EspM Is a Conserved Transcription Factor That Regulates Gene Expression in Response to the ESX-1 System

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ABSTRACT Pathogenic mycobacteria encounter multiple environments during macrophage infection. Temporally, the bacteria are engulfed into the phagosome, lyse the phagosomal membrane, and interact with the cytosol before spreading to another cell. Virulence factors secreted by the mycobacterial ESX-1 (ESAT-6-system-1) secretion system mediate the essential transition from the phagosome to the cytosol. It was recently discovered that the ESX-1 system also regulates mycobacterial gene expression in Mycobacterium marinum (R. E. Bosserman, T. T. Nguyen, K. G. Sanchez, A. E. Chirakos, et al., Proc Natl Acad Sci USA 114:E10772–E10781, 2017, https://doi.org/10.1073/pnas.1710167114), a nontuberculous mycobacterial pathogen, and in the human-pathogenic species M. tuberculosis (A. M. Abdallah, E. M. Weerdenburg, Q. Guan, R. Ummels, et al., PLoS One 14:e0211003, 2019, https://doi.org/10.1371/journal.pone.0211003). It is not known how the ESX-1 system regulates gene expression. Here, we identify the first transcription factor required for the ESX-1-dependent transcriptional response in pathogenic mycobacteria. We demonstrate that the gene divergently transcribed from the whiB6 gene and adjacent to the ESX-1 locus in mycobacterial pathogens encodes a conserved transcription factor (MMAR_5438, Rv3863, now espM). We prove that EspM from both M. marinum and M. tuberculosis directly and specifically binds the whiB6-espM intergenic region. We show that EspM is required for ESX-1-dependent repression of whiB6 expression and for the regulation of ESX-1-associated gene expression. Finally, we demonstrate that EspM functions to fine-tune ESX-1 activity in M. marinum. Taking the data together, this report extends the esx-1 locus, defines a conserved regulator of the ESX-1 virulence pathway, and begins to elucidate how the ESX-1 system regulates gene expression.

IMPORTANCE Mycobacterial pathogens use the ESX-1 system to transport protein substrates that mediate essential interactions with the host during infection. We previously demonstrated that in addition to transporting proteins, the ESX-1 secretion system regulates gene expression. Here, we identify a conserved transcription factor that regulates gene expression in response to the ESX-1 system. We demonstrate that this transcription factor is functionally conserved in M. marinum, a pathogen of ectothermic animals; M. tuberculosis, the human-pathogenic species that causes tuberculosis; and M. smegmatis, a nonpathogenic mycobacterial species. These findings provide the first mechanistic insight into how the ESX-1 system elicits a transcriptional response, a function of this protein transport system that was previously unknown.

KEYWORDS ESAT-6, ESX-1, Mycobacterium, protein secretion, regulation, feedback control
Following infection, pathogenic mycobacteria, including *Mycobacterium tuberculosis*, are engulfed by macrophages and reside in the phagosome (1–3). Survival in the phagosome requires regulated changes in bacterial gene expression (1, 4). Pathogenic mycobacteria use the ESX-1 secretion system (SS) to lyse the phagosome and mediate bacterial access the cytoplasm (5–13). The ESX-1 system is functionally conserved between *M. tuberculosis*, the cause of human tuberculosis, and *Mycobacterium marinum*, a pathogen of poikilothermic fish and an established model for the ESX-1 system (14–18). Phagosomal lysis releases secreted bacterial factors and triggers the host response to infection (7, 8, 19–27). In the absence of an ESX-1 system, both mycobacterial pathogens remain in the phagosome and are attenuated (7–9, 22).

