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ABSTRACT The type VII secretion systems are conserved across mycobacterial species and in many Gram-positive bacteria. While the well-characterized Esx-1 pathway is required for the virulence of pathogenic mycobacteria and conjugation in the model organism Mycobacterium smegmatis, Esx-3 contributes to mycobactin-mediated iron acquisition in these bacteria. Here we show that several Esx-3 components are individually required for function under low-iron conditions but that at least one, the membrane-bound protease MycP3, of M. smegmatis, is partially expendable. All of the esx-3 mutants tested, including the ΔmycP3ms mutant, failed to export the native Esx-3 substrates EsxHms and EsxGms, to quantifiable levels, as determined by targeted mass spectrometry. Although we were able to restore low-iron growth to the esx-3 mutants by genetic complementation, we found a wide range of complementation levels for protein export. Indeed, minute quantities of extracellular EsxHms and EsxGms were sufficient for iron acquisition under our experimental conditions. The apparent separation of Esx-3 function in iron acquisition from robust EsxGms and EsxHms secretion in the ΔmycP3ms mutant and in some of the complemented esx-3 mutants compels reexamination of the structure-function relationships for type VII secretion systems.

IMPORTANCE Mycobacteria have several paralogous type VII secretion systems, Esx-1 through Esx-5. Whereas Esx-1 is required for pathogenic mycobacteria to grow within an infected host, Esx-3 is essential for growth in vitro. We and others have shown that Esx-3 is required for siderophore-mediated iron acquisition. In this work, we identify individual Esx-3 components that contribute to this process. As in the Esx-1 system, most mutations that abolish Esx-3 protein export also disrupt its function. Unexpectedly, however, ultrasensitive quantitation of Esx-3 secretion by multiple-reaction-monitoring mass spectrometry (MRM-MS) revealed that very low levels of export were sufficient for iron acquisition under similar conditions. Although protein export clearly contributes to type VII function, the relationship is not absolute.

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One of the many strategies evolved by Mycobacterium tuberculosis to prevent clearance by the host is protein export via Esx-1 (1–3), a specialized secretion system that is also required for conjugation in Mycobacterium smegmatis (4, 5). There are four paralogous esx loci in the M. tuberculosis genome (6–8), but the functions of these Esx systems are just beginning to be revealed (9–19, 55). Whereas Esx-1 is essential for the in vivo growth of pathogenic mycobacteria, there is strong evidence that Esx-3 is essential for in vitro growth (12, 13, 19, 20). Building on observations that esx-3 expression responds to iron and zinc availability (21, 22), we and others have demonstrated that Esx-3 is required for mycobacterial growth in low iron (12, 13, 19). Mycobacteria acquire iron by at least two siderophore pathways—exochelin, present in fast-growing species, such as M. smegmatis, and mycobactin, present in nearly all species (23–25)—in addition to a porin-based, low-affinity iron transport system (26) and the heme uptake system (27, 28). Epistasis experiments using M. smegmatis strains with deficiencies in Esx-3 and in the production of exochelin or mycobactin show that Esx-3 functions in iron acquisition via the mycobactin pathway (13). Moreover, addition of purified, iron-bound mycobactin does not rescue the low-iron growth defect, suggesting that Esx-3 is required for optimal utilization of the siderophores (13). The organizations and contents of the esx-1 and esx-3 loci are similar (8). Both encode small, secreted proteins; Esx-1 contains EsxB (Cfp-10) and EsxA (Esat-6), and Esx-3 contains the paralogous EsxG and EsxH proteins (Fig. 1). We use the systematic nomenclature proposed by Bitter et al. (29). Genes that flank esxB/A and esxG/H include those encoding EccC3 (a putative FtsK/ SpoIIIIE ATPase that is paralogous to EccCa1/EccCb1 where the “a” and “b” suffixes indicate the parts of the split gene and the subscript number refers to the esx-1 gene cluster), EspG3 (a putative soluble protein of unknown function that is paralogous to EspG1), EccD3 (paralogous to the hypothesized secretion channel...
protein EccD1, and MycP3 (paralogous to the membrane-bound protease MycP1) (8).

