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

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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 (EspBM) 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 EspBM. Mutants that fail to secrete only EspBM or only CFP-10 are less attenuated in macrophages than mutants failing to secrete both substrates. EspBM physically interacts with Mh3879c; the M. tuberculosis homolog, EspB7, physically interacts with Rv3879c; and mutants of EspBM 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 [1–6]. The homologous M. marinum ESX-1 is required for virulence in zebrafish, growth in macrophages, cytosis and cytotoxicity, and cell-to-cell spread, in addition to ESAT-6 and CFP-10 secretion [7,8]. In zebrafish embryo infections, M. marinum ESX-1 is required for macrophage aggregation and granuloma formation [9]. In M. smegmatis, ESX-1, in addition to being required for secretion of ESAT-6 and CFP-10, modulates conjugal DNA transfer [10,11]. In contrast, most strains of M. ulcers, 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 [12,13]. 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 [6]. 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.

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Abbreviations: BMDM, bone marrow–derived macrophage; CFP-10, culture filtrate protein 10; EspB, ESX-1 substrate protein B; ESAT-6, early secretory antigenic target 6; ESX-1, ESAT-6 system 1; extRD1, extended region of difference 1; FAP, fibronectin attachment protein; OD, optical density; RD1, region of difference 1
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Currently, a third ESX-1 substrate, EspA (Rv3616c), was identified [14]. 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 [15–18]. 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 [1–3]. These core components include at least two putative SpoIIE/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 [14,19]. 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 [11]; an M. bovis mutant disrupted for the expression of the genes homologous to Rv3867 through Rv3869 fails to secrete ESAT-6 and CFP-10 [20]; 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) [7].

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 [15]. 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 glycine-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.
to *M. marinum* growth in macrophages when it is expressed along with *espB* from the bacterial chromosome. In contrast, *espB* 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::tn* were probed with a mouse polyclonal antibody raised against a 100 amino acid fragment of EspBT extending from amino acid 234 to 333 (Figure 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::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*ₙ 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 [21], 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, extending from amino acid 234 to 333 (Figure 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::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.

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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 2C). Although EspBM was found in both the cell lysate and culture filtrate fractions of most mutants, EspBM was not detected in the culture filtrates of MmRD1, Mh3868::tn, Mh3879c::tn, or Mh3871::tn. The Mh3868::tn mutants failed to accumulate protein in the pellet, suggesting that 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 EspBM secretion. In contrast, none of the mutants secreted ESAT-6 [7], 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 [7]. Complementation of Mh3879::tn and espB::tn restored EspBM 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 [22], was not disturbed in any of the extRD1 mutants. Thus, the product of the espBM 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. EspBM 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 S2), 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 EspBM to this mutant, suggesting parallel functions for the genes in the two species.

Figure 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.

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substrate in the secretion of ESAT-6 and EspBM, while the Rv3871 wild-type M. tuberculosis species of and its secretion requires core ESX-1 components in both is a secreted protein in both or in contrast, the Rv3871 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.

M. ΔRD1, espBΔc::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, espBΔc::tn, and Mh3871::tn were disrupted for the secretion of all three substrates: ESAT-6, CFP-10, and EspBM. In contrast, the Mh3879::tn mutant was disrupted only for the secretion of ESAT-6 and EspBM, 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, M. marinum Mh3876::tn mutant showed significant EspBM secretion (Figure 2C), any interaction between Rv3876 and EspBT is not likely to be required for EspB secretion and thus was not pursued.

