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<td>Published Version</td>
<td>doi:10.1128/mBio.01073-14</td>
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Mycobacterial Esx-3 Requires Multiple Components for Iron Acquisition

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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.

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 M. 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 responses 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-B 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 EccCa/EccCb3, 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...
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, EccCa1/EccCb1, EccD1, and MycP3 (paralogous to the membrane-bound protease MycP1) (8).

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EspG1, EccCa1/EccCb1, EccD1, and MycP3 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.

**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.,
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 ΔeccC3ms mutant grows significantly more slowly than the fxbA strain in low-iron medium (13). Although the fxbA ΔexxGHms, fxbA ΔespG3ms, and fxbA ΔeccD3ms mutants display similar low-iron growth deficiencies, the fxbA ΔmycP3ms 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 EccC3ms, EsxGms/EsxHms, EspG3ms, and EccD3ms, with a more minor contribution from MycP3ms.

**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 Δexx-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 eccC3ms, exxGHms, espG3ms, or eccD3ms 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 mycP3ms has a more modest effect (Fig. 3). We conclude that the Esx-3 components EccC3ms, EsxGms/EsxHms, EspG3ms, and EccD3ms, are critical to the function of the M. smegmatis Esx-3 system in mycobactin-mediated iron acquisition.

**Secretion of EsxHms and EsxGms 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 EccC3ms, EsxGms/EsxHms, EspG3ms, and EccD3ms are required for function in mycobactin-mediated iron acquisition and that MycP3ms 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
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 eccC<sub>3ms</sub>, esxGH<sub>ms</sub>, espG<sub>3ms</sub>, or eccD<sub>3ms</sub> 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 mycP<sub>3ms</sub> mutation causes a much smaller decrease in iron acquisition than other mutants (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 M. smegmatis (Fig. 4 and S5). To test whether the observed lack of secretion by the esx-3 mutants reflects a general decrease in protein expression, we also compared the whole-cell extracts of the wild type, ΔeccC<sub>3ms</sub>, ΔespG<sub>3ms</sub>, and ΔmycP<sub>3ms</sub> mutants to the whole-cell extract of the wild type. The first observation is not unexpected; although MycP<sub>1mt</sub> fails 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 predicted to be nonessential for in vitro growth (20, 47, 48). The Esx-1 paralogs EccCa1/EccCb1, EspG<sub>1</sub>, and EccD<sub>1</sub> are required for virulence in pathogenic mycobacteria and conjugation in M. smegmatis 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 Esx-3 component, MycP<sub>3</sub>, that is necessary for EsxG and EsxH export (Fig. 4 and 5) but not absolutely required for mycobactin-mediated iron acquisition (49). More recent work corroborates an in vivo growth defect from loss of MycP<sub>3mt</sub> (41), but the lack of a direct comparison to other esx-1 mutant strains precluded analysis of the relative defect. Like the other esx-3 genes, mycP<sub>3ms</sub> is essential for M. tuberculosis growth in vitro (47). Given that MycP<sub>1</sub> and MycP<sub>3</sub> likely have different substrate specificities.

![FIG 3](image-url) Loss of Esx-3 components is not rescued by exogenous mycobactin. Growth of fxbA, fxbA mbtD, and fxbA esx-3 mutants in unsupplemented, low-iron medium or in low-iron medium containing 20 μg/ml mycobactin S at 48 h. The experiment was performed at least three times in triplicate. Representative data are shown and are expressed as percentages of the fxbA mutant’s growth in low-iron medium. Error bars indicate the standard errors of the proportions.
FIG 4  EsxG_m (A) and EsxH_m (B) abundances in wild-type and esx-3 mutant supernatants. Protein concentrations for EsxG_m and EsxH_m 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_m 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_m-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 ΔmycPms mutant, as neither mycPms alone nor mycPms alongside the downstream ecxGms restored EsxHms-FLAG export (not shown). Transcription of the esx-3 locus varies according to iron and zinc availability (13, 21, 22). It is possible that expression of the complementing genes from a heterologous, constitutive promoter altered the stoichiometry of Esx-3 components, which in turn resulted in suboptimal protein secretion.