Multiple studies on the Esx-1 system support a model in which the FtsK/SpoIIIE ATPase EccCa1/EccCb1 provides energy to propel EsxB and EsxA across the cytoplasmic membrane via a translocation pore composed of EccD1 (30–32). It is not yet clear how type VII substrate proteins cross the cell envelope. Esx-1 may simply export these proteins across the cell envelope into the extracellular space. Alternatively, it may act only across the cytoplasmic membrane and require a mechanism for driving substrates across the remainder of the thick mycobacterial cell wall. Such a structure may be composed of yet-unidentified components or of EsxA, EsxB, and possibly other unlinked Esx-1 substrates (33, 34).

EspG1, EccCa 1/EccCb1, EccD 1, and MycP 1 are required for both EsxB and EsxA export and Esx-1 function in most mycobacterial species tested (3, 5, 35–41), prompting early speculation that EsxB and EsxA are the effector proteins of the secretion system. However, there have since been reports of several Esx-1 mutations that abolish function without affecting EsxB and EsxA export (4, 35, 42, 43), as well as two genetic perturbations that prevent M. tuberculosis EsxB (EsxBmt) and EsxAmt secretion but do not alter M. tuberculosis virulence (44). These studies suggest that the relationship between protein export and Esx function may be more complicated than previously assumed.

Esx-3 exports EsxH (13) and, as we show here, EsxG. We found that several M. smegmatis Esx-3 components were individually required for export of EsxHms and EsxGms and for iron acquisition. However, we also observed low or even no detectable secretion for some strains that were able to grow to wild-type levels in low iron. The apparent separation of the phenotypes suggests new models for the associations between Esx structure and function.

RESULTS

Esx-3 components are required for low-iron growth. We have previously shown that Esx-3 is required for mycobacterial growth in low-iron medium via the mycobactin pathway (13). The secretion system is essential for growth in M. tuberculosis (12, 13, 20), however, complicating efforts to test the contributions of individual Esx-3 components to the function of the entire system. The model organism M. smegmatis can grow without functional Esx-3 in normal growth medium. We therefore constructed unmarked, in-frame deletions of esx-3 genes in M. smegmatis (see Fig. S1 in the supplemental material). Because M. smegmatis has partially redundant siderophore-based iron acquisition mechanisms, i.e.,

**FtsK/SpoIIIE-like ATPase**  
- AAA+ chaperone  
- Inner membrane  
- Serine protease  
- Transmembrane  
- Unknown

**FIG 1** Schematic diagram of gene conservation in mycobacterial esx-1 and esx-3 loci. **∗**, the M. smegmatis esx-1 locus has a slightly different organization than the M. tuberculosis region from espJ to espB (5, 8); **∗∗**, there is no MSMEG_0625, mycP3ms is MSMEG_0624, and eccE3ms is MSMEG_0626. We use the nomenclature proposed by Bitter et al. (29). Briefly, the terms ecc and esp, respectively, stand for esx conserved component and Esx-1 secretion-associated protein. The alphabetic suffix of conserved esx genes follows the gene order in the esx-1 locus. The numerical subscript at the end of the gene name refers to the esx cluster to which the gene belongs. We also provide the standard M. tuberculosis and M. smegmatis gene numbers at the beginning and end of each locus for comparison. Although not listed in the NCBI or SmegmaList databases, MSMEG_0620 is esxGms, and MSMEG_0621 is esxHms.
the mycobactin and exochelin pathways (24), we combined each esx-3 deletion with an insertional mutation in fxbA, which encodes a formyl transferase required for exochelin synthesis. Previously, we found that the fxbA \(\Delta\)eccC\(_{3ms}\) mutant grows significantly more slowly than the fxbA strain in low-iron medium (13). Although the fxbA \(\Delta\)esxGH\(_{ms}\), fxbA \(\Delta\)espG\(_{3ms}\), and fxbA \(\Delta\)eccD\(_{3ms}\) mutants display similar low-iron growth deficiencies, the fxbA \(\Delta\)mycP\(_{3ms}\) strain has a less pronounced defect (Fig. 2 and 3). These strains are rescued by the presence of iron (Fig. S2) and upon reintroduction of the corresponding esx-3 gene (13) (Fig. 2). Thus, \(M.\) smegmatis growth in low iron requires the Esx-3 components EccC\(_{3ms}\), EsxG\(_{ms}\)/EsxH\(_{ms}\), EspG\(_{3ms}\), and EccD\(_{3ms}\), with a more minor contribution from MycP\(_{3ms}\).