Several ESX-1 conserved components (Ecc’s) form a complex in the cytoplasmic membrane (CM). The ESX-1 membrane complex recognizes ESX-1 substrates and provides the energy and the pore for the export of ESX-1 substrates across the CM (28, 29). The protein substrates are then translocated across the periplasm and mycolate outer membrane via an unknown process (30). ESX-1 substrates can be localized to the cell surface and/or secreted from the bacterial cell into the extracellular environment (31–34). We recently demonstrated that, in addition to transporting proteins, the presence or absence of the ESX-1 membrane complex in the CM elicits a widespread transcriptional response, a previously unrecognized function of the ESX-1 system (35). ESX-1-dependent gene expression has since been confirmed in *M. marinum* and reported in *M. tuberculosis* (36, 37).

The ESX-1-dependent transcriptional response includes a negative-feedback mechanism linking the levels of ESX-1 substrates to the presence or absence of the ESX-1 membrane complex (35). WhiB6 is a stress-responsive transcription factor (38, 39) that directly activates ESX-1 substrate gene expression in *M. marinum* and in *M. tuberculosis* (38, 39). The ESX-1 system regulates *whiB6* gene expression both in *M. marinum* and in *M. tuberculosis* (35–37). In the presence of the ESX-1 membrane complex, the *whiB6* gene is expressed, and there is WhiB6-dependent expression of the genes encoding ESX-1 substrates. In the absence of the ESX-1 membrane complex, *whiB6* gene expression, as well as the expression of ESX-1 substrate genes, is significantly reduced (35, 36). How the ESX-1 membrane complex regulates *whiB6* gene expression is unknown.

On the basis of our published data and of those published previously by independent groups, ESX-1-dependent changes in gene expression cannot be explained by the loss of the WhiB6 transcription factor alone (35–37). Therefore, we hypothesized that additional transcription factors regulate genes in response to the presence of the ESX-1 membrane complex.

**RESULTS**

The EspM protein binds upstream of the *whiB6* gene. To identify transcription factors that regulate genes in response to the ESX-1 membrane complex, we focused on the regulation of the *whiB6* gene. The 1 kb of DNA upstream of the *whiB6* gene is sufficient for regulation of *whiB6* gene expression by the ESX-1 membrane complex (35). We used a DNA pulldown to enrich proteins from *M. marinum* lysate that specifically bind the 1 kb of DNA upstream of the *whiB6* gene (“*whiB6* promoter bait,” Fig. 1A; bp 6577326 to 6578305 in the *M. marinum* genome). Using liquid chromatograph-tandem mass spectrometry (LC-MS/MS)-based quantitative proteomics on the proteins eluted from the DNA, we identified several proteins that were specifically and reproducibly enriched for binding the *whiB6* promoter bait relative to binding nonspecific DNA (*rpoA* bait; see Table S1 in the supplemental material). MMAR_5438 was enriched for binding the *whiB6* promoter bait 64.0-fold ± 0.4-fold relative to the *rpoA* bait (Fig. 1B). We propose renaming the MMAR_5438 gene “espM,” consistent with current ESX-1 nomenclature (40). We generated an *M. marinum* strain with an unmarked deletion of the espM gene (ΔespM; Fig. S1) and a complementation strain with an integrated constitutive espM expression plasmid (ΔespM/p<sub>espM</sub>). The EspM protein was not identified in the DNA pulldown performed with lysate from the ΔespM strain and was further enriched for *whiB6* promoter bait binding compared to the *rpoA*
We also identified several *M. marinum* proteins with known DNA binding activity that were not significantly or reproducibly enriched for binding the *whiB6* promoter bait relative to the *rpoA* bait (Table S1). For example, the *M. marinum* DNA-binding protein Hu homolog HupB (MMAR_1728) bound the two baits comparably following incubation with any *M. marinum* lysate (Fig. 1B).

