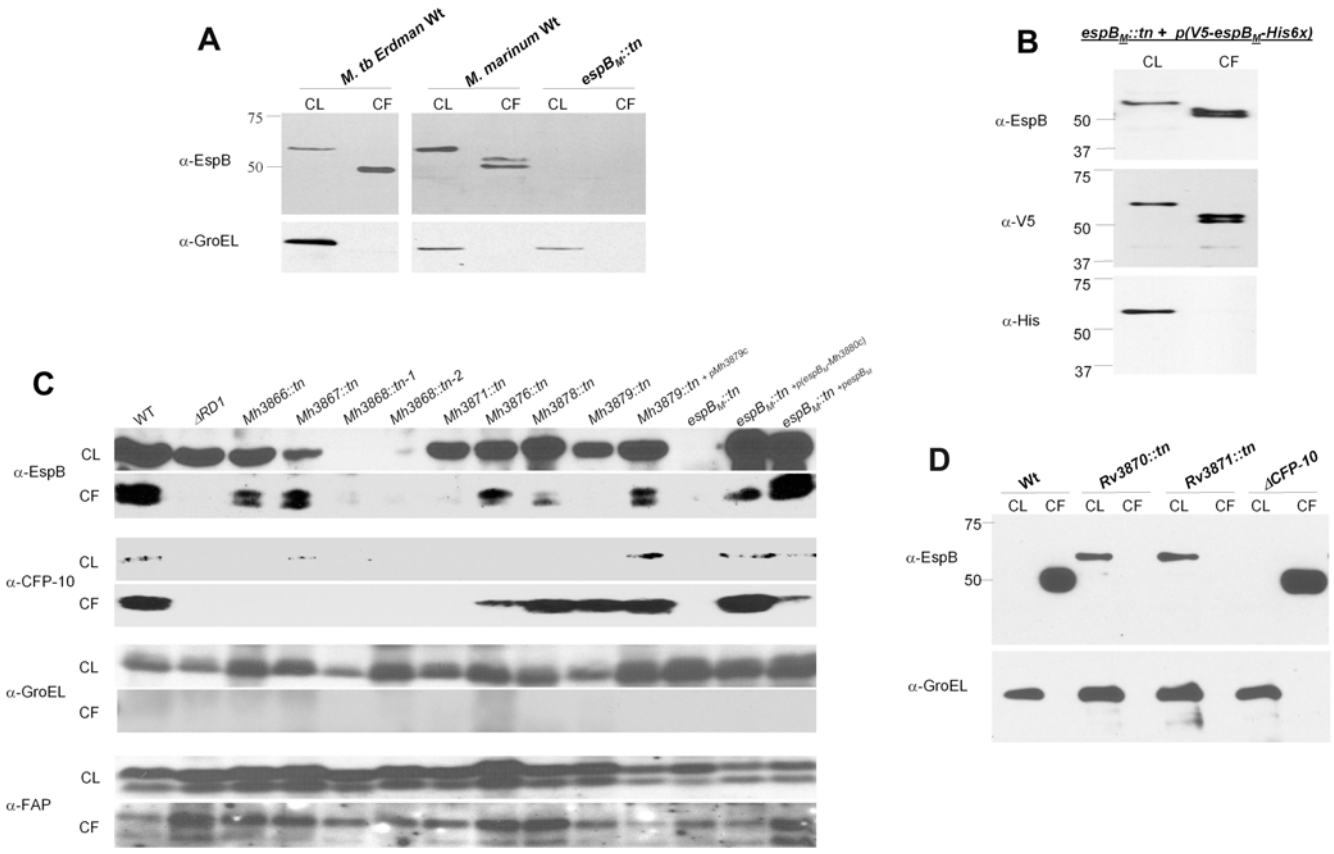

A
M. tb Erdman Wt
M. marinum Wt
espBM::tn
CL
CF
75
50
α-EspB
α-GroEL
B
espBM::tn + p(V5-espBM-His6x)
α-EspB
α-V5
α-His
50
37
C
WT
ΔRD1
Mh3866::tn
Mh3867::tn
Mh3868::tn-1
Mh3868::tn-2
Mh3871::tn
Mh3876::tn
Mh3878::tn
Mh3879::tn
Mh3879::tn + pMh3879c
espBM::tn
α-CFP-10
α-FAP
D
Wt
Rv3870::tn
Rv3871::tn
ΔCFP-10

Figure 2.3 Growth of *M. marinum* Secretion Mutants in BMDMs

BMDMs were infected with *M. marinum* strains as described in Materials and Methods at a multiplicity of infection of 1, and growth of bacteria was monitored over time as in Figure 1. Data are summarized from three independent experiments. Strains differed significantly by one-way ANOVA after 24 h, 48 h, 72 h, 96 h, and 120 h ($p < 0.001$ for each).

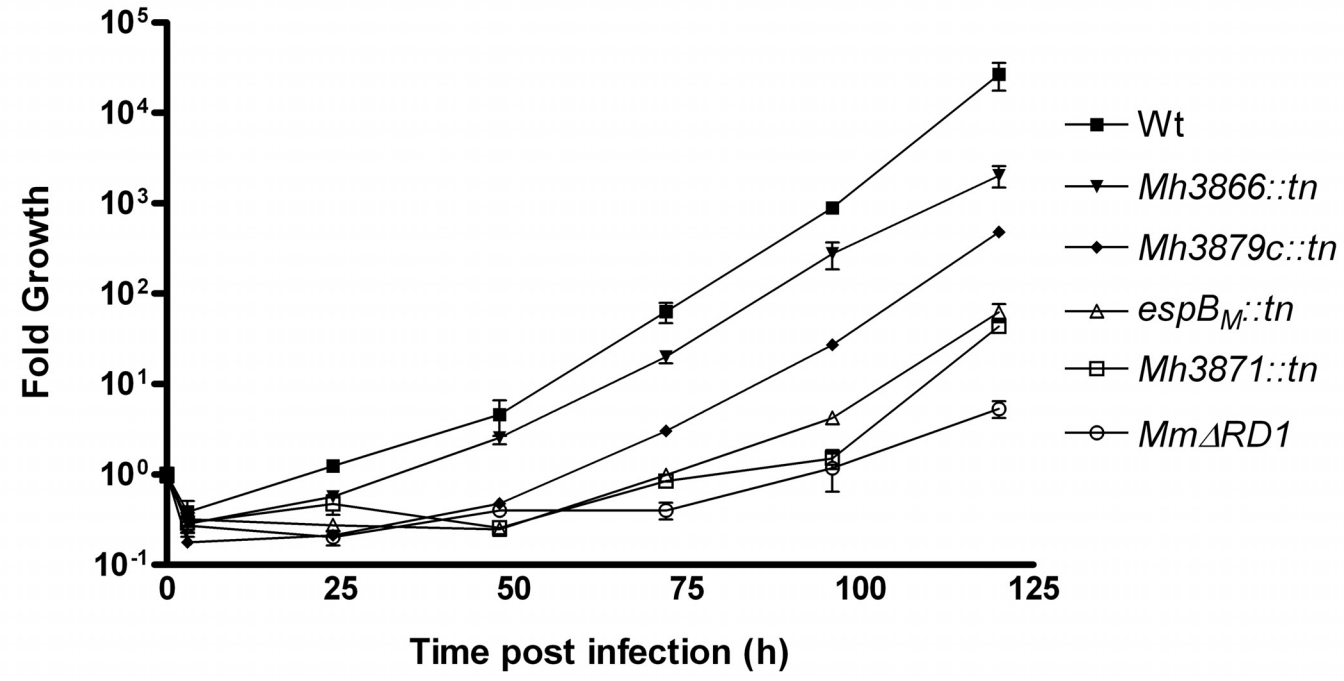

Fold Growth
10^5
10^4
10^3
10^2
10^1
10^0
10^-1
0
25
50
75
100
125
Time post infection (h)
Wt
Mh3866::tn
Mh3879c::tn
espB_M::tn
Mh3871::tn
MmΔRD1

Figure 2.4 Bacterial Two-Hybrid Analysis of Interaction of $EspB_T$ with Proteins of extRD1

The target plasmid containing $EspB_T$ fused to the RNA polymerase alpha subunit was co-transformed with each of the bait plasmids containing the indicated extRD1 proteins fused to the lambda repressor into the reporter validation strain. Shown is the ratio of growth of the co-transformants on selective (+5 mM 3AT) versus non-selective plates. The experiment depicted is representative of three independent determinations.

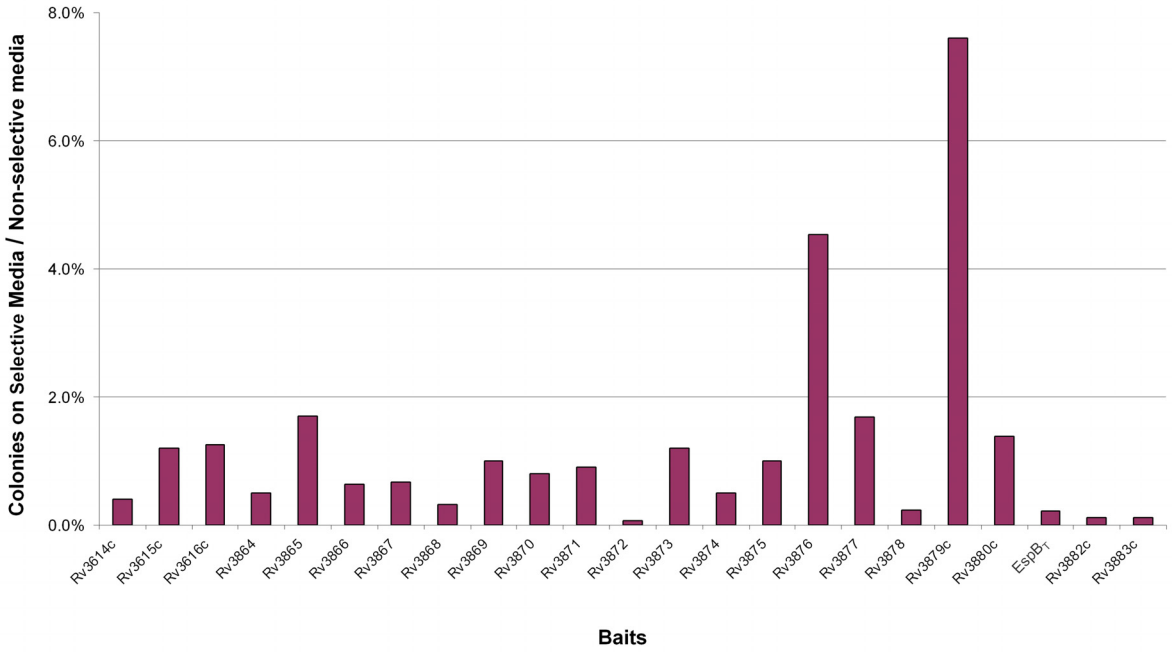
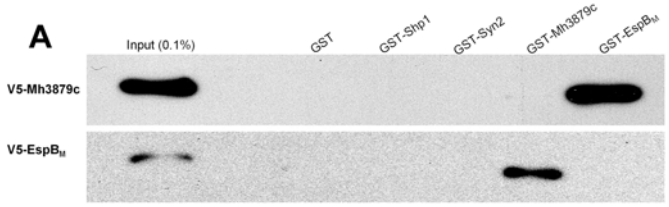

Colonies on Selective Media / Non-selective media
8.0%
6.0%
4.0%
2.0%
0.0%
Rv3614c
Rv3615c
Rv3616c
Rv3864
Rv3865
Rv3866
Rv3867
Rv3868
Rv3869
Rv3870
Rv3871
Rv3872
Rv3873
Rv3874
Rv3875
Rv3876
Rv3877
Rv3878
Rv3879c
Rv3880c
EspB$_T$
Rv3882c
Rv3883c
Baits

Figure 2.5 GST Pulldown Analysis of EspB and Rv3879 Interactions

(A) Agarose beads with immobilized GST, GST-SHP1, GST-syntaxin2, GST-Mh3879c, and GST-$EspB_M$ were incubated with lysates of *E. coli* expressing V5-Mh3879c and V5-$EspB_M$.

(B) Agarose beads with immobilized GST, GST-SHP1, GST-syntaxin2, GST-Rv3879c, or GST-$EspB_T$ were incubated with lysates of *E. coli* expressing V5-Rv3871, V5-Rv3879c, and V5-$EspB_T$. Proteins from cell lysates retained on the beads after washing were separated by SDS-PAGE and detected by western blotting with an antibody against V5. To the right of each set of pull-downs, 0.1% of the input *E. coli* lysate was analyzed. $EspB_M$ physically interacts with Mh3879c, $EspB_T$ physically interacts with Rv3879c, and Rv3879c also interacts with Rv3871.


A
Input (0.1%)
GST
GST-Shp1
GST-Syn2
GST-Mh3879c
GST-EspB$_M$
V5-Mh3879c
V5-EspB$_M$

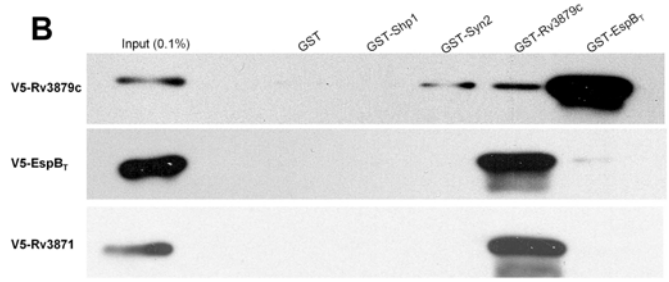

B
Input (0.1%)
GST
GST-Shp1
GST-Syn2
GST-Rv3879c
GST-EspB$_T$
V5-Rv3879c
V5-EspB$_T$
V5-Rv3871

Figure 2.6 Deletion Analysis of $EspB_M$ Secretion and Interaction with Mh3879c

(A) The *M. marinum* $espB_M$*::tn* mutant was transformed with a non-integrating plasmid expressing N-terminally V5-tagged $EspB_M$ full length, $EspB_M$ Δ(2-31), $EspB_M$ Δ(264-271), or $EspB_M$ Δ(400-454). Cell lysates (CL) and culture filtrates (CF) were prepared from the indicated strains as described in Materials and Methods. The samples of each strain are normalized by OD readings. Of the total CL and CF fractions collected for each strain from one experiment, 3% of the CL was loaded in each lane and 15% of the total CF was loaded into each lane, separated by SDS-PAGE and detected by western blotting with an antibody against V5.