**Esx-3 components contribute to optimal mycobactin utilization.** Previously, we constructed an \(M.\) smegmatis strain that contains insertions in both fxbA, described above, and mbtD, which encodes a polyketide synthase required for mycobactin synthesis (13). This mutant, which lacks both means of high-affinity iron uptake, does not grow in iron-depleted medium but can be rescued by the addition of purified, iron-bound mycobactin or carboxymycobactin (13). However, the siderophores fail to rescue the fxbA \(\Delta\)esx-3 mutant, suggesting that Esx-3 is required for optimal utilization of iron bound to mycobactins (13). In the absence of the exochelin pathway, deletion of the esx-3 gene eccC\(_{3ms}\), esxGH\(_{ms}\), espG\(_{3ms}\), or eccD\(_{3ms}\) impairs iron-bound mycobactin utilization in \(M.\) smegmatis to an extent similar to that after removal of the entire Esx-3 system, whereas deletion of mycP\(_{3ms}\) has a more modest effect (Fig. 3). We conclude that the Esx-3 components EccC\(_{3ms}\), EsxG\(_{ms}\)/EsxH\(_{ms}\), EspG\(_{3ms}\), and EccD\(_{3ms}\), are critical to the function of the \(M.\) smegmatis Esx-3 system in mycobactin-mediated iron acquisition.

**Secretion of EsxH\(_{ms}\) and EsxG\(_{ms}\) requires Esx-3 components.** Secretion of EsxB and EsxA is generally linked to Esx-1 function; that is, most mutations that abolish export of these proteins also inhibit virulence (\(M.\) tuberculosis and \(M.\) marinum) or conjugation (\(M.\) smegmatis) (3–5, 35–40). Our work on the Esx-3 system demonstrates that EccC\(_{3ms}\), EsxG\(_{ms}\)/EsxH\(_{ms}\), EspG\(_{3ms}\), and EccD\(_{3ms}\) are required for function in mycobactin-mediated iron acquisition and that MycP\(_{3ms}\) plays a more limited role (Fig. 2 and 3). Previously, we showed that export of heterologously expressed, myc-tagged EsxH depends on iron levels and on the presence of an intact Esx-3 locus (13). To test whether the loss of individual Esx-3

**FIG 2** Esx-3 components contribute to low-iron growth. The growth of the fxbA and fxbA esx-3 \(M.\) smegmatis mutants in low-iron medium was monitored by determining their optical densities at 600 nm. pJ, pJEB402 vector; pJ-[gene name], pJEB402 containing the indicated \(M.\) smegmatis gene. The experiments were performed 2 to 6 times in triplicate. Representative data are shown, and error bars represent the standard deviations from the replicates.
components similarly influences protein export, we monitored the abundance of representative EsxG<sub>ms</sub> and EsxH<sub>ms</sub> peptides in culture filtrates and selected whole-cell extracts by targeted, quantitative mass spectrometry (MS). Assays for peptides from each of these proteins were constructed using stable-isotope dilution MS (SID-MS) and multiple-reaction-monitoring MS (MRM-MS) (45, 46). For these experiments, we grew strains with intact exochelin production in medium with a level of iron chelation that induces EsxH secretion (13) but does not produce differences in growth. Deletion of the esx-3 gene <i>eccC<sub>3ms</sub></i>, <i>exxGH<sub>3ms</sub></i>, <i>espG<sub>3ms</sub></i>, or <i>eccD<sub>3ms</sub></i> results in supernatant levels of EsxH<sub>ms</sub> and EsxG<sub>ms</sub> that are below the limit of quantitation (LOQ) across two biological replicates (Fig. 4 and S3 to S5 in the supplemental material). Interestingly, although the <i>mycP<sub>3ms</sub></i> mutation causes a much smaller decrease in low-iron growth than the other mutations (Fig. 2), there is a comparable decrease in supernatant peptides in three of the four data sets to levels below the LOQ (Fig. 4 and S3 to S5).