To confirm the interaction of the EspM protein with the *whiB6* promoter region, we expressed and purified an N-terminally 6×His-tagged EspM<sub>MM</sub> fusion protein from *Escherichia coli* (the *MM* subscript refers to the protein from *M. marinum* [40]) (see Fig. S2 in the supplemental material) and performed electrophoretic mobility shift assays (EMSAs). We observed a specific shift in mobility of the *whiB6* promoter probe

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*FIG 1*  Identification of MMAR_5438 (EspM) as a DNA-binding protein in *M. marinum*. (A) The *whiB6* gene is separated from the *esx-1* locus by the MMAR_5438 gene. The biotinylated 1-kb probe (circles) for the DNA pulldown is indicated. The 500-bp probe for the EMSA analysis is indicated in panel C. (B) MS analysis of the DNA pulldown showing the enrichment of the EspM and HupB proteins. The scale represents normalized MS peak area intensity levels. a.u., arbitrary units. (C and D) EMSAs performed with increasing concentrations of the 6×His-EspM protein from *M. marinum* (EspM<sub>MM</sub>) (C) or the 6×His-EspM protein from *M. tuberculosis* (EspM<sub>MT</sub>) (D). The control probe used as indicated in both panels was 500 bp of the *rpoA* open reading frame (ORF) (bp 1309999 to 1310499) from *M. marinum*. (E) Schematic of the 6×His-EspM<sub>MM</sub> proteins affinity purified from *E. coli* used in the EMSAs. (F) EMSA performed with the *whiB6-espM* probe with increasing amounts of EspM<sub>MM</sub>, EspM<sub>NT</sub>, and EspM<sub>CT</sub> from *M. marinum*. 

bait in lysates from the complemented strain (Fig. 1B).
was significantly higher in the \( \Delta \) espMNT (aa 1 to 133) and EspMCT (aa 127 to 363) levels (\( \text{ EspM}_{\text{MM}} \)). We hypothesized that the C-terminal half of the EspM protein mediated DNA binding. We expressed and purified 6\(|\text{His}\)-tagged \( \text{ EspM}_{\text{MT}} \) in \( \text{ E. coli } \) (the MT subscript refers to the EspM protein from \( \text{ M. tuberculosis } \)) (Fig. S2). We amplified the 500 bp upstream of the \( \text{ whiB6} \) gene from \( \text{ M. tuberculosis } \) Erdman and tested if \( \text{ EspM}_{\text{MT}} \) specifically bound the \( \text{ whiB6} \) promoter region using EMSAs. Increasing concentrations of 6\(|\text{His}\)-tagged \( \text{ EspM}_{\text{MT}} \) protein led to a specific mobility shift of the \( \text{ whiB6} \) promoter probe and to a corresponding loss of free probe (Fig. 1D). Although bound rpoA probe was not observed at the highest concentrations of 6\(|\text{His}\)-tagged \( \text{ EspM}_{\text{MT}} \) protein, the free probe was reduced, indicating weak binding at the highest protein concentrations. Together, these data indicate that EspM, from both \( \text{ M. marinum } \) and \( \text{ M. tuberculosis } \), directly and specifically bound the \( \text{ whiB6}-\text{espM} \) intergenic region.

\( \text{ espM} \) is a predicted conserved regulatory protein (42), but the corresponding function has not been investigated. The \( \text{ EspM}_{\text{MM}} \) protein is predicted to have an \( \text{N-terminal forkhead-associated (FHA) domain (amino acids [aa] 32 to 89) and a C-terminal helix-turn-helix domain (Fig. 1E). We hypothesized that the C-terminal half of the protein mediated DNA binding. We expressed and purified 6\(|\text{His}\)-tagged \( \text{ EspM}_{\text{MT}} \) (aa 1 to 133) and \( \text{ EspM}_{\text{CT}} \) (aa 127 to 363) \( \text{ M. marinum } \) proteins from \( \text{ E. coli } \) (Fig. S2). We tested the ability of each protein to bind the \( \text{ whiB6} \) promoter probe using EMSA. The 6\(|\text{His}\)-tagged \( \text{ EspM}_{\text{MT}} \) protein did not shift the mobility of the \( \text{ whiB6} \) promoter probe (Fig. 1F). Incubation with increasing concentrations of the 6\(|\text{His}\)-tagged \( \text{ EspM}_{\text{CT}} \) protein caused a shift in mobility of the \( \text{ whiB6} \) promoter probe and a loss of free probe. We conclude that the C-terminal half of the EspM protein is required for DNA binding.