(B) Agarose beads with immobilized GST-Mh3879c were incubated with *E. coli* lysates expressing V5-tagged $EspB_M$ full length, $EspB_M$ Δ(2-31), $EspB_M$ ⊗(264-271), or $EspB_M$ ⊗(400-454). The input lysate (0.1%) and the material from the cell lysates that bound to the beads was run on SDS-PAGE and detected by western blotting with an antibody against V5.

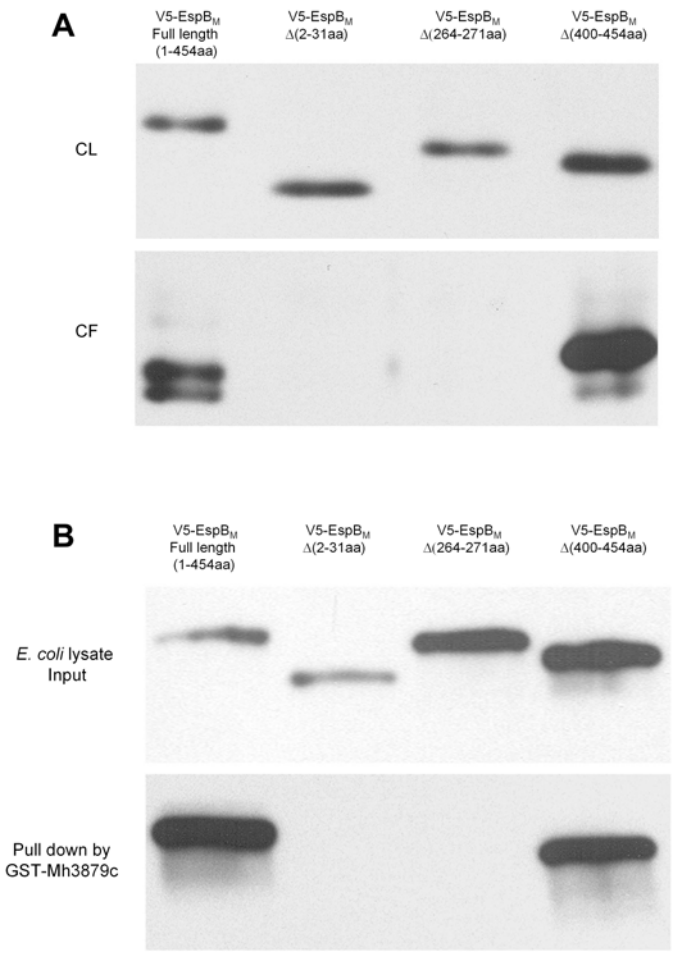

A
V5-EspB$_M$ Full length (1-454aa)
V5-EspB$_M$ Δ(2-31aa)
V5-EspB$_M$ Δ(264-271aa)
V5-EspB$_M$ Δ(400-454aa)
CL
CF
B
V5-EspB$_M$ Full length (1-454aa)
V5-EspB$_M$ Δ(2-31aa)
V5-EspB$_M$ Δ(264-271aa)
V5-EspB$_M$ Δ(400-454aa)
E. coli lysate Input
Pull down by GST-Mh3879c

Figure 2.7 Model for EspB Secretion

Depicted are the core ESX-1 components Rv3870, Rv3871, and Rv3877, as well as the ESAT-6/CFP-10 and EspB/Rv3879c complexes. Both cytosolic complexes require interaction with Rv3871 for substrate secretion.

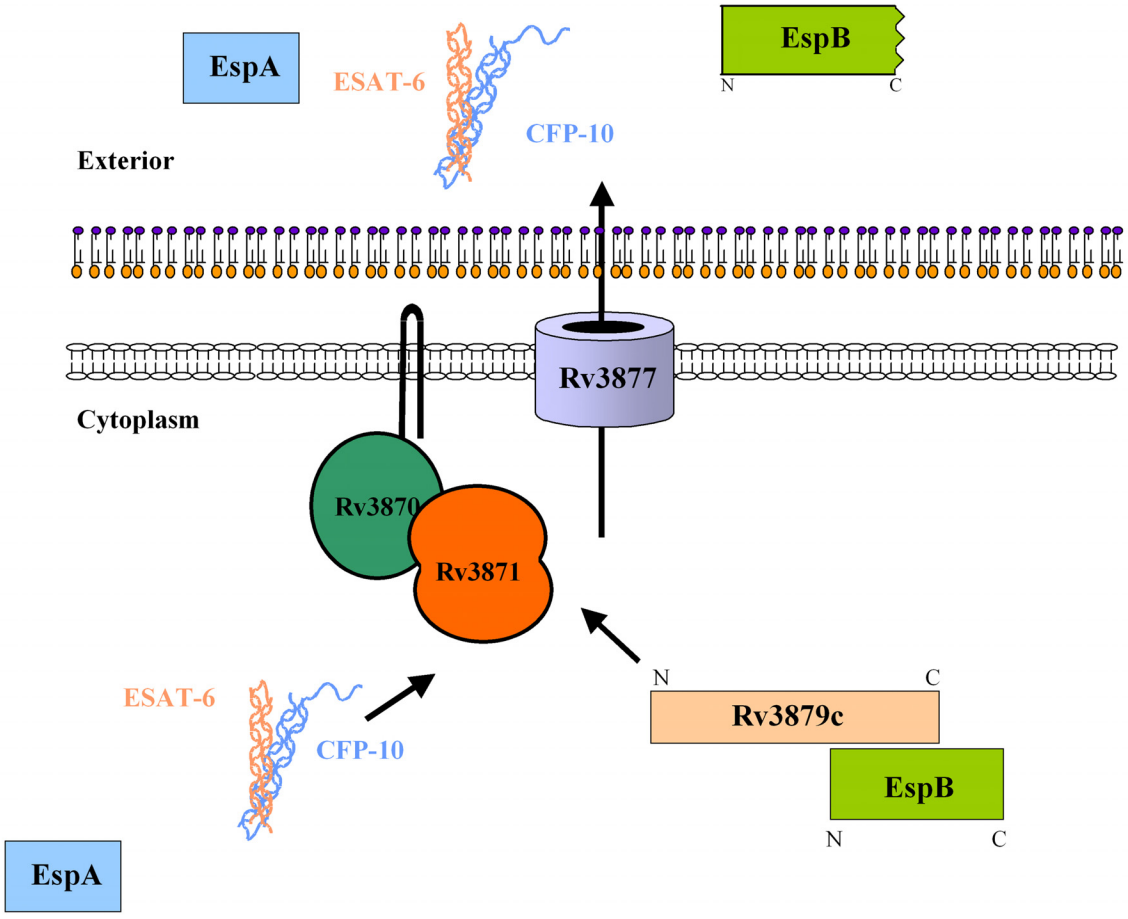
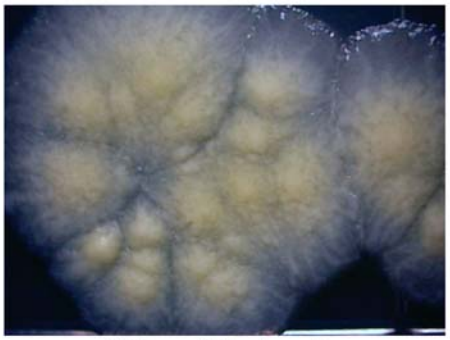

EspA
ESAT-6
CFP-10
EspB
N
C
Exterior
Rv3877
Cytoplasm
Rv3870
Rv3871
ESAT-6
CFP-10
N
C
Rv3879c
EspB
N
C
EspA

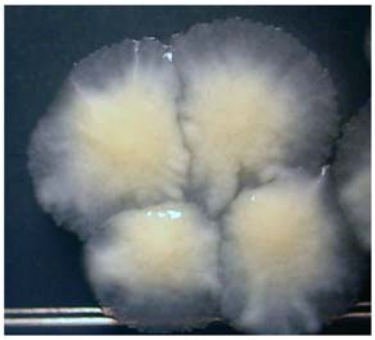
Figure 2.8 Colony Morphology of *espB$_M$::tn*

Dilutions of each strain were grown on 7H10 agar, without antibiotics, and imaged after 12 d.



Wt *attB::hyg*^{r}

espB$_{M}$*::tn attB::hyg*^{r}



espB$_{M}$*::tn*
attB::espB$_{M}$

espB$_{M}$*::tn*
attB::espB$_{M}$*-Mh3880c*

espB$_{M}$*::tn*
attB::Mh3883-3880c

espB$_{M}$*::tn*
+ p(Mh3883-3880c)

Figure 2.9 *Mh3871* Is Required for the Secretion of $EspB_M$

Cell lysates (CL) and culture filtrates (CF) were prepared from wild-type *M. marinum* strain 1218 and isogenic mutants as described in Materials and Methods. CL and CF proteins were separated by SDS-PAGE and the indicated proteins detected by western blot. Cultures of each strain were inoculated into Sauton's medium at an optical density (OD) of 0.5 and grown for 36 h. Therefore, the samples of each strain are normalized by OD readings. Of the total CL and CF fractions collected for each strain from one experiment, 3% of the CL was loaded in each lane and 15% of the total CF was loaded into each lane.

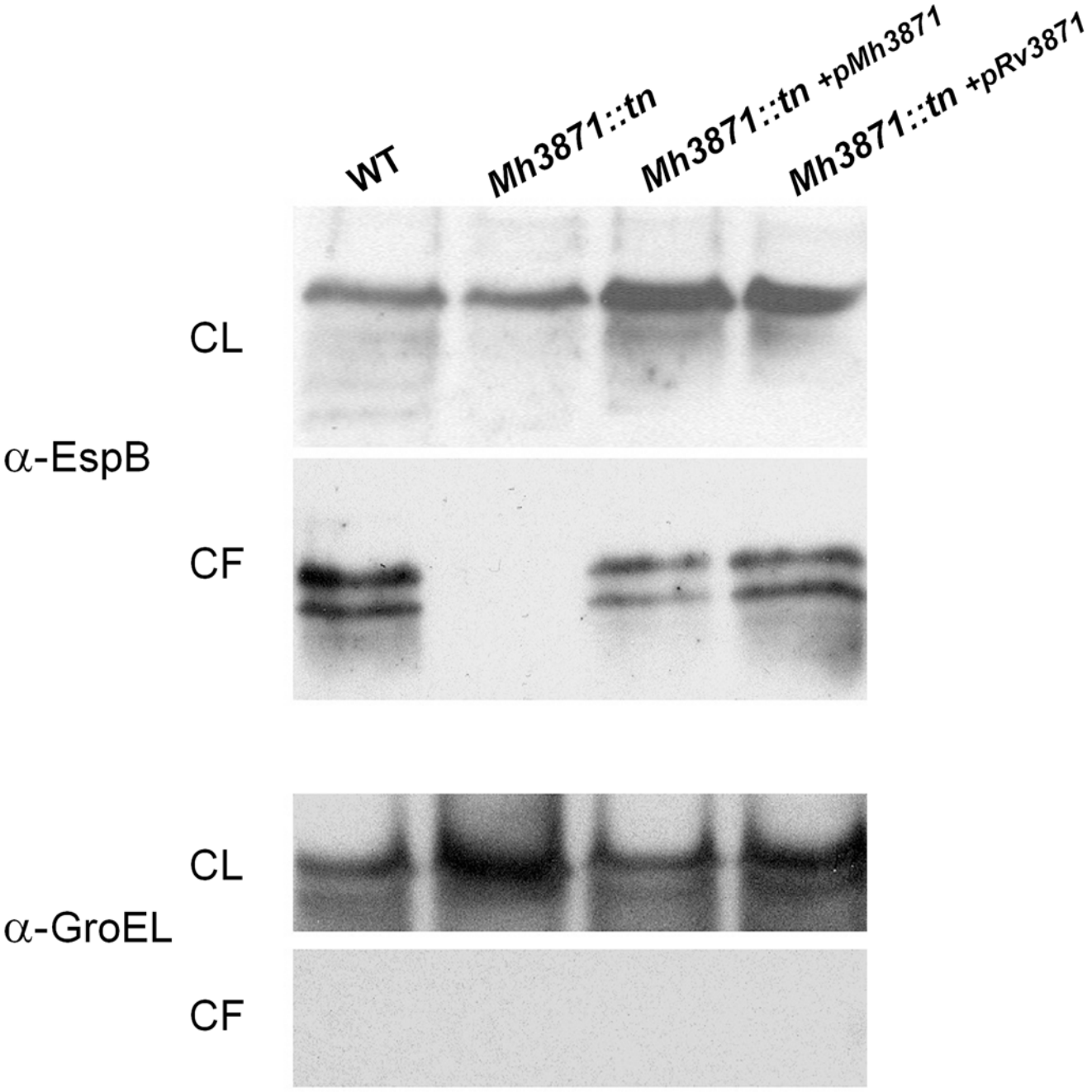
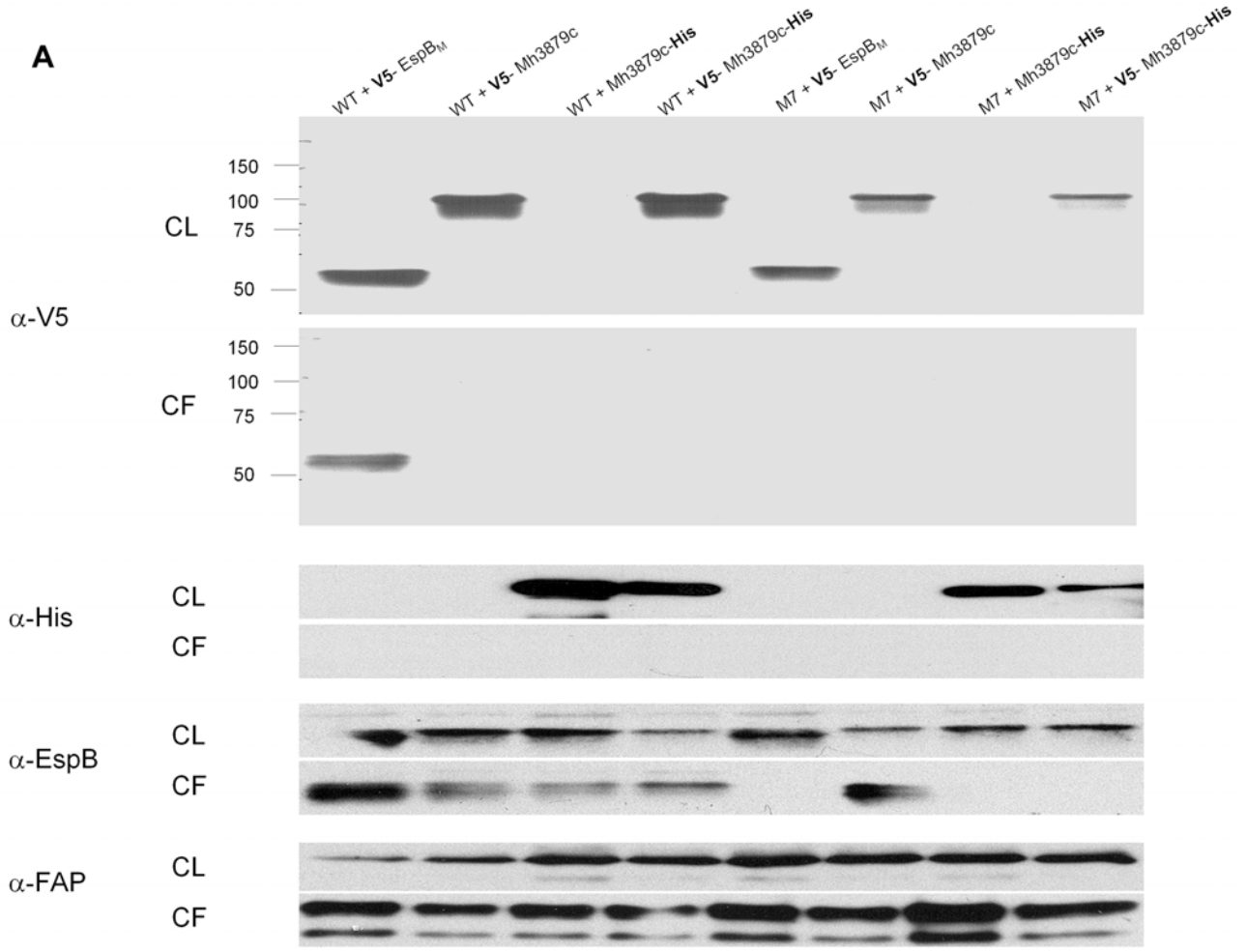