We attempted to measure secreted proteins using native antibodies and Western blotting. However, the antisera that we were able to obtain had low affinity and poor specificity, making quantitation 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 surprising dynamic range in the levels of EsxG and EsxH export that support iron acquisition. We were able to restore esx-3 mutant growth to wild-type levels by adding iron to the growth medium (Fig. S2) or by complementing the deleted genes (Fig. 2). However, there are now numerous reports of Esx-1 mutants, as neither mycPms nor mycPms alongside the downstream ecxGms restored EsxHms-FLAG export (not shown). Transcription of the esx-3 locus varies according to iron and zinc availability (13, 21, 22). It is possible that expression of the complementing genes from a heterologous, constitutive promoter altered the stoichiometry of Esx-3 components, which in turn resulted in suboptimal protein secretion.

Indeed, there is growing appreciation that the relationship between Esx protein export and function is more complex than initially assumed. There are now numerous reports of Esx-1 mutations that attenuate pathogenic mycobacteria without impacting secretion. For example, supplementation of the natural esx-1 mutant M. microti (Fig. 1) with a panel of cosmids containing intact or mutant versions of the M. tuberculosis esx-1 region revealed that espFms and espGms are required for virulence but not EsxBms and EsxAms export (35). Deletion of these genes from M. tuberculosis also resulted in attenuation without impacting the secretion of EsxBms and EsxAms (42). In a different example, disruption of disulfide bond formation in the Esx-1 substrate EspA attenuated M. tuberculosis virulence but had no effect on EsxBms or EsxAms secretion (43). Finally, transposon insertions in espIms, espKms, and espBms impaired conjugation but not EsxBms export (4). In aggregate, these studies show that secretion of EsxB and EsxA is not sufficient for Esx-1 function in virulence.

Recently, Chen and coworkers isolated two point mutations of EspAms that block EsxBms and EsxAms 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 mycPms, blocked the export of native EsxGms and EsxHms and FLAG-tagged EsxHms (Fig. 4 and 5), yet the fkhA ΔmycPms 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 reinitate low-iron growth to some of our complemented esx-3 mutants (Fig. 2 and 4). Importantly, disruption of the esx-3 system in the model organism M. smegmatis allowed us to compare the two phenotypes, EsxG and EsxH export and mycobactin-mediated iron acquisition, under 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 functions (8). One reason may be the existence of multiple Esx substrates, each with its own requirements for export and contributions to function. The roles of EspG and EspB are particularly informative in this regard. Inactivation of the paralogous espGms, espGms, or, as we show in Fig. 4, espGms gene generally prevents the export of Ess substrate proteins (11, 17, 37, 42). However, in M. tuberculosis, loss of EspGms did not produce an obvious phenotype (55), while EspGms was required for full virulence but not EsxBms or EsxAms secretion (35, 42). Interestingly, EspG and EspG have been shown to interact specifically with cognate Pro-Glu (PE)/Pro-Pro-Glu (PPE) proteins (56, 57) and have been proposed 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 Esx-1 substrate EspB appear to be independent of EsxA (58, 59) and EsxB (60).

Our data are consistent with at least three general models. It is conceivable that loss of Esx-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 esx-3 mutants do not have altered sensitivities to SDS, vancomycin, rifampin (Fig. S5), ampicillin, or kanamycin (19), at least when iron is not limiting, we are unable to rule out this model. A second possibility is that Esx-3 secretes a protein or proteins necessary for iron acquisition and that this protein requires the core components EccCms, EccDms, and EspGms, but not EssGms, EssHms, or MycPms for export. Although it is possible that Esx-3 function in iron acquisition does not require any EssGms and EssHms secretion, we think that this is unlikely given that the fkhA ΔessGms strain has a complementable growth phenotype in low iron. A third model is that both EssGms and EssHms are substrates and structural components of or chaperones for the Esx-3 machinery. In this scenario, EssGms and EssHms are exported across the cytoplasmic membrane via EccDms into the periplasm, where they are poised to deliver yet-unidentified effectors of fermentative mycobactin uptake across the remainder of the cell wall (Fig. 6). If EssGms and EssHms 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...
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*$_{ms}$, *espG*$_{3ms}$, *eccD*$_{3ms}$, *mycP*$_{3ms}$, 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* inserational 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 pJBE402 plasmid (38). The construct containing *esxGms* and *esxHms* 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 pSPY (62).