\textbf{EspM is a conserved regulator of \text{ whiB6} and \text{ esx-1} gene expression.} We confirmed that the \( \text{ espM} \) transcript was absent in the \( \Delta \text{espM} \) \( \text{ M. marinum } \) strain using quantitative reverse transcription-PCR (qRT-PCR) (Fig. 2A). The \( \text{ espM} \) expression level was significantly higher in the \( \Delta \text{espM}/\text{p}_{\text{mm}}\text{espM} \) complemented strain than in the wild-type (WT) strain \( (P < 0.0001) \). These data indicate that the complementation strain is an \( \text{ espM} \) overexpression strain. We did not observe a significant reduction of \( \text{ espM} \) gene expression in the \( \Delta \text{eccCb1} \) strain relative to the WT strain. These data confirm that \( \text{ espM} \) expression is not regulated by the ESX-1 system in \( \text{ M. marinum } \), consistent with our previously published transcriptomic analysis (35).

Because EspM bound the region upstream of the \( \text{ whiB6} \) gene, we tested if EspM regulates \( \text{ whiB6} \) gene expression. We measured \( \text{ whiB6} \) gene expression in \( \text{ M. marinum } \) using qRT-PCR. Consistent with our prior findings (35), \( \text{ whiB6} \) gene expression was significantly reduced in the \( \Delta \text{eccCb1} \) strain compared to the WT strain (Fig. 2B, inset, \( P < 0.0001 \)). Deletion of the \( \text{ espM} \) gene resulted in a significant increase in \( \text{ whiB6} \) expression relative to the WT strain \( (P < 0.0001) \). Overexpression of the \( \text{ espM} \) gene resulted in \( \text{ espM} \) expression that was not significantly different from that seen with the WT strain. We conclude that EspM is a repressor of \( \text{ whiB6} \) gene expression.

We measured the levels of WhiB6 protein in the presence and absence of the \( \text{ espM} \) gene (Fig. 2C). The parental \( \text{ M. marinum} \) strain for these strains includes a \( \text{ whiB6} \) gene with a C-terminal FLAG epitope tag (WhiB6Fl [35]). Consistent with our previously published data (35), the WhiB6Fl protein was absent from the lysate generated from the \( \Delta \text{eccCb1} \) strain (Fig. 2C, lane 2). Consistent with the expression data (Fig. 2B), deletion of the \( \text{ espM} \) gene resulted in increased WhiB6Fl protein levels relative to those seen with the WT strain (Fig. 2C, compare lane 3 to lane 1). The WhiB6Fl protein levels in the \( \Delta \text{espM}/\text{p}_{\text{mm}}\text{espM}_{\text{MM}} \) complemented strain (\( \text{ espM} \) overexpression) were lower than those
EspM is a conserved regulator of whiB6 and esx-1 gene expression. (A) qRT-PCR measuring the levels of espM expression relative to sigA expression. A one-way ordinary analysis of variance (ANOVA) \( P < 0.0001 \), followed by a Dunnett's multiple-comparison test relative to the WT strain, was performed. ****, \( P < 0.0001 \). (B) qRT-PCR measuring the levels of whiB6 expression relative to sigA expression. A one-way ordinary ANOVA \( P < 0.0001 \), followed by a Sidak's multiple-comparison test relative to the WT strain, was performed. ****, \( P < 0.0001 \). The inset shows just the comparison between the WT and \( \Delta cccC_b1 \) strains. A Student's unpaired, two-tailed t test was used to define the significance of the results of the comparisons between the two strains. For panels A and B, the data represent averages of results from at least three biological replicates, each performed in technical triplicate. (C) Western blot analysis of 10 \( \mu g \) of protein per lane. Anti-Mpt32 was used as the loading control. All \( M. \) marinum strains indicated in this panel contained a C-terminal FLAG epitope tag on the whiB6 gene. Samples were resolved on an 18% Tris-glycine gel. The Western blot shown is representative of at least three independent biological replicates. (D) Conservation of the EspM proteins (percent identity at the amino acid level) from \( M. \) tuberculosis, \( M. \) marinum, and \( M. \) smegmatis. (E) Scatterplot of genes differentially expressed in the \( \Delta espM \) strain versus the \( \Delta espM/pmsp\_espM \) complemented strain. Genes indicated in red had a q value of \( < 0.05 \). Regions enriched with the esx-1 locus or mce6 locus are highlighted. Full gene lists are available in Table S3. (F) Heat map of esx-1 locus genes that are significantly differentially regulated in the \( \Delta espM \) strain versus the \( \Delta espM/pmsp\_espM \) complemented strain compared to genes expressed in the \( \Delta espM, \Delta whiB6, \) or \( \Delta cccC_b1 \) mutant strains relative to the WT strain.
in the WT strain (Fig. 2C, lane 4 versus lane 1). Together, these data strongly support the conclusion that EspM represses \textit{whiB6} gene expression in \textit{M. marinum}.