WT
Mh3871::tn
Mh3871::tn +pMh3871
Mh3871::tn +pRv3871
CL
α-EspB
CF
CL
α-GroEL
CF

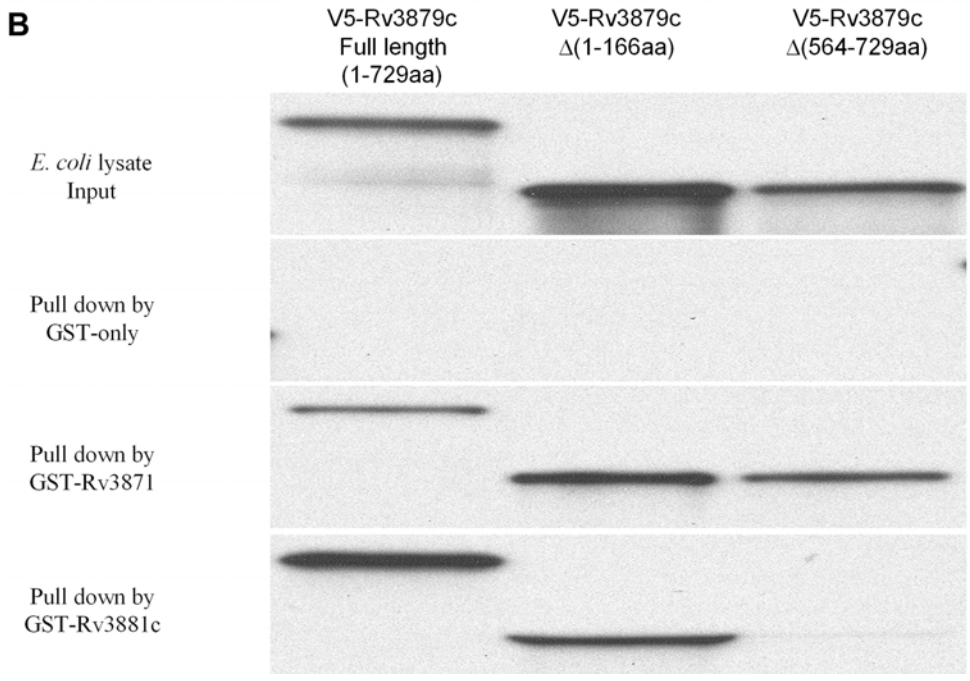
Figure 2.10 Mh3879c Is Not Secreted and the Carboxyterminus of Rv3879 Is Required for Interaction with EspB

Cell lysates (CL) and culture filtrates (CF) were prepared from the indicated strains as described in Materials and Methods. Proteins were separated by SDS-PAGE and the indicated proteins were detected by western blot strains as described in Materials and Methods.

(A) Cultures of each strain were inoculated into Sauton's medium at an OD of 0.5 and grown for 36 h. Therefore, the samples of each strain are normalized by OD readings. Of the total CL and CF fractions collected for each strain from one experiment, 3% of the CL was loaded in each lane and 15% of the total CF was loaded into each lane.

(B) Agarose beads with immobilized GST only, GST-Rv3871, or GST-$EspB_T$ were incubated with *E. coli* lysates expressing V5-tagged Rv3879c full-length (1-729), Rv3879c ⊗(1-166), or Rv3879c ⊗(564-729). The input lysate (0.1%) and the material from the cell lysates that bound to the beads was run on SDS-PAGE and detected by western blotting with an antibody against V5.


A
WT + V5- EspB$_M$
WT + V5- Mh3879c
WT + Mh3879c-His
WT + V5- Mh3879c-His
M7 + V5- EspB$_M$
M7 + V5- Mh3879c
M7 + Mh3879c-His
M7 + V5- Mh3879c-His
150
100
75
50
CL
CF
α-V5
α-His
α-EspB
α-FAP


B
V5-Rv3879c
Full length
(1-729aa)
V5-Rv3879c
Δ(1-166aa)
V5-Rv3879c
Δ(564-729aa)
E. coli lysate
Input
Pull down by
GST-only
Pull down by
GST-Rv3871
Pull down by
GST-Rv3881c

Chapter 3

The role of EspB in the virulence and morphology of *Mycobacterium*

ABSTRACT

The ESX-1 secretion system is a major determinant of virulence in *Mycobacterium*, but the mechanisms by which ESX-1 effectors manipulate their host environment is unknown. The ESX-1 substrate, EspB, is required for virulence and growth in macrophages and appears to function in altering host pathways that are distinct from those altered by the most the abundant ESX-1 subtrates, CFP-10 and ESAT-6. While investigating the pathogenic mechanisms of $EspB_M$ function, we found that the carboxyterminus of EspB is dispensable for EspB secretion, but is required for *M. marinum* growth in macrophages and for hemolytic activity. While an *$espB_M$::tn* mutant has a smooth colony morphology and was found to have a subtle cording defect it did not exhibit increased sensitivity to acidic conditions or reactive nitrogen intermediates in vitro assays nor was it altered for the transcription of other extRD1 genes. We found that the survival profile of an *$espB_M$::tn* mutant in macrophages diverged from that of wild-type after 24 hours of infection. We propose that EspB is both an essential component and substrate of the ESX-1 secretion system, that a carboxyterminal alpha helix of EspB is required for its role in virulence, and that it enables *Mycobacterium marinum* to alter the macrophage environment shortly after infection.

INTRODUCTION

Many pathogens have evolved strategies for evading immune responses, as well as hijacking host cells in ways that benefit microbial growth. *Mycobacterium tuberculosis* is a highly successful pathogen of humans, in part, because it has evolved mechanisms to suppress the host immune response to infection, avoid phagolysosome fusion, disseminate, and persist within its hosts in a dormant state[15,19,23,35]. Similarly, *Mycobacterium marinum* is a highly successful pathogen of its natural hosts -fish and amphibians- avoiding phagolysosome fusion, persisting in caseous granulomas, and disseminating to various tissues and organs[3,6].

In both species of *Mycobacterium*, the ESX-1 secretion system appears to be a significant pathway for the secretion of virulence factors and the manipulation of the host environment. In mice infected with *Mycobacterium tuberculosis*, ESX-1 likely secretes bacterial products into the macrophage that lead to the induction of IFN-β; which in turn might suppress the production of other cytokines, allowing the infection to progress[35]. In zebrafish embryo infections, *M. marinum* ESX-1 effectors appear to elicit macrophage aggregation and granuloma formation[6]. ESX-1 enables *Mycobacterium tuberculosis* to damage macrophage mitochondrial inner membranes, decrease the level of macrophage intracellular ATP, and cause tissue necrosis in the lungs of infected mice[36]. ESX-1 is also required for *Mycobacterium tuberculosis* to arrest the progression of nascent phagosomes into phagolysosomes, allowing replication in a compartment that resembles early endosomes[23]. Interestingly, the ESX-1 substrates ESAT-6 and CFP-10 are not required to prevent lysosome fusion, suggesting one or more of the other ESX-1 substrates carry out

that function. Further, *Mycobacterium tuberculosis* phagocytosed by human monocyte derived dendritic cells or macrophages, which are then cultured for seven days, and imaged by electron microscopy of cryosections, lack an encapsulating electron lucent zone; while ESX-1 mutants visualized in this assay do display an encapsulating electron lucent zone. From this result, it is hypothesized that *Mycobacterium tuberculosis* escapes from phagosomes and does so in an ESX-1 dependent manner[37].

While the most abundant substrates of ESX-1 are the proteins ESAT-6 and CFP-10, ESX-1 secretes at least four other proteins –EspA, EspB, EspC, and EspR[10,11,13,14]. ESAT-6 forms a 1:1 dimer with CFP-10 and the pair is chaperoned through ESX-1 via the interaction of the C-terminus of CFP-10 with Rv3871[15,16]. In vitro, at low pH or upon acetylation, ESAT-6 can dissociate from CFP-10, and the dissociated ESAT-6 is believed to interact with and cause lysis of phospholipid membranes[28,38]. Once beyond the mycobacterial cell wall then, ESAT-6 could dissociate and enable phagosome escape or cytolysis. Secreted EspC interacts with itself as well as a number of other proteins including EspA[11]. EspC is not evenly expressed across a population of cells nor on the perimeter of individual cells [11]. The secreted EspC complex confers sphingomyelinase activity to *Mycobacterium tuberculosis* that might generate ceramide to signal for apoptosis or disrupt lipid rafts and signaling through toll-like receptors. Finally, EspR activates the expression of EspA and EspC, and appears to be secreted in order to negatively regulate ESX-1 activity[14].

The function of EspB remains to be elucidated. Unlike EspA and EspC, its secretion is not mutually dependent on the secretion of the ESAT-6/CFP-10 dimer[3,13]. In particular, the *M. marinum* mutant *Mh3879::tn* secretes CFP-10 but does not secrete

ESAT-6 or EspB. Further, a *Mycobacterium tuberculosis ΔCFP-10* mutant continues to secrete EspB, but does not secrete any of the four substrates: EspA, EspC, ESAT-6, or CFP-10[13]. In contrast to other ESX-1 mutants that lack EspB secretion, the *ΔCFP-10* mutant still arrests phagosome maturation[23]. Therefore, it is tempting to speculate that EspB functions specifically to block phagosome maturation.

The phenotypes of the *espB$_M$::tn* mutant offer other hints as to the function of EspB. The *espB$_M$::tn* mutant is attenuated for growth in mouse macrophages and in zebrafish, forms smooth colonies on agar, lacks the contact dependent hemolytic activity of wild-type, and fails to secrete ESAT-6 and CFP-10[3]. Both EspB$_M$ and the *Mycobacterium tuberculosis* homologue of EspB$_M$, complement *espB$_M$::tn*. The altered surface of *espB$_M$::tn* suggests that EspB influences the composition of lipids or polysaccharides in the cell wall and may indirectly function in protecting the bacilli from acid or other harsh environmental conditions. The ESAT-6 and CFP-10 secretion defect indicates that EspB, in addition to being a substrate for secretion, may also be a component of the secretion machine. The loss of hemolysis activity could be an indirect consequence of the failure to secrete ESAT-6. Alternatively, EspB secretion, while not sufficient for hemolytic activity, could be required.

Finally, the secondary structure of EspB provides data for speculation as to EspB function. EspB$_M$ is 455 amino acids with a predicted molecular weight of 47 kda. The amino-terminus and the middle of the protein are required for interaction with Rv3879c, for delivery of EspB to the core ESX-1 machinery, and for secretion. The carboxyterminus is cleaved either during or after secretion and this cleaved portion contains a somewhat amphipathic alpha helix that might enable interaction with lipids or

membranes. Cleavage might thus serve to release the amino terminus of EspB from the lipids to which it is bound. The middle of the protein contains a putative phosphopantetheine attachment site. Phosphopantetheine is the prosthetic group of acyl carrier proteins (ACP) and multienzyme complexes, such as polyketide synthases. Therefore, this motif along with the smooth colony morphology of *espB$_M$::tn* suggests that EspB might function in synthesizing lipids or lipo-polysaccharides components of the cell wall.

In this work we find that the carboxyterminus of EspB, while dispensable for interaction with Rv3879 and EspB secretion, is required for *M. marinum* growth in macrophages and for hemolytic activity. We show that the putative phosphopantetheine biding site in EspB does not contribute to virulence. The *espB$_M$::tn* mutant displays wild-type sensitivity to acidic conditions and reactive nitrogen intermediates in in-vitro assays, but has a subtle defect in cording in addition to its smooth colony morphology. The *espBM::tn* mutant is not altered for the transcription of *extRD1* genes other than *espB* and *Mh3880c*. Finally, the survival profile of an *espB$_M$::tn* mutant in macrophages diverges from that of wild-type after 24 hours of infection.

RESULTS

The putative phosphopantetheine binding site in EspB does not contribute to virulence

A simple PROSITE protein motif search identified one putative functional domain within EspB$_M$, a phosphopantetheine attachment site. This domain is characterized by a conserved sequence of 14 amino acids and exists between the 374th

and the 389th amino acids of $EspB_M$. Phosphopantetheine is the prosthetic group of acyl carrier proteins and some multienzyme complexes, including polyketide synthases and fatty acid synthases. It acts as a 'swinging arm' for the attachment of activated fatty acid and amino-acid groups. Phosphopantetheine is covalently attached to the hydroxyl group of a serine residue in the center of a phosphopantetheine binding domain. Therefore, the 378th amino acid (serine) of $EspB_M$ is predicted to bind to phosphopantetheine. While a phosphopantetheine attachment site domain is predicted within $EspB_M$, this domain is not predicted within $EspB_T$ because two amino acid differences between $EspB_M$ and $EspB_T$ amino acids in this region prevent $EspB_T$ correspondence with the predicted motif. Below capital letters represent amino acids that fit the pattern and lower case letters represent amino acids that do not fit the pattern. Importantly, the center serine, which is absolutely essential, is conserved in both homologues and thus we hypothesized that both proteins could bind phosphopantetheine.