We find most of the detectable EsxH<sub>ms</sub> and EsxG<sub>ms</sub> in the supernatant fraction of wild-type <i>M. smegmatis</i> (Fig. 4 and S5). To test whether the observed lack of secretion by the <i>esx-3</i> mutants reflects a general decrease in protein expression, we also compared the whole-cell extracts of the wild type, <i>ΔespG<sub>3ms</sub></i> mutant, and complemented <i>ΔespG<sub>3ms</sub></i> strain. The amounts of EsxH<sub>ms</sub> and EsxG<sub>ms</sub> were similar across the samples (Fig. S5), suggesting that the observed change in supernatant abundance accurately reports an export defect. The general lack of protein accumulation in the whole-cell extract, furthermore, implies that the cell tightly regulates the abundance of what we hypothesize is a small, cytoplasmic pool of EsxH<sub>ms</sub> and EsxG<sub>ms</sub>.

These data were consistent with our previous findings that <i>esx-3</i> is required for the export of EsxH<sub>ms</sub>-myc (13). Unlike native EsxG<sub>ms</sub> and EsxH<sub>ms</sub>, however, the tagged protein accumulates in the bacterial cytoplasm to robust levels (13). Therefore, we confirmed the mass spectrometry findings by constructing a new plasmid that constitutively expresses <i>esxG<sub>ms</sub></i> and FLAG-tagged <i>esxH<sub>ms</sub></i> and comparable amounts of EsxH<sub>ms</sub>-FLAG in cell-associated and supernatant fractions of wild-type and <i>esx-3</i> mutant <i>M. smegmatis</i> strains. In agreement with the MRM-MS results, we were consistently unable to detect EsxH<sub>ms</sub>-FLAG from the supernatants of the <i>ΔeccC<sub>3ms</sub></i>, <i>ΔespG<sub>3ms</sub></i>, <i>ΔeccD<sub>3ms</sub></i>, and <i>ΔmycP<sub>3ms</sub></i> mutants or from any of the whole-cell extracts (Fig. 5).

**DISCUSSION**

We found that EccC<sub>3ms</sub>, EspG<sub>3ms</sub>, and EccD<sub>3ms</sub> are core <i>esx</i>-3 components that are required for both mycobactin-mediated iron acquisition and EsxG<sub>ms</sub> and EsxH<sub>ms</sub> export. The <i>M. tuberculosis</i> homologs EccC<sub>3mt</sub>, EspG<sub>3mt</sub>, and EccD<sub>3mt</sub> are all predicted to be necessary for <i>in vitro</i> growth (20, 47, 48). The <i>esx-1</i> paralogs EccCa<sub>1</sub>/EccCb<sub>1</sub>, EspG<sub>1</sub>, and EccD<sub>1</sub> are required for virulence in pathogenic mycobacteria and conjugation in <i>M. smegmatis</i> and, with the exception of EspG<sub>1mt</sub> (35, 42), for EsxB and EsxA export (3–5, 35–40).

We have also identified a potential accessory <i>esx-3</i> component, MycP<sub>3y</sub>, that is necessary for EsxG and EsxH export (Fig. 4 and 5) but not absolutely required for mycobactin-mediated iron acquisition (Fig. 2 and 3). The first observation is not unexpected, as mutants that lack MycP fail to secrete EsxB and EsxA (36, 41). The data on the contribution of MycP to Esx function are less clear; although MycP<sub>1mt</sub> is required for DNA transfer in <i>M. smegmatis</i> to the same or greater extent as other <i>esx-1</i> components (4, 36), <i>mycP<sub>1mt</sub></i> disruption in <i>M. tuberculosis</i> results in a delayed phenotype in mice compared to the phenotypes resulting from transposon insertions in other <i>esx-1</i> genes (49). More recent work corroborates an <i>in vivo</i> growth defect from loss of MycP<sub>1mt</sub> (41), but the lack of a direct comparison to other <i>esx-1</i> mutant strains precluded analysis of the relative defect. Like the other <i>esx-3</i> genes, <i>mycP<sub>3ms</sub></i> is essential for <i>M. tuberculosis</i> growth in <i>in vitro</i> (47). Given that MycP<sub>1</sub<y and MycP<sub>3</sub>y likely have different substrate specificities...
**FIG 4** EsxG<sub>ms</sub> (A) and EsxH<sub>ms</sub> (B) abundances in wild-type and esx-3 mutant supernatants. Protein concentrations for EsxG<sub>ms</sub> and EsxH<sub>ms</sub> were approximated, respectively, by measuring the concentration of the FVEVSAK peptide alone and by summing the individual concentrations above the LOD of three of the methionine forms of AMATTHEQNTMAMSAR peptides (Fig. S4). Dotted lines show the LOQ (Fig. S4). The experiments were performed in technical replicate across two biological replicates. The protein abundance data from one of the biological replicates are shown here in graphical and table format, and the data from the other replicate are reported in the Fig. S5 table. comp, complemented; ND, none detected.