The \textit{espM} gene is syntenic in \textit{M. marinum}, \textit{M. tuberculosis}, and \textit{M. smegmatis} (Fig. S3). \textit{M. smegmatis} is a nonpathogenic, soil-dwelling mycobacterial species that uses the ESX-1 system to mediate conjugation (43, 44). The EspM orthologs in all three species are conserved at the protein level (Fig. 2D; see also Fig. S3). The \textit{M. smegmatis} ortholog (MSMEG\_0052; EspM\textsubscript{\textit{MS}}) lacks the N-terminal FHA domain. Aligning the C-terminal halves of the EspM\textsubscript{\textit{MT}} and EspM\textsubscript{\textit{MS}} proteins with EspM\textsubscript{\textit{MS}} revealed that the \textit{M. marinum} and \textit{M. tuberculosis} C-terminal halves are 81.20\% identical at the amino acid level. EspM\textsubscript{\textit{MS}} is 63.10\% and 62.20\% identical to the C-terminal half of EspM\textsubscript{\textit{ASM}} and EspM\textsubscript{\textit{MTP}} respectively.

Because EspM proteins are conserved across three mycobacterial species, we hypothesized that the repression of \textit{whiB6} expression by EspM would be functionally conserved. We generated integrating plasmids constitutively expressing the \textit{espM} genes from \textit{M. tuberculosis} Erdman (\textit{espM\textsubscript{\textit{MT}}}) and \textit{M. smegmatis} mc\textsuperscript{c}\textsubscript{155} (\textit{espM\textsubscript{\textit{MS}}}) and introduced each plasmid into the Δ\textit{espM} strain. As shown in Fig. 2C, overexpression of the EspM\textsubscript{\textit{MT}} protein or EspM\textsubscript{\textit{MS}} protein reduced WhiB6\textsubscript{Fl} protein levels in the Δ\textit{espM} \textit{M. marinum} strain (Fig. 2C; compare lanes 5 and 6 with lane 3), similarly to the complemented strain overexpressing the \textit{espM\textsubscript{\textit{MM}}}, gene (Fig. 2C, lane 4). These data demonstrate that repression of \textit{whiB6} expression is functionally conserved between the EspM orthologs in \textit{M. marinum}, \textit{M. tuberculosis}, and \textit{M. smegmatis}.