To test for an analogous interaction between EspBM and Mh3879c and to confirm the potential interaction between EspBΔc 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 5A, GST-tagged EspBM, but none of the GST controls, bound specifically to V5-tagged Mh3879c. In the reciprocal experiment, GST-tagged Mh3879c bound specifically to V5-EspBM. Similarly, as shown in Figure 5B, GST-tagged EspBT bound specifically to V5-tagged Rv3879c, and GST-tagged Rv3879c bound specifically to V5-tagged EspBT. These data demonstrate that recombinant EspBT 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 EspBΔc or Rv3879c. GST-tagged Rv3879c bound to V5-tagged Mh3871, whereas the GST controls and GST-EspBT did not bind to Rv3871. This suggests that Rv3879c may facilitate EspBT 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 EspBM deletion mutants with N-terminal V5 tags and expressed them in the espBMΔc::tn mutant strain using the espBM promoter. As shown in Figure 6A, V5-tagged full-length EspBM was secreted. This N-terminally tagged protein, like native EspBM, underwent C-terminal truncation either during or after secretion. EspBM deletion mutant constructs Δ(2–31), Δ(264–271), and Δ(400–454) were stably expressed in M. marinum, but only EspBM Δ(400–454) accumulated in the culture filtrate. The secreted EspBM Δ(400–454) had a higher apparent molecular weight than the secreted full-length EspBM, 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 EspBM is dispensable for secretion, but N-terminal and internal amino acids are required. Next, we tested these EspBM mutants interacted with Mh3879c. Lysates of E. coli that express V5-tagged EspBM mutants were incubated with GST-Mh3879c. While full-length EspBM and EspBM Δ(400–454) bound to GST-Mh3879c, the stably expressed but non-secreted EspBM Δ(2–31) and EspBM Δ(264–271) constructs did not bind to GST-Mh3879c (Figure 6B). These data support a model in which EspB interacts with Rv3879c, which in turn interacts with Rv3871, to facilitate the secretion of EspB.

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 2D). Secretion of EspB 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.

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 4). An advantage of the bacterial two-hybrid system is that it can allow detection of interactions of membrane-bound proteins [23]. 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 EspBΔc target with an empty bait. In contrast, the Rv3879c bait and EspBΔc target resulted in a ratio of 7.6%, an increase of more than 75-fold. An Rv3876 bait also showed interaction above background with EspBT, but since the M. marinum Mh3876::tn mutant showed significant EspBM secretion (Figure 2C), any interaction between Rv3876 and EspBT is not likely to be required for EspB secretion and thus was not pursued.

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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 secretion defect of the Mh3879c::tn mutant, but Mh3879c-His6x and V5-Mh3879c-His6x failed to complement the secretion defect (Figure S3A). In wild-type M. marinum, V5-Mh3879c and V5-EspBM were expressed at nearly identical levels in the cell lysate, but only V5-EspBM was detected in the culture filtrate (Figure S3A). To determine whether failure of secretion reflected inefficient competition of V5-tagged protein with native protein, the V5-EspBM 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 Mh3879c::tn mutant, also was detected only in the cell lysate. Since Mh3879c-His6x failed to complement the EspB 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 that express V5-tagged Rv3879c mutants were incubated with GST alone, GST-Rv3879, or GST-EspB1. While full-length Rv3879 and Rv3879 Δ(1–166) bound to GST-EspB1, Rv3879 Δ(564–729) failed to bind to GST-EspB1 (Figure S3B). None of the constructs bound to GST alone, and all three constructs bound to GST-Rv3879. 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 EspBM. Further, we showed protein complex formation between EspB and Mh3879c, as well as identical behavior of their M. tuberculosis homologs. Two mutants of EspBM that were stable after synthesis but failed to bind Mh3879c were not secreted, while a large carboxyterminal deletion did not interfere with either Mh3879c binding or secretion. Additionally, the carboxyterminus of Rv3879c/Mh3879c is required for interaction with and secretion of EspB. These results suggest that the EspB/Mh3879c protein complex is required for EspBM 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 EspBM 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-6CFP-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 EspB1, 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 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 [7]. 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 espBM 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 (Mm.ARD1 and

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**Figure 5.** GST Pulldown Analysis of EspB and Rv3879 Interactions

(A) Agarose beads with immobilized GST, GST-SHP1, GST-syntaxin2, GST-Mh3879c, and GST-EspBM were incubated with lysates of E. coli that express V5-Mh3879c and V5-EspBM.