**FIG 5** Export of epitope-tagged EsxH<sub>ms</sub> in the presence or absence of Esx-3 components. Anti-FLAG immunoblotting of whole-cell lysates (wcl) and culture supernatants (sup) from wild-type and esx-3 M. smegmatis containing pSYMP-esxGH<sub>ms</sub>-FLAG in low-iron medium. All strains contain either the empty pJEB402 vector or pJEB402 containing the complementing gene. The antibody against the intracellular protein RNAP is a loading and lysis control. The experiment was performed twice with similar results.
(41, 50, 51), it may be that the relative importance of MycP as an 
Esx component varies according to the secretion apparatus with 
which it associates.

Despite our attempts to avoid polar effects by constructing 
in-frame, unmarked, full gene deletions, it is possible that the 
mutations altered the expression of downstream genes. However, 
this does not appear to be the case for at least the ΔmycP3ms 
muntant, as neither mycP3ms alone nor mycP3ms alongside the down-
stream espF1mt restored EsxHms-FLAG export (not shown). Tran-
scription of the esp-3 locus varies according to iron and zinc 
availability (13, 21, 22). It is possible that expression of the com-
plementing genes from a heterologous, constitutive promoter al-
tered the stoichiometry of Esx-3 components, which in turn re-
sulted in suboptimal protein secretion.

We attempted to measure secreted proteins using native anti-
odies and Western blotting. However, the antisera that we were 
able to obtain had low affinity and poor specificity, making quan-
tification difficult. Given the tendency of secretion systems to be 
 refractory to reporter fusions (52), we turned to MRM-MS to 
measure bacterial protein export. This label-free method is highly 
specific and sensitive and is likely to have broad applicability to 
bacterial protein secretion studies (53).

Precise quantitation of secretion by MRM-MS revealed a sur-
prising dynamic range in the levels of EsxG and EsxH export that 
support iron acquisition. We were able to restore esp-3 mutant 
growth to wild-type levels by adding iron to the growth medium 
(Fig. S2) or by complementing the deleted genes (Fig. 2). How-
ever, the same genetic constructs varied widely in their abilities to 
restore EsxGms and EsxHms export (Fig. 4 and 5). Only a fraction of 
secreted wild-type EsxGms and EsxHms levels appeared necessary 
for complementation; we observed robust low-iron growth 
(Fig. 2) concomitant with protein export that spanned 3 orders of 
magnitude, from <1% to approximately 40% of wild-type levels 
(Fig. 4). We recently found that the essential drug targets dihydro-
folate reductase and b-alanine racemase are present in excess (54). 
It is possible that wild-type M. smegmatis exports EsxGms and 
EsxHms at levels much greater than those needed to support low-
iron growth. This raises the possibility that the locus has multiple 
fractions, each with different secretion requirements.

Indeed, there is growing appreciation that the relationship be-
tween Esx protein export and function is more complex than ini-
tially assumed. There are now numerous reports of Esp-1 muta-
tions that attenuate pathogenic mycobacteria without impacting 
secretion. For example, complementation of the natural esp-1 mut-
ant M. microti (Fig. 1) with a panel of cosmids containing intact 
or mutant versions of the M. tuberculosis esp-1 region revealed that 
espF1ms and espG1ms are required for virulence but not EsxBmt and 
EsxAmt export (35). Deletion of these genes from M. tuberculosis 
also resulted in attenuation without impacting the secretion of 
EsxBmt and EsxAmt (42). In a different example, disruption of 
disulfide bond formation in the Esp-1 substrate EspA attenuated 
M. tuberculosis virulence but had no effect on EsxBmt or EsxAmt 
secretion (43). Finally, transposon insertions in espF1ms, espKmt, 
and espBmt impaired conjugation but not EsxBmt export (4). In 
aggregate, these studies show that secretion of EsxB and EsxA is 
not sufficient for Esp-1 function in virulence.