$EspB_M$	374-389aa	LAGGESVRPAAAGDIA
$EspB_T$	377-392aa	igGAESVRPAGAGDIA

Using a genetic approach, we asked whether the putative $EspB_M$ phosphopantetheine binding contributes to virulence. The essential serine at amino acid 378 (to which the phospopantetheine directly binds) in $EspB_M$ was changed to an alanine to create the $EspB_M$ mutant, $EspB_M$-S378A. This mutation was generated by quick-change mutagenesis and verified by DNA sequencing. The expectation was that if $EspB_M$ binds phosphopantetheine, and such an attached phosphopantetheine moiety helps $EspB_M$ contributes to virulence, then *$espB_M$-S378A* would not be able to complement the *$espB_M$::tn* mutant equivalently to *$espB_M$*.

Two C-terminally truncated forms were also constructed, $EspB_M$ Δ(371-455) and $EspB_M$ Δ(400-455). Each of three constructs, along with *$espB_M$*, was exogenously expressed in the *$espB_M$::tn* mutant, using the *$espB_M$* promoter. The strains were tested for the ability to restore hemolytic activity to the mutant as well as to grow in mouse bone marrow derived macrophages (BMDM). In both assays, *$espB_M$-S378A* complemented *$espB_M$::tn* equally as well as *$espB_M$* (Figures 3.1A and 3.1B). Therefore, if $EspB_M$ binds phosphopantetheine such attachment does not contribute to the ability of *M. marinum* to lyse red blood cells or persist and grow in macrophages.

The carboxyterminus of $EspB_M$ is required for virulence and contains an alpha helix

Unlike the $EspB_M$-S378A mutation, truncation of the carboxyterminus of $EspB_M$ disrupted $EspB_M$ function (Figures 3.1A and 3.1B). Both the polypeptides $EspB_M$ Δ(371-455) and $EspB_M$ Δ(400-455) failed to complement *$espB_M$::tn*. Consistent with this result, Xu et al., recently found that a construct $EspB_M$ Δ(332-455) fails to complement *$espB_M$::tn*[39].

Since the secondary structure of the EspB carboxyterminus includes a somewhat amphipathic alpha helix (Figure 3.2), we tested whether that helix is required for EspB to complement *$espB_M$::tn.* To do ths, an *$espB_M$* mutant was constructed with three amino acids in the alpha helix converted to prolines by quickchange mutagenesis. Specifically, the 432nd through the 435th amino acids were changed from alanine, leucine, tyrosine, threonine to proline, proline, tyrosine, proline. This mutant, *$espB_M$-432ppyp*, was transformed into the *$espB_M$::tn* mutant. A V5 aminoterminal epitope tagged version of the mutant was also constructed. The strain was grown in Sauton's media, the culture filtrate

fraction was prepared, and probed for the expression and secretion of the V5 epitope tag (Figure 3.3A). The negative controls included *espB$_M$::tn* transformed with either V5-GFP or the untagged version mutant construct *espB$_M$-432ppyp.* The positive controls included: *espB$_M$::tn* was transformed with: *V5-espB$_M$*; *V5-espB$_M$-mh3880c*; and *V5-espB$_M$-mh3880c-His.* As shown in Figure 3.3A, an antibody against the V5 epitope detects secretion of both V5-EspB$_M$ and V5-EspB$_M$-432ppyp, but neither V5-GFP nor untagged EspB$_M$-432ppyp. Therefore, V5-EspB$_M$-432ppyp is stably expressed and secreted.

Next, both the tagged and untagged versions of the carboxyterminal alpha helix mutant were tested for the ability to restore hemolytic activity to *espB$_M$::tn*. As shown in Figure 3.3B, the constructs expressing *espB$_M$* restore hemolytic activity to *espB$_M$::tn*, but neither version of the carboxyterminal alpha helix mutant restores hemolytic activity. Therefore, the carboxyterminus of EspB is dispensable for secretion but contains a somewhat amphipathic alpha helix that is required for hemolytic activity, and presumably virulence as well.

The espB$_M$::tn mutant exhibits a subtle decrease in cording in mid log phase 7H9 broth cultures

Wild type *Mycobacterium marinum*, similar to *Mycobacterium tuberculosis,* forms colonies with rough morphology when grown on 7H10 agar. In contrast, the *espB$_M$::tn* mutant, like many of the *extRD1* mutants, has a smooth, translucent, colony morphology. A rough colony morphology is partially restored to the *espB$_M$::tn* mutant upon integration of *espB$_M$*, and is fully restored upon integration of the whole operon,

espBM-Mh3880c[13]. It has been hypothesized that increased surface expression of glycopeptidolipids (GPL), a structural component of the mycobacterial cell wall, can cause a switch from a rough to a smooth colony morphology[40].

In addition to the rough colony morphology when grown on agar, both *M. marinum* and *M. tuberculosis* form structures that look like ropes, bundles or serpentine aggregates called cords when grown in liquid media. Cording is thought to depend on cell wall lipids, in particular trehalose dimycolates, and in some cases, at least, a smooth mycobacterial colony morphology correlates with decreased cording[41-44]. Thus we examined the cording ability of the *espB$_M$::tn* mutant. Phase contrast microscopy of bacilli grown in 7H9 liquid broth revealed that in late log stage of growth, wild-type *M. marinum* appears to form slightly larger corded structures than the *espB$_M$::tn* mutant (Figure 3.4A).

A subtle cording defect was also reflected in optical density (OD) readings taken on growing liquid cultures of bacteria. A heavily corded culture absorbs slightly less light than an uncorded culture of the same number of bacilli. Therefore, we expected later log phase wild-type cultures have a slighter lower OD than the *espB$_M$::tn* mutant . Consistent with this expectation, the O.D. readings of wild-type grew slower than the *espB$_M$::tn* mutant during the mid log phase of growth in liquid cultures (Figure 3.4B). When the bacilli are grown with slightly more detergent, (0.007% SDS in addition to the 0.05% Tween80 normally in our 7H9), the liquid cultures wild-type and the mutant are indistinguishable consisting of just single bacilli and small corded structures. Similarily, the OD readings taken on wild-type and *espB$_M$::tn* during growth in the 0.007% SDS, increased with congruent slopes in the mid log phase. Wild-type and *espB$_M$::tn* exhibit

equal sensitivity to higher concentrations of detergent (Figure 3.4C). Thus, our observations by phase contrast microscopy and the OD readings of liquid grown cultures suggest that *espB$_M$::tn* cords slightly less during mid log phase.

The espB$_M$::tn mutant and wild-type exhibit similar sensitivity to lysozyme, low pH, and reactive oxygen intermediates during growth in liquid culture

A smooth colony morphology also can reflect decreased cell wall integrity because of loss of lipid content or particular lipid species required for rough morphology[4]. Such a loss of cell wall integrity can cause increased sensitivity to detergent, lysozyme, reactive nitrogen intermediates, or acid. To test for such possible phenotypes, the *espB$_M$::tn* mutant was grown in 7H9 liquid media containing varying concentrations of lysozyme, $NaNO_2$, acid, or H_2O_2 (Figure 3.5). For each test, the cultures were inoculated at an OD of 0.01 and then OD readings were taken after 83 hours of growth. In this experiment the *espB$_M$::tn* mutant did not show increased sensitivity to lysozyme, $NaNO_2$, acid, or H_2O_2.

Analysis of extRD1 transcription does not reveal any novel transcription factors among the extRD1 genes

As described earlier, the ESX-1 substrate, EspR, is a transcriptional activator of the ESX-1 locus, *Rv3616c-14c*, as well as for the gene encoding the EspC interacting protein Rv0888[14]. Disruption of *espR* reduces the expression of *Rv3616c-14c,* and thereby disrupts the secretion of ESAT-6. The ESAT-6 secretion defect of the mutant is bypassed by constitutive expression of *Rv3616c-14c.* Further, expression of an active

espR mutant that is not secreted results in increased expression of *Rv3616c-14c.* Together, those results indicate that EspR activity is negatively regulated by secretion through the ESX-1 system and raises the question of whether other ESX-1 substrates are also secreted to achieve negative regulation.

Quantitative RT-PCR (QPCR) was used to measure mRNA levels of *extRD1* genes in wild type, ten extRD1 mutants, and $espB_M$*::tn* complemented with $espB_M$ during growth in 7H9 media (Figure 3.6). As shown, the *ΔRD1* strain does not transcribe any of the genes *Mh3872-Mh3879c*. The individual transposon insertion mutants have significantly reduced transcription of the genes that are disrupted and depending on the direction and position of the transposon insertion, the rest of the genes in their operon as well. The transcription of *sodA* was used as a control, and among the mutants it varied at most 20% from wild-type. None of the disrupted extRD1 genes, represented by the mutants tested, diminished the expression of extRD1 outside of their own operons, during the mid log phase of growth in 7H9 media. This result does not exclude the possibility that *extRD1* genes could function as transcriptional regulators upon infection of macrophages, persistence in a granuloma, or during growth in different media.

Intraphagosomal growth profiles of wild-type M. marinum and $espB_M$::tn diverge around 24 hours post infection

ESX-1 is required for the arrest of phagosome maturation as well as phagosomal escape[23,45]. *Mycobacteria* appear to actively prevent phagolysosome fusion early after infection, as the *M. marinum $espB_M$::tn* mutant is found in acidic compartments within just 2 hours [39]. Phagosome escape takes place later, with actin tails first appearing 15

hours after macrophage infection, and after 48 hours after infection still just 20% of all intracellular bacteria demonstrate actin tails[45]. We speculate that EspB functions in the arrest of phagosome maturation while ESAT-6 might enable phagosome escape, and thus we attempted to discern the relative importance of these two events for intracellular growth of *M. marinum*.

In this experiment, BMDM were infected with both wild-type *M. marinum* and the *espB$_M$::tn* mutant, but the media during and after infection contained 5μg/mL of gentamicin sulfate. Gentamicin sulphate is an aminoglycoside with a wide spectrum of antibacterial activity and high solubility, but it does not cross cell membranes efficiently. Therefore, gentamicin is unable to enter the cytoplasm, but it does reach phagosomal bacteria through fusion of pinocytotic endosomes with phagosomes [46,47]. Additionally, the antibacterial activity of gentamicin is considerably lower at a low pH [46]. Cytoplasmic bacilli, therefore, should avoid exposure to the gentamicin and bacilli in acidic phagosomes should experience minimal effect from the presence of gentamicin.

In the presence of gentamicin, if phagosomal escape alone is sufficient for intracellular proliferation, then wild-type *M. marinum* should still grow in BMDM. As shown in figure 3.7, during an infection in the presence of 5μg/mL gentamicin, the number of wild-type and *espB$_M$::tn* bacilli are equivalent until about 20 hours post infection. Then, wild-type undergoes a short burst of growth that is contemporaneous with the timing of phagosomal escape. Subsequently, though, the growth of both strains stagnates. During the stagnant stage most of the wild type bacilli presumably are in a compartment of near neutral pH to which active gentamicin is being delivered by pinocytosis. The *espB$_M$::tn* phagosome is acidic; thus the gentamicin delivered to it is

presumably inactive. From this experiment, it appears that phagosomal escape gives a short boost to intracellular growth, but that cytoplasmic bacilli are not sufficient for continued proliferation. Rather, the reservoir of bacilli in arrested phagosomes is likely to be essential for full virulence and proliferation in macrophages.

DISCUSSION

In this study, we demonstrate that the carboxyterminus of EspB, and specifically the carboxyterminal alpha helix, is required for both *M. marinum* growth in macrophages and for hemolytic activity. Presumably then the EspB carboxyterminal alpha helix is crucial in the structure or function of the ESX-1 machinery or in the extracellular activity of EspB. Such a hydrophilic alpha helix might oligomerize, perhaps to create a membrane pore, or might interact with yet other ESX-1 proteins required for the hemolytic activity of *M. marinum*. As we previously showed though, the carboxyterminus of EspB, is not required for EspB secretion[13].

We also found that the operon *$EspB_M$-Mh3880c* is required for a full wild-type level of cording during growth in liquid broth. This observation coincides with the requirement of both *$espB_M$* and *Mh3880c* for the fully rough colony morphology of wild-type[13]. Trehalose 6,6' dimycolate (TDM), or cord factor, determines the cording phenotype, but excess glycolipids in the outer layer of solvent-extractable cell wall lipids, or capsule, are also believed to prevent interactions of TDM molecules from contiguous bacteria and thus indirectly inhibit cording[44]. A smooth colony morphology may be the result of alteration in any of the several structurally diverse groups of glycolipids in the

capsule including: the lipoglycans, lipoarabinomannan (LAM), lipomannan, and related phosphatidylinositol mannosides (PIMs), glycopeptidolipids (GPLs), dimycocerosates of phthiocerol (DIMs) and related phenolic glycolipids (PGLs), triacylglycerols, as well as trehalose-containing glycolipids such as lipooligosaccharides (LOSs), sulpholipids (sulphated acyl trehaloses), cord factor (trehalose 6,6'-dimycolate), and diacyl-, triacyl- and pentaacyl-trehaloses[48]. Underneath the capsule, lie the mycolic acids, and mutations in genes involved in their biosynthesis also gives a smooth colony morphology to *Mycobacterium tuberculosis*[42]. Importantly, some smooth colony mutants, such as *kasB::tn*, have drastic cording defects, as well as sensitivity to detergent and lysozyme[4]. In contrast, *espB$_M$::tn* has a very smooth colony, but does not exhibit increased sensitivity to detergent or lysozyme and has only a very subtle reduction in cording. Therefore, it seems likely that the reduced cording phenotype of *espB$_M$::tn* is linked to its smooth colony morphology, but its cell wall and capsule could be more similar to that of wild-type than to that of smooth colony mutants like *kasB::tn*. It is interesting to speculate that the cording phenotype also is associated with virulence, since trehalose dimycolate (cording factor) has been shown to be a very potent proinflammatory molecule released by *Mycobacteria* [[49]].