**SID, MRM-MS.** (i) Labeled-peptide internal standards. Figure S3 shows the amino acid sequences of the proteins *EsxGms* and *EsxHms* 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 *M. tuberculosis* protein database (NCBI nr). Four different versions of the peptide AMATTHEQNTMAMSAR for the *EsxHms* protein were selected, all of which were observed in the discovery data.

(ii) Peptide synthesis. Five signature peptides from the two proteins, *EsxGms* (FVEYSAK) and *EsxHms* (AMATTHEQNTMAMSAR, with four forms of differing oxidized methionine states), were synthesized with a single, uniformly labeled [13C6]lysine or [13C6]arginine at their C termini by New England Peptide (Gardner, MA). Unlabeled [12C]-labeled forms of each peptide were also synthesized by New England Peptide. Synthetic peptides were purified to >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/1 μg of digested supernatant protein and a fixed amount of heavy, internal-standard peptides (1 fmol/1 μg) of the supernatant protein mix from the *D*esxGHms sample. This supernatant of the *D*esxGHms 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 three times the LOD. The blank sample consists of the *D*esxGHms 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 μl of 0.1% formic acid, and 1 μg/μl 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 μl on a Waters, packed, Reprosil, 3-μm-bead column (75-μm internal diameter [ID], 10-μm ID tip opening) with a 2.5-in by 20-μm ID spray needle. Sample was eluted at 300 nI/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 transitions were chosen based upon relative abundance and a mass-to-charge ratio (m/z) greater than the precursor m/z in the full-scan tandem MS (MS/MS) spectrum recorded on the Xevo TQ mass spectrometer. The final MRM-MS method consisted of 10 optimized transitions for each of the five selected peptides from the two target proteins. One of the four peptides (AMATTHEQNTMAMSAR) was not used for data analysis due to a weak signal.

(v) MRM-MS data analysis. Data analysis was performed using the Skyline Software Module (https://skyline.gs.washington.edu/). The relative ratios of the three transitions selected and optimized for the final MRM assay were predefined in the absence of the target proteins (i.e., in buffer) for each peptide using the [13C6]-labeled internal standards. The most abundant transition for each pair was used for quantification unless interference in this channel was observed. The 12C/13C peak area ratios were used to calculate concentrations of the target peptides in each sample by the following equation: measured concentration = peak area ratio × (1 fmol/μl 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 (OD600), 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. Tryptsin (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 C18 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.** Strains were inoculated from frozen stocks into 7H9 medium and grown to saturation. They were then diluted 1:500 in chelated Sauton’s medium, grown to saturation, and diluted 1:100 in chelated Sauton’s medium. Proteins from cell pellets and supernatants of cultures grown for 12 h in this fashion were run on 10 to 20% Tris-Tricine gels (Invitrogen) and revealed using an anti-FLAG antibody. An antibody to RNA polymerase (RNAP; Neoclone W0023), an intracellular protein, served as a loading and lysis control.

**SUPPLEMENTAL MATERIAL**

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

Figure S1, PDF file, 0.2 MB.
Figure S2, PDF file, 0.1 MB.
Figure S3, PDF file, 0.1 MB.
Figure S4, PDF file, 0.1 MB.
Figure S5, PDF file, 0.1 MB.
Figure S6, PDF file, 0.1 MB.
Figure S7, PDF file, 0.1 MB.
Figure S8, PDF file, 0.1 MB.

**ACKNOWLEDGMENTS**

We thank Colin Ratledge for providing ferri-mycobactin S and Jeff Murry for constructing the pJ1M1 vector. We gratefully acknowledge the helpful advice and insight from Meera Unnikrishnan, Sarah Fortune, and Jeff Murry. We also thank Mike Burgess for his help in running the MRM-MS assays.

This research was supported in part by the Broad Institute of MIT and Harvard (S.A.C.) and by grants to E.J.R. (NIH/NIAID grant 1P01 AI074805-01A1), J.A.P. (NIH/NIAID grant 1R01 AI087682-01A1 and a Clinical Scientist Development Award from the Doris Duke Charitable Foundation), and M.S. (Research Council of Norway grants 220836/H10 and 223255/F50).

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