Recently, Chen and coworkers isolated two point mutations of 
EspAmt that block EsxBmt and EsxAmt export in vitro but do not 
attenuate M. tuberculosis (44). Although they did not rule out a 
role for the host environment in permitting secretion in vivo, these 
data suggest that export of these proteins, at least in large amounts, 
may not be strictly required for virulence. Similarly, we found a 
mutation that inhibits Esx protein secretion but permits partial 
function: loss of mycP3ms, blocked the export of native EsxGmt 
and EsxHmt and FLAG-tagged EsxHms (Fig. 4 and 5), yet the fxbA 
ΔmycP3ms mutant retained some ability to grow in low iron 
(Fig. 2). We also found that very low quantities of EsxGms and 
EsxHms secretion were sufficient to reinstate low-iron growth to 
some of our complemented esp-3 mutants (Fig. 2 and 4). Impor-
tantly, dissection of the esp-3 system in the model organism 
M. smegmatis allowed us to compare the two phenotypes, EsxG 
and EsxH export and mycobactin-mediated iron acquisition, un-
der similar in vitro conditions.

Why are not protein secretion and iron utilization completely 
congruent in our mutant strains? This is especially puzzling given 
that the genome of M. smegmatis, unlike M. tuberculosis, does not 
encode the closely related paralogs EssR and EssS, which might 
otherwise be hypothesized to substitute for EssG and EssH func-
tion (8). One reason may be the existence of multiple Esp sub-
strates, each with its own requirements for export and contribu-
tions to function. The roles of EspG and EspB are particularly 
informative in this regard. Inactivation of the paralogous espG1, 
espG2, or, as we show in Fig. 4, espG3 gene generally prevents the 
export of Esp substrate proteins (11, 17, 37, 42). However, in 
M. tuberculosis, loss of EspG1ms did not produce an obvious phe-
notype (55), while EspG1mt was required for full virulence but not 
EsxBmt or EsxAmt secretion (35, 42). Interestingly, EspG1 and 
EspG2 have been shown to interact specifically with cognate Pro-
Glu (PE)/Pro-Pro-Glu (PPE) proteins (56, 57) and have been pro-
posed to serve as chaperones for Esx secretion of these substrates 
(57). These data suggest that the PE/PPE proteins have export 
requirements and functional contributions distinct from those of 
other type VII substrates. Similarly, the secretion and activity of 
the Esp-1 substrate EspB appear to be independent of EssA (58, 
59) and EssB (60).

Our data are consistent with at least three general models. It is 
conceivable that loss of Esp-3 function produces global changes in 
cell wall structure such that protein localization is affected by 
changes both in secretion and in the compartment into which 
translocation occurs. We note that while esp-3 mutants do not 
have altered sensitivities to SDS, vancomycin, rifampin (Fig. 55), 
ampicillin, or kanamycin (19), at least when iron is not limiting, 
we are unable to rule out this model. A second possibility is that 
Esp-3 secretes a protein or proteins necessary for iron acquisition 
and that this protein requires the core components EccC, EccD, 
and EspG, but not EssG, EssH, or MycP for export. Although it 
is possible that Esp-3 function in iron acquisition does not require 
any EssG and EssH secretion, we think that this is unlikely given 
that the fxbA ΔessGHmt strain has a complementable growth phe-
notype in low iron. A third model is that both EssG and EssH are 
substrates and structural components of or chaperones for the 
Esp-3 machinery. In this scenario, EssG and EssH are exported 
across the cytoplasmic membrane via EccD into the periplasm, 
where they are poised to deliver yet-unidentified effectors of ferri-
mycobactin uptake across the remainder of the cell wall (Fig. 6). If 
EssG and EssH indeed act within or across the mycobacterial 
envelope, some of the protein detected in the supernatants of 
broth-grown mycobacteria may represent sloughing of protein 
associated with the cell wall (33, 34).