We performed RNA-seq transcriptional profiling to determine if EspM regulates other genes in addition to \textit{whiB6} in \textit{M. marinum}. Comparison of the WT strain to the Δ\textit{espM} strain (both bearing the \textit{whiB6\textsubscript{Fl}} allele) identified 134 genes that were upregulated and 300 genes that were downregulated (\textgreater{}2-fold; false-discovery rate [\textit{q} value] of <0.05) (Fig. S4A; see also Table S3). Genes controlled by EspM are also expected to be differentially regulated in the Δ\textit{espM} strain compared to the complemented strain that overexpresses the repressor. We observed 44 genes that were upregulated and 55 genes that were downregulated in the Δ\textit{espM} strain compared to the complemented strain (\textgreater{}2-fold; \textit{q} value of <0.05) (Fig. 2E; see also Table S3B). Consistent with repression of \textit{whiB6} expression by EspM, we observed that \textit{whiB6} expression was induced 1.6-fold and 7.0-fold in the Δ\textit{espM} strain compared to the WT strain and the complemented overexpression strain, respectively. Of the 44 genes that were induced in the Δ\textit{espM} strain compared to the complemented strain, 21 genes from the \textit{esx-1} locus were identified (\textit{MMAR\_5436} to \textit{MMAR\_5457}), including 8 genes that were also induced in the Δ\textit{espM} strain compared to WT strain (Fig. 2F; see also Fig. S4B). Most of the other genes in the \textit{esx-1} locus were significantly induced in the Δ\textit{espM} strain relative to the WT strain, but with induction levels below 2-fold.

We also observed induction of unlinked \textit{esx}-associated loci in the Δ\textit{espM} strain compared to the complemented strain, including \textit{MMAR\_0187-188} (\textit{esxB\_1esxA\_1}), \textit{MMAR\_3654} (\textit{esxP2}), and the ESX-1 substrate locus \textit{MMAR\_2894} (45) (Table S3B). Several of these genes were previously shown to be regulated by WhiB6 or ESX-1 (Fig. 2F; see also Fig. S4B).

Of the 55 genes downregulated in the Δ\textit{espM} strain relative to the complemented strain, 39 were also downregulated in the Δ\textit{espM} strain relative to WT strain (Table S3). These included 24 strongly downregulated genes between \textit{MMAR\_0159} and \textit{MMAR\_0182} (Fig. 2E; see also Table S3B), which includes the \textit{mce6} locus (Fig. S4C), and genes for amino acid metabolism and lipid anabolism. Prior studies with the Δ\textit{whiB6} and \textit{eccC\_B}, mutant strains showed induction of the genes in the \textit{mce6} locus (Fig. S4C), supporting the idea of ESX-1-dependent regulation. Curiously, we also detected downregulation of an ESX-1-associated operon, \textit{MMAR\_4166} to \textit{MMAR\_4168} (\textit{esPA}, \textit{esPC}, and \textit{esPD}). Together, these data strongly support the conclusion that EspM is a regulator of genes broadly associated with the ESX-1 system in \textit{M. marinum}.

\textbf{EspM represses \textit{whiB6} expression in the absence of the ESX-1 membrane complex.} \textit{whiB6} gene expression levels are reduced in the absence of the ESX-1 membrane complex (35). We hypothesized that EspM represses \textit{whiB6} and \textit{esx-1} gene
expression in the absence of the ESX-1 membrane complex (Fig. 3A). Four ESX-conserved components (Ecc’s) reside in the CM (Fig. 3A; EccB1, EccCa1, EccD1, and EccE1 [28, 29]), and two Ecc’s (EccCa1 and EccA) are cytoplasmic (10, 46–49).

We reasoned that if EspM repressed whiB6 gene expression in the absence of the ESX-1 membrane complex, then deletion of the espM gene in strains lacking the ESX-1 membrane complex would restore whiB6 gene expression. We generated M. marinum strains bearing deletions of each ecc gene (eccA to eccE1) alone or in combination with deletion of the espM gene. Deletion of any ecc gene resulted in a loss of WhiB6Fl protein relative to the WT strain (Fig. 3B, lanes 2 to 7 versus lane 1). The deletion of the espM gene in combination with the ecc genes (ΔespM ΔeccA, ΔespM ΔeccB1, ΔespM ΔeccCa1, ΔespM ΔeccD1, and ΔespM ΔeccE1, mutant strains) resulted in levels of WhiB6Fl similar to those in the ΔespM strain (Fig. 3B, lanes 9 to 14 versus lane 8) and higher than those in the WT strain (Fig. 3B, lane 1). We further demonstrated that complementation with the eccA gene or the espM gene in the ΔespM ΔeccA strain resulted in levels of WhiB6Fl similar to those seen with the ΔespM deletion strain or the ΔeccA deletion strain, respectively (Fig. S5).