Many of the extRD1 mutants, beyond *espB$_M$::tn*, have smooth colonies and this phenotype may correlate with virulence. This correlation was shown in our complementation of *espB$_M$::tn*, and was also demonstrated by complementation of a BCG strain with a fragment of the RD1 locus containing ESAT-6, that resulted in a strain more virulent than the parent and a more rough colony morphology[33]. This link between colony morphology and virulence further intersects with the formation of mycobacterial

biofilms. The same GPLs which give smooth colony morphologies are required for both the sliding motility and biofilm formation of *M. smegmatis*[50]. In *M. marinum*, which does not produce GPLs, it seems LOSs are required for biofilms[51]. It is the presence of LOSs that give *M. canetti* and some variants of *M. kansasii* their smooth colony morphologies. While these surface glycolipids promote biofilms they seem to compromise virulence. Rough variants of *M. kansasii*, devoid of all LOSs, cause chronic systemic infections in mice, while smooth variants containing LOSs are rapidly cleared from the organs of infected animals[52]. LOSs are also absent in most clinical isolates of M. tuberculosis and the laboratory strain H37Rv. ESX-1 is certainly required for the rough colony morphology and future experiments to discern if ESX-1 has a role in biofilm formation will be interesting. Also it will be interesting to determine whether mycobacterium modulates between a rough virulent state and a smooth, less virulent, but biofilm competent state. If mycobacterium do modulate between these states it is tempting to speculate that ESX-1 participates in this regulation.

In a detailed analysis of the growth of both wild-type and $espB_M$*::tn*, in BMDMs, we found that the two growth profiles diverge around 24 hours post infection. This experiment was performed including gentamicin in the post infection media. Since gentamicin does not enter the macrophage cytoplasm but does pinocytose to phagosomes, our data thus suggests that both phagosome escape and a reservoir of bacilli in arrested phagosomes contribute to the proliferation of bacilli in a monolayer of macrophages. ESX-1 appears to contribute to both of these pathogenic events; continued characterization of the substrates will likely contribute to our understanding of both phagosome maturation and phagosome escape.

In conclusion, this work carboxyterminus of EspB, and specifically the carboxyterminal alpha helix, is required for virulence phenotypes and that EspB influences the cell surface composition of mycobacterium. One strategy in the design new *M. tuberculosis* vaccines is to make a vaccine strain that still secretes the antigenic proteins but secretes attenuated versions of those proteins. An EspB carboxyterminal truncation mutant might contribute to this strategy because it is still secreted but is no longer functional in terms of virulence.

Bacterial strains and plasmids.

All strains and plasmids used in this study are listed in Table 3.1. *M. marinum* strains were grown as previously described[2]. The designations assigned by the Sanger Institute in the annotation of the *M. marinum* genome and the corresponding DNA sequences are available at http://www.sanger.ac.uk/Projects/M_marinum/.

TABLE 3.1

Strain/ Plasmid	Name	Relevant gene product	Genotype using "Marinum homolog" of H37Rv nomenclature
M. marinum M	WT		
	ΔRD1		*Δ (Mh3871 - Mh3879)*
	M1		*Mh3866::tn*
	M2		*Mh3867::tn*
	M3		*Mh3868::tn-1*
	M4		*Mh3868::tn-2*
	M5		*Mh3876::tn*
	M6		*Mh3878::tn*
	M7		*Mh3879::tn*
	M8 (*espB $_M$ ::tn*)		*Mh3881::tn*
Mycobacterium plasmids	pBM332	V5- $EspB_M$	
	pBM400v	V5- $EspB_M$ Δ(400-454)	
	pBM209	$EspB_M$	
	pBM378	$EspB_M$ (S378A)	
	pBM372	$EspB_M$ Δ(372-454)	
	pBM400	$EspB_M$ Δ(400-454)	
	pBM207	$EspB_M$ -Mh3880c	
	pLyg206	empty non-integrating plasmid	
	pBM337	V5- $EspB_M$ -Mh3880c	
	pBM336	V5- $EspB_M$ -Mh3880c-His	
	pBM103	V5- $EspB_M$ (432ppyp)	
	pBM102	$EspB_M$ (432ppyp)	
	pBM330	V5-GFP	

Measurement of contact-dependent hemolysis

M. marinum was grown in 7H9 medium to 1.0 O.D.; 250 µl was transferred to a microcentrifuge tube; washed twice with phosphate buffered saline (PBS); and finally resuspended in 120 µl of DMEM (without phenol red). Defibrinated sheep red blood cells were washed three times with DMEM (without phenol red) and resuspended in 120 µl. The two suspensions were mixed and incubated at 32°C, 5% CO2 for 2 h; the pellets were resuspended; centrifuged; and absorbance of the supernatant was measured at 415 nm.

Measurement of cording and sensitivity to detergent

Cording of *M. marinum* was observed after growth in 7H9 broth to an O.D. of 2. Resistance to SDS was assayed by growing strains to an OD of 1 then inoculating *M. marinum* in 7H9 to an O.D. of 0.01 containing SDS (0.007, 0.05, 0.1, or 0.5%, v/v), and culturing for the times indicated at 30°C, 105rpm.

Susceptibility of M. marinum to acid, reactive oxygen intermediates, and lysozyme

M. marinum strains were inoculated at an O.D. of 0.01 in 7H9 media containing indicated concentrations of lysozyme, $NaNO_2$, H_2O_2, or HCl to indicated pH levels.

QPCR of extRD1 genes

M. marinum strains were each grown in 7H9 to OD_{600} of 1.0, pelleted, resuspended in 300 µl of TE buffer containing lysozyme (3 mg/mL), and incubated at room temperature for 5 minutes. Then 1.05 mL of buffer RLT (Qiagen,

http://www.qiagen.com) was added along with 200 µl of glass beads. Pellets were lysed using the mini-bead beater (BioSpec Products, http://www.biospec.com/) with three 30-s pulses at maximum speed and incubations on ice in between each pulse, and then centrifuged at 13,000g for 10 sec at 4 °C to remove unbroken cells. From the resulting supernatant 900 µl was collected and the RNA was purified using the Rneasy Mini kit (Qiagen) according to the manufacturer's instructions, including the optional on-column Dnase treatment. Primers used for generating cDNA were designed using the algorithm at the website of IDT (www.idtdna.com). A cocktail of all reverse primers (those hybridizing at the 3' end) representing the genes *Mh3864* through *Mh3883* as well as *sodA* were made so that each primer was at a concentration of 2 pmol/µL. Using equal amounts total RNA for each strain, the primer cocktail, and the Superscript III Rnase H-Reverse Transcriptase kit (Invitrogen) according to the manufacturer's instructions, cDNA was prepared. Then the primer pairs and the cDNA was used with the Brilliant SYBR Green QPCR Master Mix (Stratagene) and the Mx4000 QPCR machine (Stratagene) to quantify in real time the PCR. The data was analyzed using the Mx4000 software.

Macrophage infections

All macrophages used in these experiments were derived from bone marrow cells of C57BL/6 mice that were differentiated for 7 days in DMEM supplemented with 10% CMG supernatant [34]; 10% fetal bovine serum (FBS; HyClone, http://www.hyclone.com/); gentamicin (50 µg/mL). Immediately prior to infection, macrophage monolayers were washed once with FBS-free DMEM. *M. marinum* strains were each grown to OD_{600} of

1.0, prepared for infection, and incubated with macrophages as previously described [3]. All infections were performed at a multiplicity of infection of 1, for 2 h at 32 °C, in a 5% CO2, humidified environment, in 24-well plates. The time at which *M. marinum* was added to the well was designated time (T= 0 h); and the time at the end of the 2 h incubation period (T = 2 h). At the end of the incubation, infected monolayers were washed twice with DMEM. Where indicated, monolayers were incubated in DMEM containing 0.1% FBS and 200 μg of amikacin/ml for just 2 h to kill extracellular bacteria; and at the end of the antibiotic treatment, monolayers were washed twice with DMEM and incubated in DMEM containing 0.1% FBS at 32 °C and 5% CO2. Intracellular bacteria were enumerated by lysing macrophage monolayers and diluting and plating bacteria exactly as described[3].

Macrophage infections with gentamicin

Macrophages were prepared as described above. For the 2 hour infection of macrophages with *M. marinum* the media on the macrophage was simply DMEM. At the end of the infection the macrophage monolayers were washed with DMEM, twice. No amikacin incubation was performed and instead the washed macrophages were directly incubated in DMEM containing gentamicin (5 μg/mL), 0.1% FBS at 32 °C and 5% CO2.

Protein preparation and analysis.

M. marinum strains were grown in 40-mL cultures to 0.5 OD600 in 7H9 medium. The cultures were centrifuged and washed three times with 15 mL of PBS before re-suspension in 40 mL of Sauton's medium, supplemented with 0.015% Tween-80. Strains

contained non-integrating plasmids for complementation, or empty vectors, and thus were grown in Sauton's medium supplemented with Zeocin (5 μg/ml; Invitrogen, http://www.invitrogen.com/). After growth for 36 h at 30 °C, 105 rpm, in Sauton's medium, the cells were harvested by centrifugation. Supernatants were filtered through a 0.22-μm-pore-size filter and concentrated with an Amicon Ultra-15 (5,000-molecular-weight cutoff; Millipore, http://www.millipore.com/) to 200 μL, which was saved as the culture filtrate (CF) fraction. Culture filtrate fractions were separated by SDS/PAGE on 7.5% polyacrylamide gels. V5 epitope was detected with a mouse monoclonal (R960–25, Invitrogen), at a concentration of 1:5000, and these blots were developed using West Pico (Pierce).

Figure 3.1 Disruption of the $EspB_M$ putative phosphopantetheine attachment site does not alter the ability of $EspB_M$ to restore hemolytic activity or growth in macrophage to *$espB_M$::tn*.

(A) Contact dependent hemolysis was determined by measuring the release of hemoglobin at a wavelength of 415 nm (A405) **(B)** Growth of *M. marinum* Wild-type, *$espB_M$::tn*, and *$espB_M$::tn* transformed with episomal plasmids encoding: *$espB_M$*, *$espB_M$Δ(372-454), $espB_M$Δ(400-454),* or *$espB_M$ (S378A)*. BMDMs were infected at a multiplicity of infection of 1. Colony-forming units (cfu) were determined by lysing infected monolayers and plating lysates at indicated time points, with growth indicated as fold increase compared to the initial level of infection for each strain.

Figure 3.1A

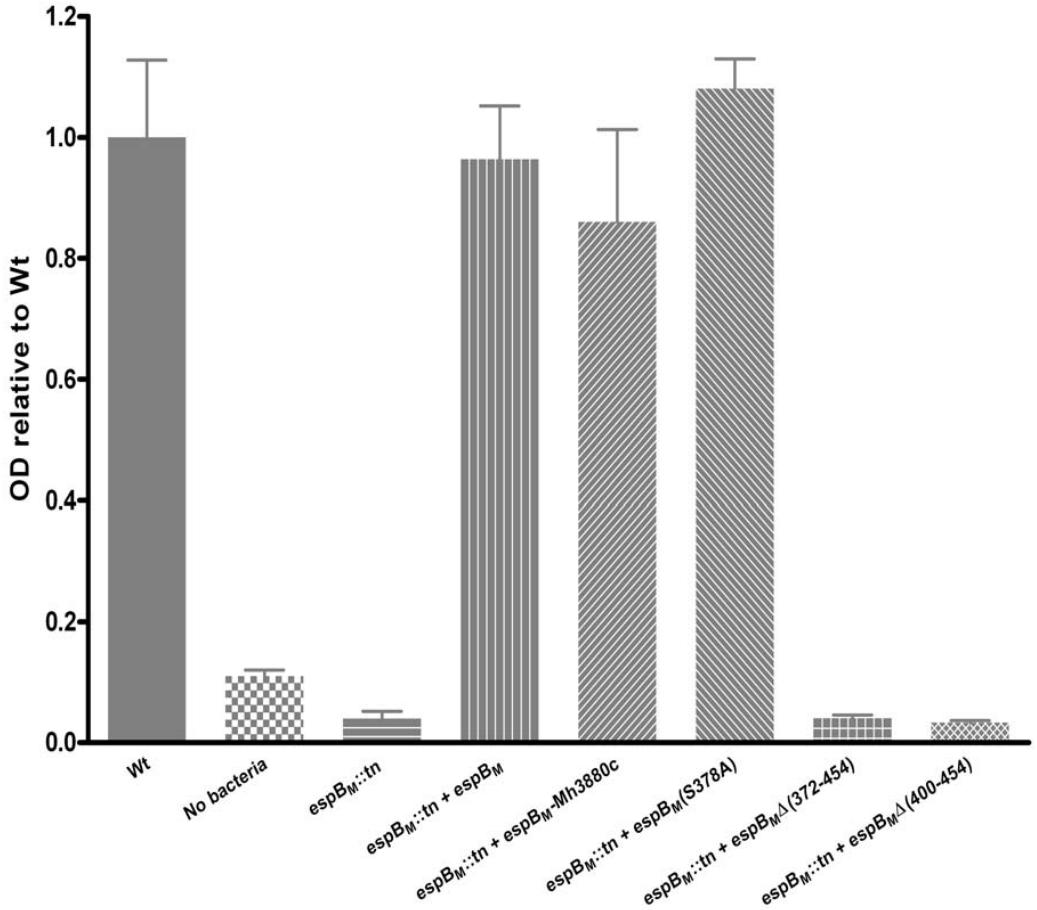

OD relative to Wt
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Wt
No bacteria
espBM::tn
espBM::tn + espBM
espBM::tn + espBM-Mh3880c
espBM::tn + espBM(S378A)
espBM::tn + espBMΔ(372-454)
espBM::tn + espBMΔ(400-454)