While type VII secretion systems have important functions

6 mBio mbio.asm.org May/June 2014 Volume 5 Issue 3 e01073-14
both in vitro and in vivo, their molecular mechanisms remain unclear. Clearly, protein export contributes to but does not entirely account for type VII function. More complete models for type VII structure-function relationships will require better characterization of secreted components and assays for protein-protein interactions that occur between Esx components in the cytosol or membrane (55, 61).

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** *M. smegmatis* was cultured in chelated Sauton’s medium containing 60 ml glycerol, 0.5 g KH$_2$PO$_4$, 2.2 g citric acid monohydrate, 4 g asparagine, and 0.05% Tween 80 per liter. After adjusting the pH to 7.4, the medium was stirred for 1 to 2 days at room temperature with 10 g Chelex 100 resin (Sigma). The medium was filtered, and 1 g MgSO$_4$·7H$_2$O was added as a sterile solution. For iron starvation experiments, bacteria were first inoculated from frozen stocks into 7H9 medium, subcultured once in chelated Sauton’s medium, and then diluted 1:1,000 in chelated Sauton’s medium without antibiotics containing either 100 μM 2,2’-dipyridyl or 12.5 μM FeCl$_3$. For mycobactin complementation experiments, bacteria were directly diluted 1:1,000 from a 7H9 starter culture into chelated Sauton’s medium with or without ferri-mycobactin S.

**Strain construction.** To create the *M. smegmatis* esx-3 gene deletions, 1-kb regions flanking eccC$_3$ms, espG$_3$ms, eccD$_3$ms, mycP$_3$ms, and esxGH$_{ms}$ were amplified from *M. smegmatis* genomic DNA, stitched together by PCR, and cloned into the suicide vector pJM1. The pJM1 vector contains a hygromycin-chloramphenicol resistance cassette and the counterselectable marker sacB. *M. smegmatis* transformants were screened by PCR using primers specific to the flanks as well as to regions within the putative deletion. Candidates were confirmed by PCR using multiple primers outside the flanks. To construct fxbA insertional mutants, the esx-3 deletion strains were transformed with the pSES-fxbA suicide vector (13) and screened by standard methods. The absence of exochelin production was confirmed for candidate mutants by patching them to chrome azurol (CAS) agar.

Complementing constructs for each mutant were constructed by am-
plifying the regions from genomic *M. smegmatis* DNA and cloning them under the MOP promoter in the integrative pJEB402 plasmid (38). The construct containing *esxG*<sub>ns</sub> and *esxH*<sub>ns</sub> C-terminally tagged with FLAG was generated by amplifying the region from genomic *M. smegmatis* DNA and cloning it under the control of the *hsp60* promoter of pSYMP (62).

### SID, MRM-MS. (i) Labeled-peptide internal standards. Figure S3 shows the amino acid sequences of the proteins *EsxG*<sub>ns</sub> and *EsxH*<sub>ns</sub> and the peptides that were selected for quantitative analysis of these proteins by multiple-reaction-monitoring mass spectrometry (MRM-MS). A peptide from each of the proteins was selected based on their detection in the discovery data by high electrospray MS signal responses and because they have unique sequences as determined by a search of the nonredundant protein database (NCBI nr). Four different versions of the peptide AMATTHEQNTMAMSAR for the *EsxH*<sub>ns</sub> protein were selected, all of which were observed in the discovery data.

(ii) Peptide synthesis. Five signature peptides from the two proteins, *EsxG*<sub>ns</sub> (PFEVSAK) and *EsxH*<sub>ns</sub> (AMATTHEQNTMAMSAR, with four forms of differing oxidized methionine states), were synthesized with a single, uniformly labeled \({\text{[13C}_6\text{]}}\)lysine or \({\text{[13C}_6\text{]}}\)arginine at their C termini by New England Peptide (Gardner, MA). Unlabeled \({\text{[13C}_6\text{]}}\)-labeled forms of each peptide were also synthesized by New England Peptide. Synthetic peptides were purified to \(\geq 99\%\) purity and analyzed by amino acid analysis (New England Peptide). Calculations of concentrations were based upon amino acid analysis.