We reasoned that overexpression of the espM gene might be sufficient to repress whiB6 expression to levels similar to those seen with the ΔeccCb1 strain. We generated a strain bearing a whiB6 transcriptional reporter in M. marinum. We replaced the whiB6 gene with the lacZ gene, creating a strain lacking the whiB6 gene and with a reporter fusion to the whiB6 promoter (ΔwhiB6: lacZ; Fig. 3C). We generated an isogenic ΔeccCb1 strain (no ESX-1 membrane complex [30]), as well as an isogenic WT strain overexpressing the espM\textsubscript{MM} gene. The level of β-galactosidase activity was significantly reduced in the ΔeccCb1 strain compared to the WT strain (Fig. 3D; \(P < 0.0001\)), confirming that the whiB6: lacZ\textsuperscript{+} reporter fusion was regulated by the ESX-1 membrane complex (35). Overexpression of the espM gene in the WT strain significantly reduced the levels of β-galactosidase activity compared to the WT strain levels (\(P < 0.0001\)).
levels of β-galactosidase activity in the ΔeccCb1 and espM overexpression strains were not significantly different from each other (P = 0.9915), demonstrating that overexpression of espM is sufficient to repress whiB6 gene expression in M. marinum. Collectively, our data demonstrate that EspM is required for repression of whiB6 gene expression in the absence of the ESX-1 membrane complex. Moreover, because the reporter strain lacks the whiB6 gene, these data indicate that EspM represses whiB6 expression in a WhiB6-independent manner.

The EspM and WhiB6 regulators coordinately control gene expression. The espM and whiB6 genes are divergently organized in mycobacterial genomes (Fig. 4A). Because the whiB6 and espM genes share an intergenic region which likely controls the expression of both genes (Fig. 4A, pink), we sought to further define the relationship between the EspM and WhiB6 regulators.

We generated espM-lacZ+ and whiB6-lacZ+ integrating transcriptional reporters (Fig. 4). The espM-lacZ+ reporter resulted in significantly increased β-galactosidase activity in the ΔespM strain compared to the WT strain (P < 0.0001; Fig. 4A). Loss of the whiB6 gene did not significantly impact β-galactosidase activity relative to the WT strain (P = 0.1195). Deletion of both the espM and whiB6 genes (ΔespM ΔwhiB6 mutant strain) resulted in β-galactosidase activity comparable to that seen with the WT M. marinum strain (P = 0.9305). We conclude from these data that espM expression is negatively autoregulated. Moreover, in the absence of EspM, WhiB6 is required for the observed increased espM gene expression. We confirmed that WhiB6 binds the whiB6-espM intergenic region by expressing and purifying a C-terminally 6×His-tagged WhiB6MM fusion protein from Escherichia coli (Fig. S2) and performing EMSAs (Fig. S6). We observed a specific shift in mobility of the whiB6 promoter probe (Fig. 1A, “EMSA probe”) and a concomitant loss of free whiB6 promoter probe with increasing concentrations of the WhiB6MM-6×His protein (Fig. S6). We did not observe a mobility shift of the rpoA probe, confirming the specific binding of the WhiB6MM protein to the whiB6 promoter probe.