Figure 3.1B

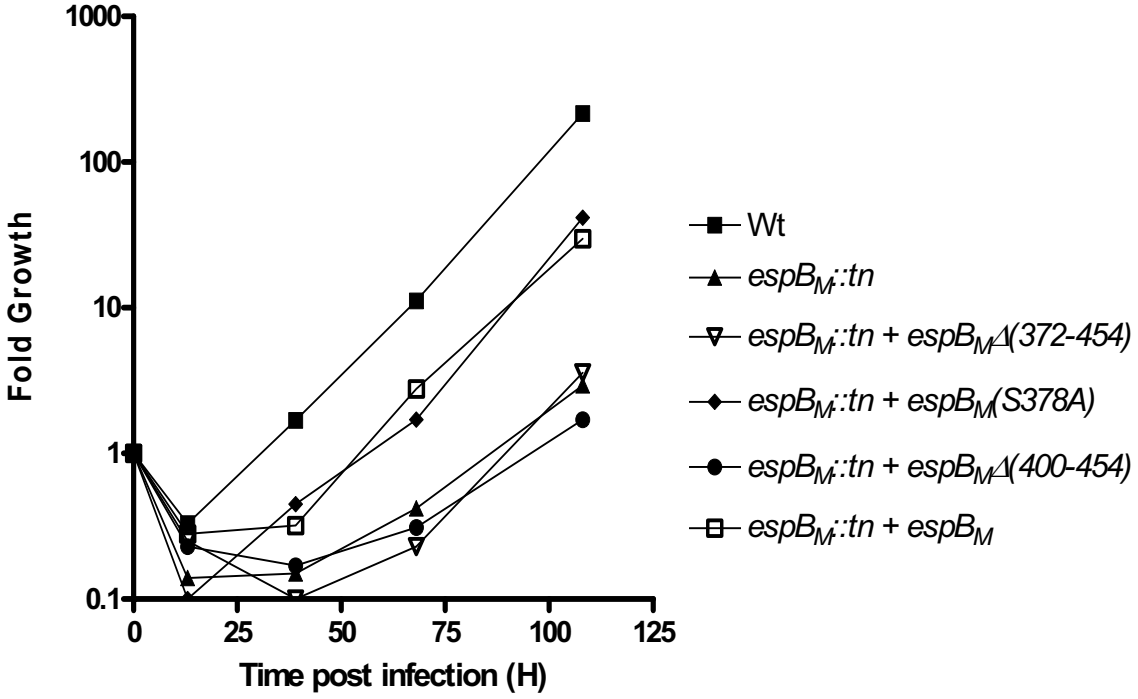
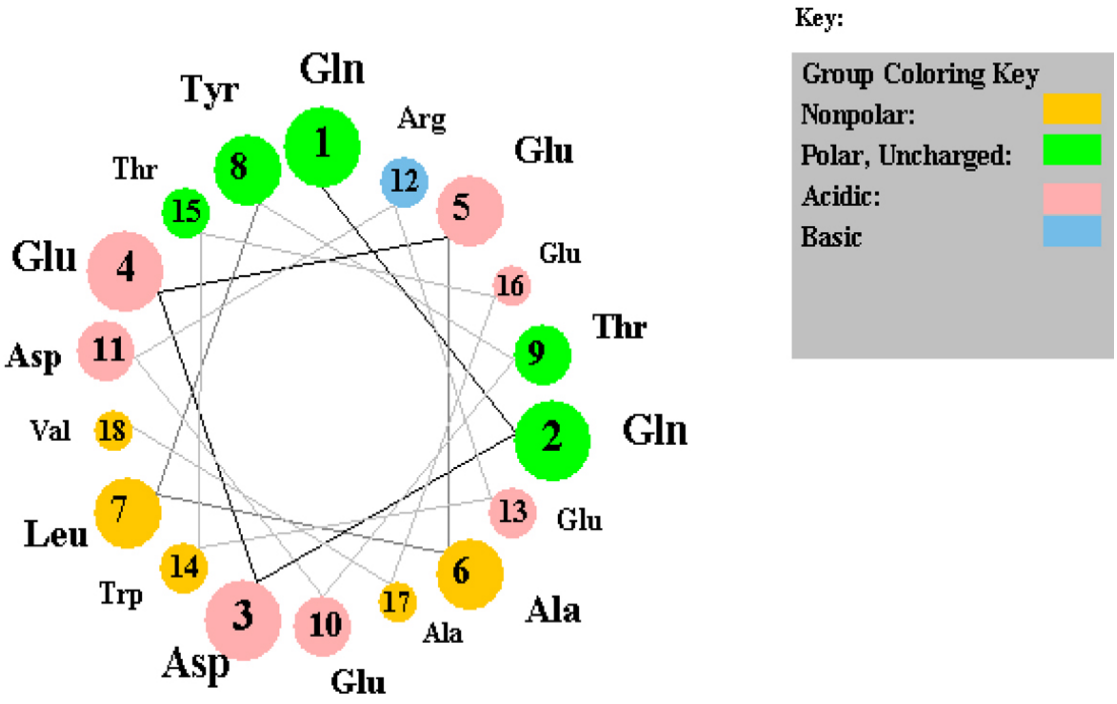

1000
100
10
1
0.1
Fold Growth
0
25
50
75
100
125
Time post infection (H)
Wt
espBM::tn
espBM::tn + espBMΔ(372-454)
espBM::tn + espBM(S378A)
espBM::tn + espBMΔ(400-454)
espBM::tn + espBM

Figure 3.2 The carboxyterminus of EspB contains an amphipathic alpha helix. Depicted is a helical wheel representation to emphasize that one face is non-polar. The helical wheel was generated using the applet on the website: http://cti.itc.virginia.edu/~cmg/Demo/wheel/wheelApp.html

Figure 3.2



….QQDEEALYTEDREWTEAVIGNRRR…

432aa 450aa

Figure 3.3 Disruption of the EspB carboxyterminal alpha helix does not effect expression and secretion, but renders the protein unable to restore hemolytic activity to *espB*$_M$*::tn*. **(A)** Culture filtrates (CF) were prepared from the indicated strains as described in Materials and Methods. Proteins were separated by SDS-PAGE, and the V5 epitope detected by western blot as described in Materials and Methods. Cultures of each strain were grown in 7H9 to an OD of 0.5, washed, and then inoculated into Sauton's medium at an OD of 0.5 and grown for 36 h. Therefore, the samples of each strain are normalized by OD readings. **(B)** Contact dependent hemolysis for each of the indicated strains was determined by measuring the release of hemoglobin at a wavelength of 415 nm (A405)

Figure 3.3A

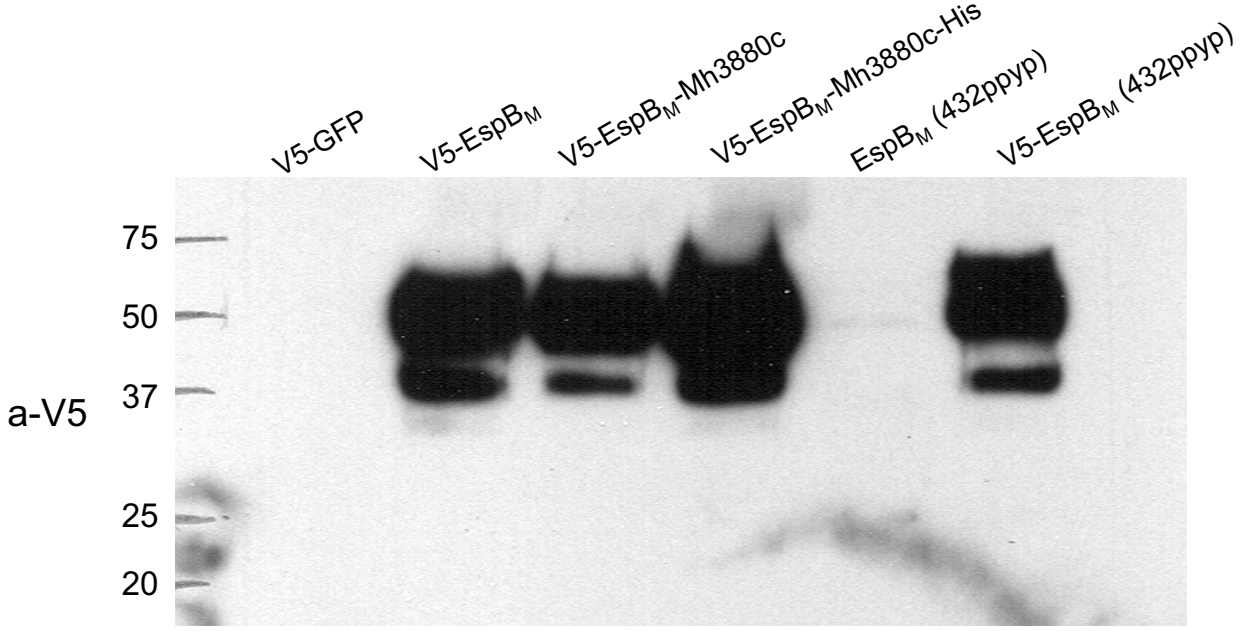

V5-GFP
V5-EspB$_M$
V5-EspB$_M$-Mh3880c
V5-EspB$_M$-Mh3880c-His
EspB$_M$ (432ppyp)
V5-EspB$_M$ (432ppyp)
75
50
37
25
20
a-V5

Figure 3.3B

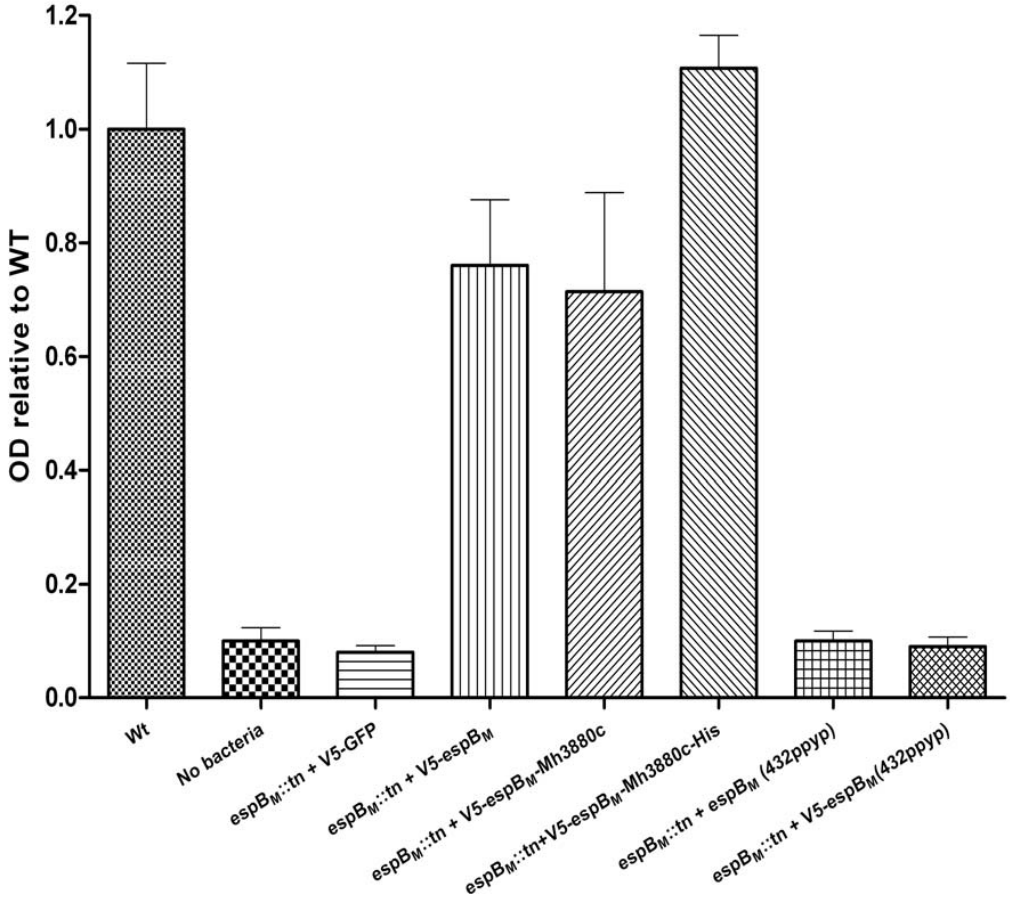
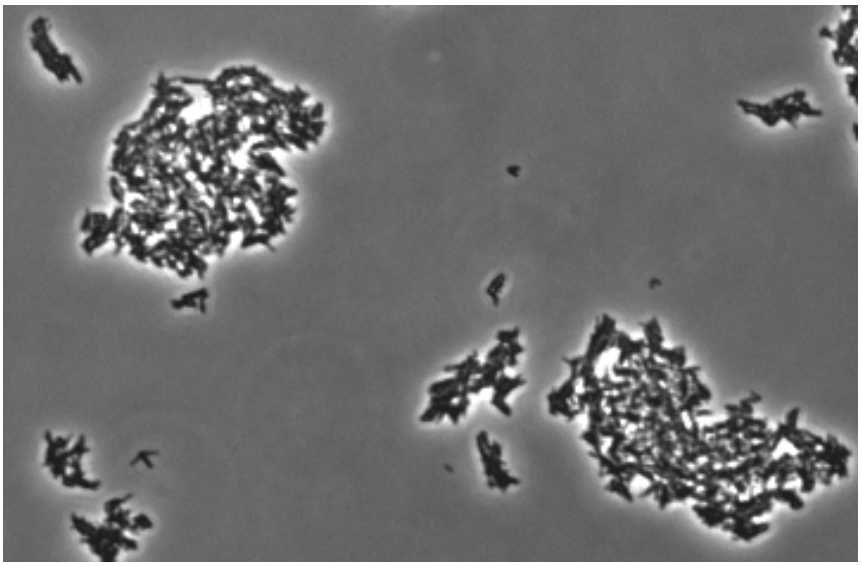

OD relative to WT
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Wt
No bacteria
espBM::tn + V5-GFP
espBM::tn + V5-espBM
espBM::tn + V5-espBM-Mh3880c
espBM::tn+V5-espBM-Mh3880c-His
espBM::tn + espBM (432ppyp)
espBM::tn + V5-espBM(432ppyp)

Figure 3.4 The *espB*$_M$*::tn* mutant displays a subtle defect in cording**. (A)** Cording of *M. marinum* strains after growth in 7H9 broth to an OD of 2. **(B)** Strains were grown to an OD of 1 then inoculated into fresh 7H9 at an O.D. of 0.01 containing SDS (0.007%) or no SDS. OD_{600} readings were taken at the time points indicated. **(C)** Strains were grown to an OD of 1 then inoculated at an O.D. of 0.01 into fresh 7H9 containing the indicated concentrations of SDS. OD_{600} readings were taken at 62 hours and 87 hours after the inoculation into the fresh 7H9.