(B) Agarose beads with immobilized GST, GST-SHP1, GST-syntaxin2, GST-Rv3879c, or GST-EspBT were incubated with lysates of E. coli that express V5-Rv3871, V5-Rv3879c, and V5-EspBc. Cells lysates retained on the beads after washing were separated by SDS-PAGE and detected by western blotting with an antibody against V5. The right of each set of pulldowns, 0.1% of the input E. coli lyasate was analyzed. EspBM physically interacts with Mh3879c, EspBc physically interacts with Rv3879c, and Rv3879c also interacts with Rv3871.

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**Figure 6.** Deletion Analysis of EspBM Secretion and Interaction with Mh3879c

(A) The M. marinum espBM::tn mutant was transformed with a non-integrating plasmid expressing N-terminally V5-tagged EspBM full length, EspBM Δ(2–31), or EspBM 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-Mh3879c and V5-EspBM. 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. EspB M physically interacts with Mh3879c, EspB T physically interacts with Rv3879c, and Rv3879c also interacts with Rv3871.

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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 1. M. marinum strains were grown as previously described [24]. 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/

The transposon insertion in the mutant espBAC::tn lies between the 175th and 176th base pairs of the espBM gene, and the kanamycin gene within the transposon is transcribed opposite to the direction of transcription of the espBM gene. The strains M. marinum M attB::hgy and espBAC::tn attB::hgy were constructed by transforming the strains M. marinum M WT and espBAC::tn with the plasmid pMV306.hgy. The strains espBAC::tn attB::espB hgy and espBAC::tn attB::espBM-Mh3880c hgy were constructed by ligating 250 bp upstream of espB along with espBM or espBM-Mh3880c into pMV306.hgy and then transforming the resulting plasmids, pBM264 and pBM202, into espBAC::tn. The strain espBAC::tn attB::Mh3880c-Mh3880hgy was constructed by ligating 345 bp upstream of Mh3880c along with Mh3880c-Mh3880hgy into pMV306.hgy and then transforming the resulting plasmid, pBM265, into espBAC::tn. To construct the plasmids pBM41, pBM540, and pBM810, the genes Rv3871, Rv3879c, and Rv3880c were PCR amplified from the cosmids RD1-2F9 [25] and ligated into pBM510, a derivative of pET22b where the N-terminal His tag was replaced with the V5 epitope tag. To construct the plasmids pBM845 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 espBM, then the V5 epitope, then the espB gene, and finally, in the case of the pBM336 plasmid, the His6x epitope. The plasmids pBM809, 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 pBM336, re-ligation of the truncated gene fragments back into pBM332, while made in a manner synonymous to that of pBM332 and pBM336, where the Mh3879 promoter and gene were used. The plasmids pBM809, 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 pBM336, re-ligation of the truncated gene fragments back into pBM332, while made in a manner synonymous to that of pBM332 and pBM336, where the Mh3879 promoter and gene were used. The plasmids pBM809, 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 pBM336, re-ligation of the truncated gene fragments back into pBM332, while made in a manner synonymous to that of pBM332 and pBM336, where the Mh3879 promoter and gene were used. The plasmids pBM809, 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 pBM336, re-ligation of the truncated gene fragments back into pBM332, while made in a manner synonymous to that of pBM332 and pBM336, where the Mh3879 promoter and gene were used.
Table 1. List of Bacterial Strains and Plasmids Used in This Study