(iii) MRM-MS assay configuration. The limits of detection and quantification (LOD and LOQ, respectively) for the signature peptides used to obtain quantitative measurements in each of the 22 samples (5 mutants, 5 complemented mutants and 1 wild-type strain in each of two biological process replicates) are shown in Fig. S4. A 12-point response curve was generated by spiking light peptide versions of the 5 analyte peptides over a range of 0 to 50 fmol/\(\mu\)g of digest supernatant protein and a fixed amount of heavy, internal-standard peptide (1 fmol/\(\mu\)g) of the supernatant protein mix from the \(\Delta_{\text{esxGHns}}\) sample. This supernatant of the \(\Delta_{\text{esxGHns}}\) sample was used as the background matrix for response curve generation, as it does not express the proteins of interest. Each concentration point was analyzed by liquid chromatography (LC)–MRM-MS on a Waters Xevo TQ mass spectrometer (Milford, MA) in three technical replicates. The LOD was determined by the Linnet statistical method, and the lower LOQ was calculated as 3 times the LOD. The blank sample consists of the \(\Delta_{\text{esxGHns}}\) sample with only the isotopically labeled (heavy) peptides spiked in.

(iv) Nano-LC–MRM-MS. Tryptic peptides were prepared from culture supernatants (see below) and reconstituted in 80 \(\mu\)l of 0.1% formic acid, and 1 \(\mu\)g/\(\mu\)l of each sample was used for MRM-MS analysis. Nano-LC–MRM-MS was performed on a Xevo TQ mass spectrometer (Waters Corporation, Milford, MA) coupled to a Nano Acuity LC system. Chromatography was performed with solvent A (0.1% formic acid) and solvent B (100% acetonitrile in 0.1% formic acid). Each sample was injected with a full-loop injection of 1 \(\mu\)l on a Waters, packed, Reprosil, 3-\(\mu\)m bead column (75-\(\mu\)m internal diameter [ID], 10-\(\mu\)m ID tip opening) with a 2.5-in by 20-\(\mu\)m ID spray needle. Sample was eluted at 300 nl/min, with a gradient of 3 to 7% solvent B for 8 min, 7 to 40% solvent B for 34 min, and 40 to 90% solvent B for 3 min, with a total data acquisition time of 80 min. Data were acquired with a stable column temperature of 35°C. Collision energy (CE) was optimized for the maximum transmission and sensitivity of each MRM transition by LC–MRM-MS. Three transitions were monitored per peptide and acquired at unit resolution and sensitivity of each MRM transition by LC–MRM-MS. Three replicate injections were performed for each sample. The limits of detection and quantification were determined by the following equation: measured concentration = peak area ratio \(\times\) (1 fmol/\(\mu\)l internal standard).

### Sample preparation for MRM-MS analysis. Strains were inoculated from frozen stocks in 7H9 medium with appropriate antibiotics and grown with shaking for 48 h. The cells were then washed twice in chelated Sauton’s medium, normalized by their optical densities at 600 nm (OD<sub>600</sub>), and inoculated 1:50 into chelated Sauton’s medium. Cultures were grown for 48 h to log phase, and 8 OD units were harvested. Bacteria in the pellets were lysed by bead beating, and the lysates were stored at \(-80°C\). Protein from the supernatant was precipitated by the trichloroacetic acid (TCA) method and dissolved in urea-ammonium-bicarbonate buffer (8 M urea, 50 mM ammonium bicarbonate). Protein concentration was measured by the Bradford assay of diluted samples.

One hundred micrograms of protein was reduced by 20 mM dithiothreitol (DTT) and alkylated using 50 mM iodoacetamide. Prior to being digested with trypsin, samples were diluted to a urea concentration of 0.6 M by the addition of 50 mM ammonium bicarbonate. Trypsin (Promega Gold) digestion was carried out at an enzyme-to-substrate ratio of 1:50. The peptides were desalted using Sep-Pak cartridges (Sep-Pak C<sub>18</sub> 1-cc [50-mg] Vac cartridges; Waters) as described by the manufacturer. In the final step, samples were eluted in 80% acetonitrile and 0.1% formic acid and evaporated to complete dryness in a vacuum centrifuge.

### Immunoblotting

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01073-14/-/DCSupplemental.

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