Figure 3.4A

Wildtype



espB$_M$::tn

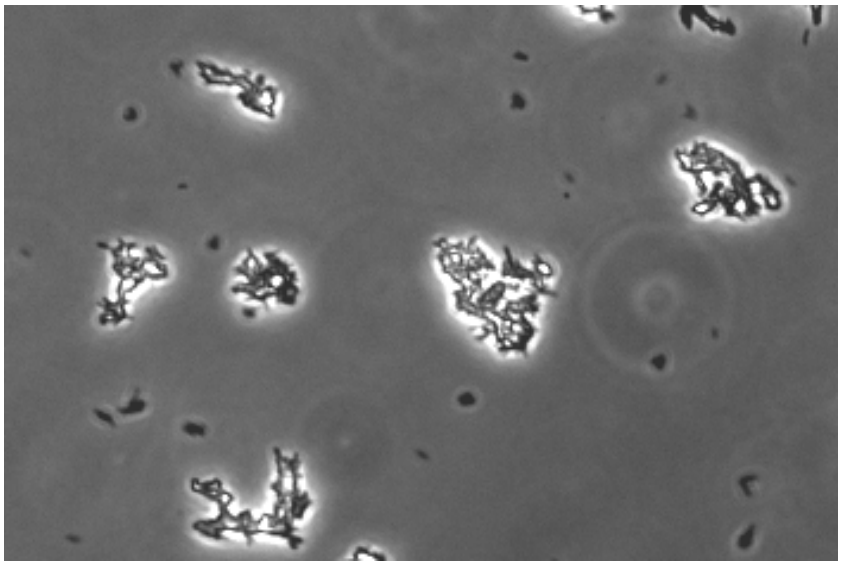
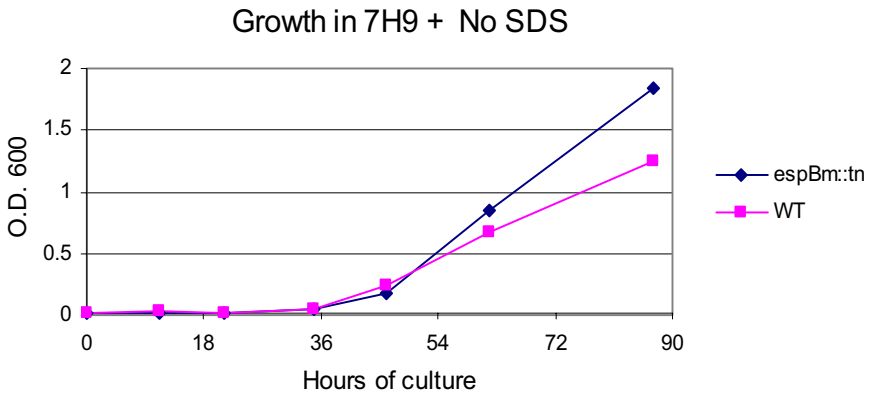


Figure 3.4B


Growth in 7H9 + No SDS
O.D. 600
2
1.5
1
0.5
0
0
18
36
54
72
90
Hours of culture
espBm::tn
WT

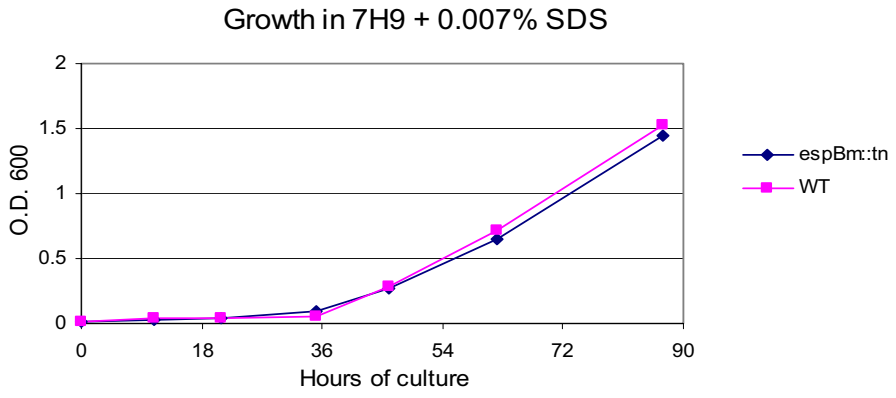
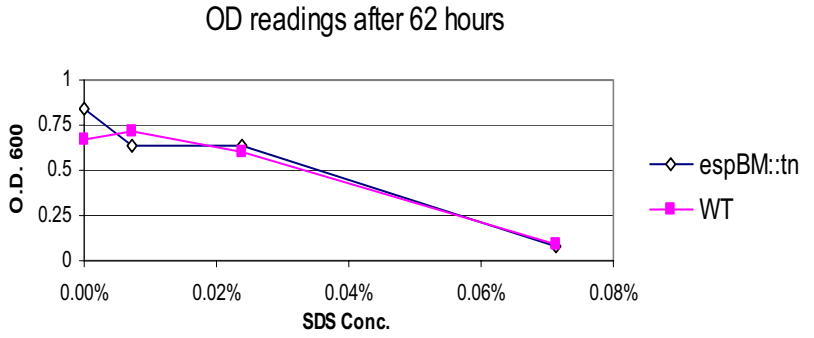

Growth in 7H9 + 0.007% SDS
O.D. 600
2
1.5
1
0.5
0
0
18
36
54
72
90
Hours of culture
espBm::tn
WT

Figure 3.4C


OD readings after 62 hours
O.D. 600
1
0.75
0.5
0.25
0
0.00%
0.02%
0.04%
0.06%
0.08%
SDS Conc.
espBM::tn
WT

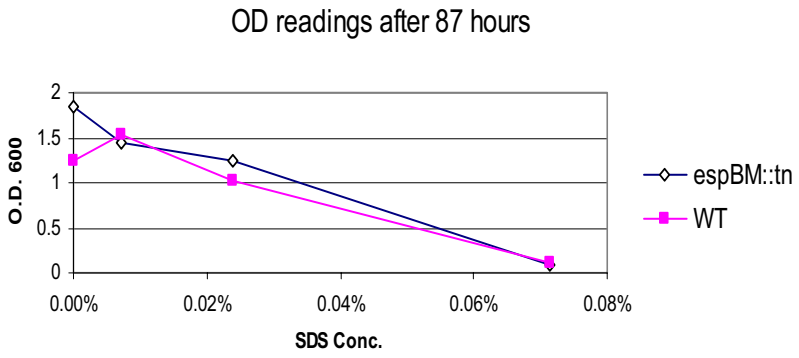
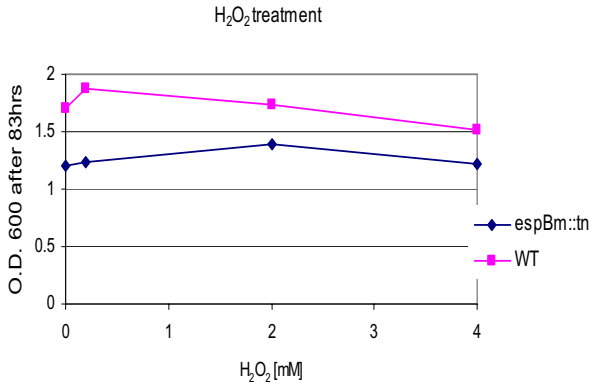
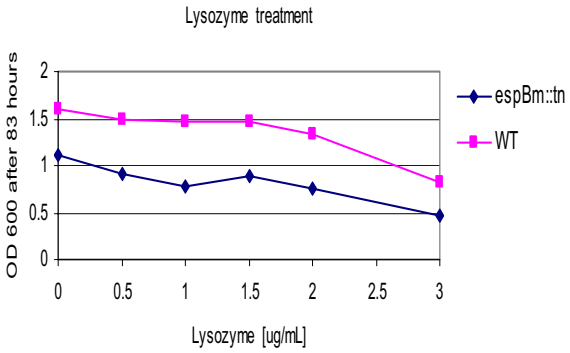
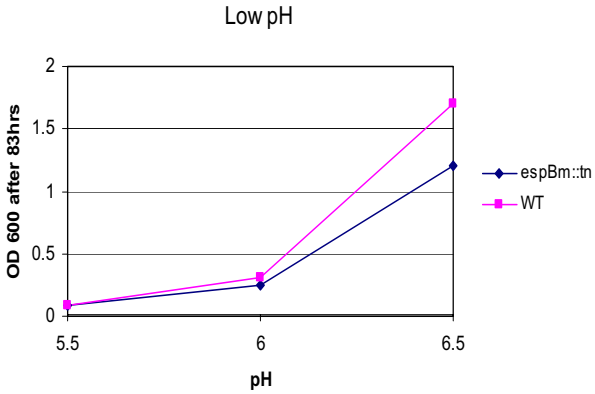

OD readings after 87 hours
O.D. 600
2
1.5
1
0.5
0
0.00%
0.02%
0.04%
0.06%
0.08%
SDS Conc.
espBM::tn
WT

Figure 3.5 The *espB$_M$::tn* mutant displays wild-type sensitivity to lysozyme, low pH, H_2O_2 and $NaNO_2$ in 7H9 liquid media. Equal volumes of wild-type and *espB$_M$::tn* were inoculated into 7H9 containing the indicated concentrations of lysozyme, $NaNO_2$, H_2O_2, or pH level. OD_{600} readings were taken at 83 hours after the inoculation into 7H9.

Figure 3.5


H_2O_2 treatment
O.D. 600 after 83hrs
2
1.5
1
0.5
0
0
1
2
3
4
H_2O_2 [mM]
espBm::tn
WT


Lysozyme treatment
OD 600 after 83 hours
2
1.5
1
0.5
0
0
0.5
1
1.5
2
2.5
3
Lysozyme [ug/mL]
espBm::tn
WT


Low pH
OD 600 after 83hrs
2
1.5
1
0.5
0
5.5
6
6.5
pH
espBm::tn
WT

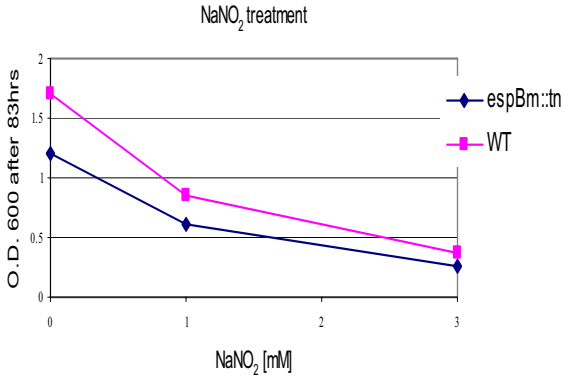

$NaNO_2$ treatment
O.D. 600 after 83hrs
2
1.5
1
0.5
0
0
1
2
3
$NaNO_2$ [mM]
espBm::tn
WT

Figure 3.6 Expression of *extRD1* genes in *extRD1* mutants during growth in 7H9. Each strain was grown in 7H9 broth to an OD of 1.0, total RNA was prepared as described in Materials and Methods, the RNA was reverse transcribed, and then the resulting cDNA was used in SYBR green based QPCR. Shown is the amplification of each gene in each mutant relative to the amplification of the respective gene in wild-type.

Figure 3.6

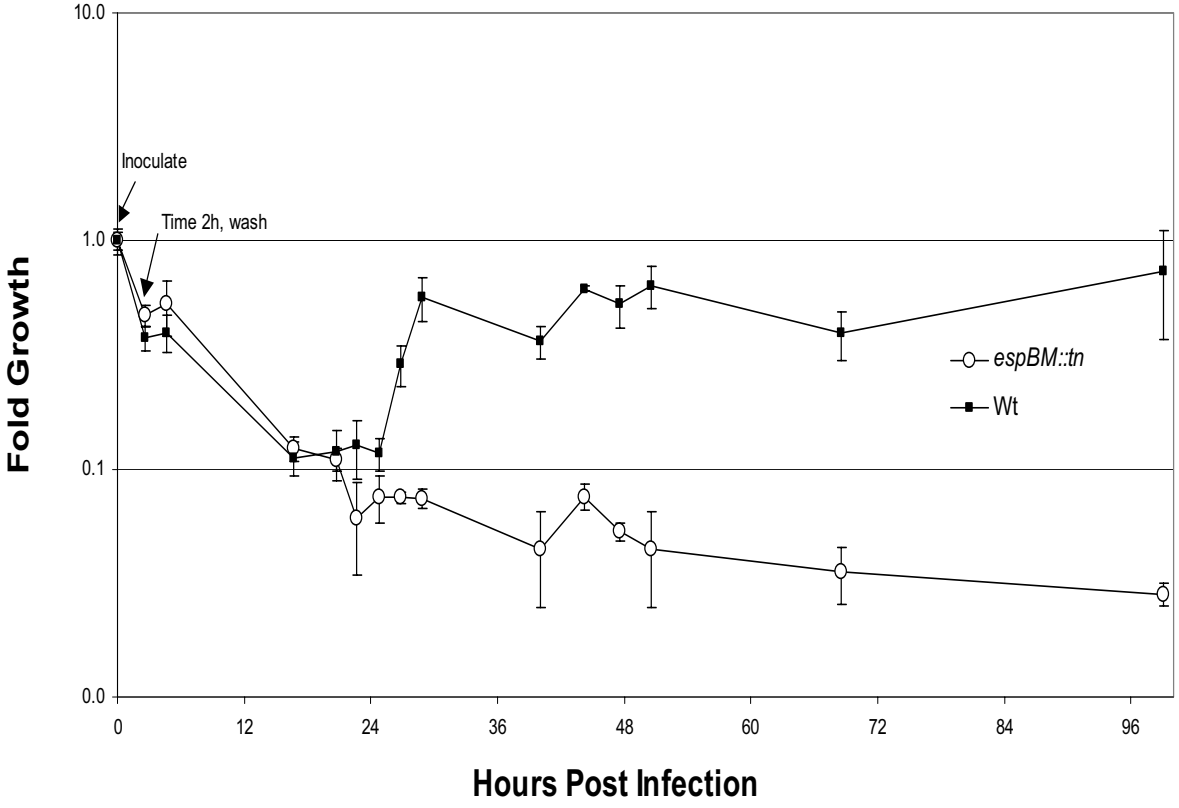
	SodA	Mh3865	Mh3866	Mh3867	Mh3868	Mh3869	Mh3870	Mh3871	Mh3872	Mh3873	Mh3874	Mh3875	Mh3876	Mh3877	Mh3878	Mh3879	Mh3880	Mh3881
WT	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
ΔRD1	1.189	0.859	0.590	0.768	0.790	0.966	0.847	0.801	0.000	0.004	0.056	0.000	0.000	0.000	0.000	0.000	1.021	0.927
Mh3866::tn	1.197	0.259	0.087	0.259	0.281	0.536	0.379	0.454	0.493	0.555	0.586	0.646	1.972	1.537	0.633	0.346	0.973	0.747
Mh3867::tn	1.173	0.664	0.319	0.252	0.224	0.412	0.358	0.633	0.467	0.460	0.514	0.669	1.932	1.591	0.540	0.266	0.966	0.927
Mh3868::tn-1	1.141	0.768	0.451	0.578	0.087	0.369	0.281	0.448	0.444	0.435	0.578	0.629	1.414	1.537	0.555	0.283	0.946	0.895
Mh3868::tn-2	0.908	0.753	0.387	0.611	0.001	0.463	0.237	0.334	0.473	0.423	0.633	0.642	2.085	1.670	0.536	0.382	0.986	1.079
Mh3868::tn-3	1.181	0.914	0.559	0.790	0.847	0.642	1.141	0.664	0.603	0.646	0.742	0.742	2.412	1.741	0.712	0.547	1.050	1.266
Mh3876::tn	1.181	0.812	0.323	0.642	0.707	0.559	1.189	1.021	0.595	0.758	0.763	0.702	2.014	0.521	0.423	0.470	1.014	1.197
Mh3878::tn	1.102	0.807	0.480	0.753	0.807	0.717	0.959	1.548	0.717	0.901	0.901	0.747	1.986	1.266	0.000	0.514	0.946	1.165
Mh3879::tn	1.094	0.763	0.551	0.753	0.824	0.722	0.801	1.181	0.712	0.883	0.933	0.742	2.028	1.366	0.559	0.136	0.973	1.454
espB $_M$::tn	1.117	0.877	0.500	0.812	0.877	0.758	0.847	1.206	0.688	0.841	0.742	0.712	1.828	1.338	0.768	0.432	0.085	0.077
espB $_M$::tn + EspB $_M$	1.064	0.727	0.423	0.540	0.559	0.642	0.578	0.835	0.536	0.532	0.563	0.702	1.404	1.057	0.486	0.310	0.060	2.158