| Strain/Plasmid | Name | Relevant Gene Product | Genotype Using “Marinum Homolog” of H37Rv Nomenclature | Genotype Using Sanger Institute Nomenclature | Reference |
|----------------|------|-----------------------|-------------------------------------------------------|-------------------------------------------|-----------|
| **M. marinum** | WT   |                       |                                                       |                                           | [7]       |
|                | ΔRD1 |                       | Δ(mh3871-mh3879)                                       | Δ(MMS446-MMS455)                         | [9]       |
| M1             | Mh3866::tn |                       |                                                       | MMS447::tn                              | [7]       |
| M2             | Mh3867::tn |                       |                                                       | MMS442::tn                              | [7]       |
| M3             | Mh3868-::tn-1 |                       |                                                       | MMS443::tn                              | [7]       |
| M4             | Mh3868-::tn-2 |                       |                                                       | MMS442::tn                              | [7]       |
| M5             | Mh3876::tn |                       |                                                       | MMS451::tn                              | [7]       |
| M6             | Mh3878::tn |                       |                                                       | MMS454::tn                              | [7]       |
| M7             | Mh3879::tn |                       |                                                       | MMS455::tn                              | [7]       |
| M8 (esp88::tn) | Mh3881::tn |                       |                                                       | MMS457::tn                              | [7]       |
| M9             | Mh3871::tn |                       |                                                       | MMS446::tn                              | This study |
| **M. marinum 1218** | WT   |                       |                                                       |                                           | [8]       |
| MRS1459        | Mh3871::tn |                       |                                                       | MMS446::tn                              | [8]       |
| MRS1459 + pRv3871 | Mh3871::tn + Rv3871 |                       |                                                       | MMS446::tn + Rv3871                      | [8]       |
| MRS1459 + pMh3871 | Mh3871::tn + Mh3871 |                       |                                                       | MMS446::tn + Mm5446                      | [8]       |
| **M. marinum** | WT   | attB::hygr'             |                                                       |                                           | This study |
|                | esp88::tn, attB::hygr' |                       |                                                       |                                           | This study |
|                | esp88::tn, attB::esp88, hygr' |                       |                                                       |                                           | This study |
|                | esp88::tn, attB::esp88-Mh3880c hygr' |                       |                                                       |                                           | This study |
| **M. tuberculosis** | WT   |                       |                                                       |                                           | [1]       |
| Rv3870::tn     | Mh3871::tn |                       |                                                       | MMS446::tn                              | [1]       |
| Rv3871::tn     | Mh3871::tn |                       |                                                       | MMS446::tn                              | [1]       |
| ΔCFP-10        | Mh3871::tn |                       |                                                       | MMS446::tn                              | [1]       |
| **E. coli**    | pBM856 | GST-Mh3879c            |                                                       |                                           | This study |
| pBM553         | GST-Rv3879c |                       |                                                       |                                           | This study |
| pBM550         | GST-EspB M |                       |                                                       |                                           | This study |
| pBM551         | GST-EspB T |                       |                                                       |                                           | This study |
| pBM843         | VS-Mh3879c |                       |                                                       |                                           | This study |
| pBM540         | VS-Rv3879c |                       |                                                       |                                           | This study |
| pBM504         | VS-EspB M |                       |                                                       |                                           | This study |
| pBM610         | VS-EspB T |                       |                                                       |                                           | This study |
| pBM841         | VS-Rv3871 |                       |                                                       |                                           | This study |
| pGST-Shp1      | GST-Shp1 |                       |                                                       |                                           | This study |
| pGST-syntaxin2 | GST-syntaxin2 |                       |                                                       |                                           | [27]      |
| pBM589         | VS-EspB M Δ(1–166) |                       |                                                       |                                           | This study |
| pBM399e        | VS-EspB M Δ(264–271) |                       |                                                       |                                           | This study |
| pBM400e        | VS-EspB M Δ(400–454) |                       |                                                       |                                           | This study |
| pBM1010        | VS-Rv3879c, M1–166 |                       |                                                       |                                           | This study |
| pBM1013        | VS-Rv3879c Δ(564–729) |                       |                                                       |                                           | This study |
| **Mycobacterium** | pBM332 | VS-EspB M |                       |                                           | This study |
| plasmids       | pBM336 | VS-EspB M-His6x |                       |                                           | This study |
| pBM367         | VS-EspB M Δ(1–30) |                       |                                           |                                           | This study |
| pBM398         | VS-EspB M Δ(264–271) |                       |                                           |                                           | This study |
| pBM400v        | VS-EspB M Δ(400–454) |                       |                                           |                                           | This study |
| pBM869         | VS-Mh3879c |                       |                                           |                                           | This study |
| pBM870         | Mh3879c-His6x |                       |                                           |                                           | This study |
| pBM871         | VS-Mh3879c-His6x |                       |                                           |                                           | This study |
| pMV306hurg     | Empty integrating plasmid |                       |                                           |                                           | [29]      |
| pLyg206        | Empty non-integrating plasmid |                       |                                           |                                           | [7]       |
| p(esp88-Mh3880c) | EspB M |                       |                                           | EspB M and Mh3880c                       | [7]       |
| p(Mh3883c-Mh3880c) | Mh3883c through Mh3880c |                       |                                           |                                           | This study |
| p(espB T-Rv3880c) | EspB T |                       |                                           |                                           | [7]       |
ligation of 5' phosphorylated hybridized oligos that restored the frame and created the Rv3879 deletions Δ1–160 and Δ564–729. To construct the plasmid p(3H3883-3H3880), the 345 bp upstream of M. tuberculosis Rv3883 along with M. tuberculosis Rv3880 was cut by restriction digest from the plasmid pBM203 and inserted into pLYG206. To construct the plasmid pEsPT-Rv3880, 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 OD₆₀₀ in 7H9 medium. The cultures were centrifuged and washed three times with 15 mL of PBS before resuspension 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, 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 protamine inhibitory 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 incubated between each pulse for 5 min on ice 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 [1]. Total protein concentrations were determined by a Bradford assay.