Opcr primer pair is to the 3' side of the transposon insertion or on either side of the transposon insertion

Opcr primer pair was to the 5' side of the transposon insertion

Figure 3.7 Detailed analysis of wild-type and *espB$_M$::tn* growth in BMDM in media containing gentamicin. BMDM were infected with *M. marinum* wild-type and *espB$_M$::tn* at a multiplicity of infection of 1. After 2hr incubation and washing of infected monolayers, a media of DMEM containing 1% FBS, 1% CMG, and 5μg/mL gentamicin was placed on the monolayers. Colony-forming units (cfu) were determined by lysing infected monolayers and plating lysates at indicated time points, with growth indicated as fold increase compared to the initial level of infection for each strain.

Figure 3.7


10.0
1.0
0.1
0.0
Fold Growth
Inoculate
Time 2h, wash
espBM::tn
Wt
0
12
24
36
48
60
72
84
96
Hours Post Infection

Chapter 4

Conclusion and Perspectives

A unique substrate of the ESX-1 secretion system

Multiple genes encoding components of the ESX-1 secretion system are essential for the virulence of the *M. tuberculosis* complex as well as other species of the genus, including *M. marinum*. The most well characterized effectors, ESAT-6 and CFP-10, are among the most abundant proteins in the culture filtrate fraction of *M. tuberculosis* and *M. marinum* and the most abundant substrates of ESX-1.

Among the puzzles of ESX-1 are the identities of the less abundant substrates, the mechanism by which the components required for ESX-1 secretion function together to target and translocate substrates across the bacterial cytosolic membrane and the cell wall, and what role the ESX-1 system plays in pathogenesis. Other curiosities include the mutually dependent secretion of EspA, EspC, ESAT-6 and CFP-10 as well as the roles of the multiple ESX-1 paralogs. The current models for the mechanisms of ESX-1 substrate secretion principally derive from studies on ESAT-6 and CFP-10 and their interaction with ESX-1 components encoded within RD1. ESAT-6 and CFP-10 interact to form a tight dimer [15,29], and in the mycobacterial cell, these two proteins are interdependent on each other for stability. Rv3870 interacts in yeast two hybrid experiments with Rv3871, a cytosolic protein, and together these two proteins are thought to function as an AAA ATPase of the SpoIIIE/FtsK family. Presumably they act in concert with the proximal AAA ATPase, Rv3868, which is required for the stability and secretion of ESX-1 substrates, as well as with Rv3877, a multitransmembrane protein, which may make up the pore that spans the cytosolic membrane [15,16]. The CFP-10 carboxyterminal seven amino acids interact with Rv3871 and seem to function as a secretion signal through

which Rv3871 can recognize the ESAT-6/CFP-10 dimer, via CFP-10, and target the complex to the secretion machine.

The studies presented in Chapter 2, describe EspB as a novel substrate of the ESX-1 secretion system, which requires the *Mh3879c* and *Mh3871* genes for secretion. Further complex formation between $EspB_M$ and Mh3879c, as well as identical behavior of the *M. tuberculosis* homologs, was shown. Two mutants of $EspB_M$ that were stable after synthesis but failed to bind Mh3879c were not secreted, while mutations that did not interfere with Mh3879c binding did not disrupt secretion. Further, the carboxyterminus of Rv3879c/Mh3879c was found to be required for interaction with and secretion of EspB. Together the results suggest that an EspB/Mh3879c protein complex is required for $EspB_M$ secretion.

Complex formation between ESAT-6 and CFP-10 is required for their secretion as a heterodimer by *M. tuberculosis*. In contrast, only $EspB_M$ of the $EspB_M$/Mh3879c complex appears to be secreted. The caveat of our data is the possibility that the results merely reflect that aminoterminus of Mh3879c is quantitatively removed during or immediately after secretion, since we do not have and could not probe with antibodies to the native protein. Nonetheless, we hypothesize that Mh3879c acts as a cytosolic chaperone to deliver $EspB_M$ to the secretion machine, that Mh3879c/Rv3879c interacts directly with Mh3871/Rv3871 and that Mh3871/Rv3871, in addition to being required for the secretion of ESAT-6/CFP-10, is required for the secretion of EspB. We propose that the mechanisms of EspB and CFP-10/ESAT-6 secretion intersect at binding to Rv3871.

The requirements for the secretion of EspB by ESX-1 are distinct from the requirements for the secretion CFP10 and ESAT-6

The results presented in this thesis demonstrate a requirement for the *Mh3879c* and *Mh3871* genes in the secretion of $EspB_M$, a requirement for *Mh3868* in the stability of $EspB_M$, and suggest that the genes *Mh3866, Mh3867, Mh3876, Mh3878*, and *Mh3880c* are all dispensable for secretion of $EspB_M$. Recently, Xu et al., also found that the EspB protein is secreted in an ESX-1 dependent manner, but their results led them to propose that ESAT-6 is crucial for the secretion of EspB whereas we propose that *Mh3879c* and *Mh3871* are crucial for the secretion of EspB. Among the novel findings of Xu et al.were: 1. *M. marinum Δ(CFP-10/ESAT-6)* and *ΔESAT-6* mutants stably express EspB in the cell lysate, but do not secrete EspB; 2. A carboxyterminal truncation mutant of EspB fails to secrete ESAT-6 and CFP-10; and 3. After three hours of co-incubation with macrophages, 95% of the *$espB_M$::tn* mutant bacilli localize in an acidic compartment, while at that same time point only 23% of wild-type and 42% of *Δ(CFP-10/ESAT-6)* localize in an acidic compartment.

The most significant discrepancy between our data and their data is that we found the mutants *Mh3866::tn*, *Mh3867::tn*, and *Mh3876::tn* all secrete EspB, whereas Xu et al. did not find secretion of EspB from any of those mutants. The discrepancy here may stem from our culture filtrates being prepared from cultures that were at lower OD readings and that were more recently transferred from 7H9 media. The use of different primary antibodies might also be the cause of the discrepancy but since both antibodies react with the N-terminal portion of the protein, a difference in the preparation of culture

filtrates is the more likely explanation. A reconciliation of the two sets of results with this explanation would require us to qualify our conclusion to say that, "*in our conditions* EspB secretion is independent of CFP-10 secretion."

We found that the N-terminus of EspB interacts with Mh3879c, while Xu et al., claim to have found that the carboxyterminus of EspB binds to ESAT-6. The claim that EspB and ESAT-6 interact is based on a co-immunoprecipitation from mycobacterium cell lysates where an antibody against EspB also pulls out ESAT-6 along with EspB. The negative control, *$espB_M$::tn + espB Δ(333-455)*, did not pull down ESAT-6. These same authors showed that ESAT-6 is less stable in the cell lysate of the *espB Δ(333-455)*mutant strain. Therefore, this experiment should have been performed taking caution to normalize the amount of ESAT-6, and hence the conclusions that EspB and ESAT-6 interact were hastily drawn. Additionally, a co-immunoprecipitation with an ESAT-6 antibody does not pull out EspB along ESAT-6. Thus, the evidence suggesting that EspB and ESAT-6 interact is not convincing.

In a puzzling twist, Xu et al., found that *M. marinum Δ(CFP-10/ESAT-6)* and *ΔESAT-6* mutants stably express EspB in the cell lysate, but do not secrete EspB, while we found *M. tuberculosis ΔCFP-10* does secrete EspB. By dogma, ESAT-6 and CFP-10 depend on each other for their stability and thus each single mutant should behave identically to the double *Δ(CFP-10/ESAT-6)* mutant. ESX-1 may function differently in the two species at this particular juncture. Or else maybe the dogma is incorrect, as the *Mh3879c::tn* mutant stably secretes CFP-10 but not ESAT-6. These superficially incongruent results warrant further experimentation.

Beyond ESAT-6, CFP-10, and EspB, ESX-1 secretes at least 3 other proteins – EspA, EspC, and EspR. The secretion of EspA, EspC, ESAT-6 and CFP-10 is mutually dependent. In contrast, we found that the secretion of EspB does not require ESAT-6 or CFP-10 secretion and CFP-10 secretion does not require EspB secretion. More recently, ESAT-6 was shown to be secreted in the absence of EspR, provided that EspA and EspC are constitutively expressed[14]. One further suggestion of independent secretion of ESX-1 substrates is that the *M. marinum Δ(CFP-10/ESAT-6)* and *ΔESAT-6* mutants form rough colonies on 7H10 while *espB$_M$::tn, Mh3871::tn,* and ΔRD1 all form smooth colonies.

While EspB secretion is not mutually dependent on ESAT-6 and CFP-10 secretion the *Δ(CFP-10/ESAT-6)* and *ΔESAT-6* mutants have some sort of a functional relationship as the *espB$_M$::tn* mutant fails to secrete both ESAT-6 and CFP-10. Unfortunately, Xu et al., proclaimed co-dependent secretion while still excluding the *Mh3879c::tn* mutant from their published analysis. This may be because the *Mh3879c::tn* mutant did not fit into their model. While a relationship between ESAT-6, CFP-10, and EspB exists the mutants *Mh3879c::tn*, *Mh3866::tn* and *Mh3867::tn* grown in our growth conditions, show that the secretion of CFP-10 and EspB is not mutually dependent at least during growth in our conditions. Recently, Colorado State University has made available a library of *M. tuberculosis* transposon insertional mutants, which promise to be a valuable resource in identifying the genetic requirements of ESX-1. Also, since the gene homologous to *Rv3879c* in *M. africanum* is a psuedogene the secretion profile of *M. africanum* is of interest. Deciphering these complex relationships, and the interactions of the other ESX-1 substrates will certainly be important to better understand ESX-1–mediated secretion and mycobacterial pathogenesis.

What are the functions of ESX-1

ESX-1 is a major determinant of virulence but the way in which this system affects the biology of the host cell is unknown. Disruption of ESX-1 secretion leads to a range of diverse phenotypes, including defective growth during infection of mice and macrophages, altered phagosome trafficking, defective immune modulation, and according to one report a failure to escape phagosomes like wild-type[15]. *M. marinum* recruits macrophages to form granulomas in zebrafish embryos in an ESX-1-dependent manner and requires ESX-1 for virulence in adult zebrafish, growth in macrophages, cytolysis and cytoxicity, and cell-to-cell spread. In *M. smegmatis*, and potentially *M. ulcerans* as well, ESX-1 negatively regulates conjugal DNA transfer [10,11]. Further, ESAT-6 may function as a toxin to directly lyse cellular membranes [19].

Although, the host cell targets of ESX-1 effectors, leading to ESX-1 mediated phenotypes, remain to be deciphered we found that mutants lacking secretion of both CFP-10 and EspB had a more marked growth defect in macrophages than the mutants lacking secretion of only one substrate. This suggests that the different substrates make distinct, and potentially additive, contributions to virulence.

A contribution of thoughts to the design of new vaccines

The current vaccine for tuberculosis, BCG, is a live attenuated strain of *Mycobacterium bovis*, with a near perfect safety record in the more than 4 billion doses that have been administered. Despite its pristine safety, the protection offered by BCG is limited to young children, and even then the vaccine should be administered before the

child is exposed to environmental mycobacterium. Thus, a major goal in the efforts to reduce the incidence of TB is the development of a new, more efficacious, yet still safe vaccine. Presumably, more than one vaccine will be required as the optimal antigens and methods of immunization may vary between latently infected patients, pre and post BCG vaccinated patients, and HIV+ patients. Presently, there are more than a dozen novel vaccines designs including subunit vaccines, DNA vaccines, as well as modified BCG and attenuated *M. tuberculosis*. Both the identification of EspB as an ESX-1 substrate in addition to the data that carboxyterminally truncated EspB is secreted but not functional in terms of virulence may enable further improvements on these novel vaccine designs. Specifically, designs based on BCG with a re-integrated RD1, while more immunogenic are less safe, but maybe the best of both worlds could be achieved if this strain were further engineered to secrete ESX-1 substrates that are not functional in terms of virulence. Presumably, BCG fails to secrete EspB and therefore maybe a subunit vaccine should include EspB. Finally, since ESX-1 appears to elicit granuloma formation; further characterization of the system may lead to antigens effective in vaccinating latently infected patients.

In summary, the work of this thesis emphasizes the utility of the *M. marinum* model system to gain a better understanding of *Mycobacterial* pathogenesis. We identified a novel substrate for ESX-1–dependent secretion, demonstrated interactions of this substrate with a protein encoded within RD1, showed that a carboxyterminal alpha helix is required for the virulence function of this substrate. We found that secretion of distinct ESX-1 substrates follows variable pathways to interaction with the core secretion

machinery, and that the different substrates may contribute independently to intracellular survival and growth of the bacteria. These data further our understanding of how genes within this locus contribute to this secretion pathway and extend the understanding of a major virulence mechanism of *Mycobacteria*.

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