**Western blot assay.** Pellet and culture filtrate fractions were prepared as described 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 immuno blotting with antibodies against EspB at a concentration of 1:5000 (mouse polyclonal to the 100 amino acid fragment of Rv3881c [234–333 aa], 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.cmbs.colostate.edu/microbiology/tb/top.htm) was used at a concentration of 1:50000, the blot of the culture filtrate fraction was developed using West Pico (Pierce), and the blot of the cell lysates were developed using West Dura (Pierce). Anti-GroEL (rabbit polyclonal, SPA-675 / SPS-875; Stressgen, http://www.asssaysdesigns.com/) was used at a concentration of 1:10000, and the blot of GroEL was developed using West Pico (Pierce). Anti-FAP [22] 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 1:29, and developed using West Pico (Pierce). HisO6 epitope was detected with a mouse monoclonal (Novagen, http://www.emdbiosciences.com/html/NVG/home.html) at a concentration of 1:5000, 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 on the chromosome Rv3884 through *Rv3883*, which were PCR amplified from genomic DNA, were cloned into the “bait” vector pBT (BacterialMatch II; Stratagene) in frame with Cl. *Rv3881e* 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' strain BL21 (F' λproλ (F' lacIq λproλ (lacIq λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λproλ (λprolo
CFP-10 (NP_218391), ESAT-6 (NP_178023), Rv3866 (NP_218383), Rv3867 (NP_218384), Rv3868 (NP_218385), Rv3870 (NP_218387), Rv3871 (NP_218388), Rv3876 (NP_218393), Rv3877 (NP_218394), Rv3878 (NP_218395), Rv3879c (NP_218396), Rv3880c (NP_218397), and Rv3881c (NP_218398).

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Author contributions. BM and EJB conceived and designed the experiments and analyzed the data. BM, JSC, JAM, and TLC performed the experiments. BM, JAM, FC, JSC, and EJB contributed reagents/materials/analysis tools. BM, FC, JSC, and EJB wrote the paper.

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