The presence of the whiB6-lacZ+ reporter resulted in significantly increased β-galactosidase activity in the ΔespM strain compared to the WT strain (P < 0.0001;
EspM fine-tunes ESX-1 function in *M. marinum*. Because EspM regulates *whiB6* and *esx-1* gene expression, we tested if EspM was required for ESX-1 activity. The WT strain produced the EspA and EspB substrates and secreted them into the culture supernatant during growth *in vitro* (Fig. 5A, lanes 1 and 2). Deletion of the *eccCb* gene, which is required for ESX-1 secretion (10, 12, 14), reduced production of EspA and EspB, and neither protein was secreted (Fig. 5A, lanes 3 and 4). The Δ*espM* strain exhibited at least WT levels of production and secretion of EspA and EspB (Fig. 5A, lanes 5 and 6). The *espM* complemented strains showed reduced levels of production of EspA and EspB (Fig. 5A, lanes 7 and 8, and Fig. S4D) but exhibited at least wild-type levels of secretion of EspA and EspB. To further confirm that the levels of ESX-1 proteins were altered, consistent with the observed EspM-dependent expression changes, we performed global proteomics on whole-cell lysates of the *M. marinum* strains represented in panel A (for the WT, Δ*eccCb*, Δ*espM*, and complemented strains) (Table S1E and F; see also Fig. S4E). We identified 1,881 proteins at a 1% false-discovery rate. Protein quantification was performed by using label-free quantification (LFQ). We found that, similarly to the EspA and EspB proteins, the levels of several ESX-1 substrates (EspF, EspK, and EspB) and components (EccA) and other associated proteins (EspG, EspH, and EspL) were significantly reduced in the complemented strain, consistent with the expression data (Fig. 2). These data demonstrate that EspM is required for fine-tuning the levels of ESX-1-associated proteins, including the EspA and EspB substrates, in the mycobacterial cells but not for the secretory function of the ESX-1 system.

The ESX-1 system promotes phagosomal lysis during macrophage infection (6, 9, 27). *M. marinum* lyases red blood cells (RBCs) in an ESX-1-dependent manner (14, 17, 50). Hemolysis analysis is a common way to measure the membranolytic activity of the ESX-1 system (14, 17, 50). The WT strain caused significantly increased hemolytic activity compared to the phosphate-buffered saline (PBS) control (no bacteria) (P < 0.0001; Fig. 5B). The Δ*eccCb*, strain exhibited hemolytic activity that was not significantly different from that seen with the PBS control (P = 0.9996). The Δ*espM* strain exhibited hemolytic activity not significantly different from that seen with the WT strain (P = 0.9602). The complemented strain, which overexpresses *espM* relative to the WT strain (Fig. 2B), showed significantly reduced hemolytic activity relative to the WT and Δ*espM* strains (P < 0.0001).

ESX-1-deficient *M. marinum* strains fail to lyse the phagosome and fail to lyse macrophages (7, 51). We infected RAW 264.7 cells with *M. marinum* at a multiplicity of infection (MOI) of 7 and measured macrophage lysis by visualizing and quantifying the uptake of ethidium homodimer by permeabilized macrophages (52). Consistent with previous findings (51, 53), the WT strain caused macrophage lysis (Fig. 5C). Infection with the Δ*eccCb*, strain resulted in a significant reduction in macrophage lysis compared to infection with the WT strain (P < 0.0001). In contrast, infection with the Δ*espM* strain resulted in significantly increased macrophage lysis compared to infection with the WT strain (P < 0.0001). Infection with the *espM* overexpression strain restored macrophage lysis to WT levels (P = 0.2138). These data show that in the absence of the *espM* gene, the ESX-1 system promoted higher levels of macrophage lysis. Moreover, combined with the hemolysis data, these findings indicate that the levels of EspM fine-tune the activity of the ESX-1 system in *M. marinum*.
Collectively, our findings identify EspM as a conserved transcription factor required for the ESX-1-dependent transcriptional response in pathogenic mycobacteria. Although the espM gene is adjacent to the esx-1 locus, EspM has not been previously characterized. The espM gene may not have been linked to the ESX-1 system previously because deletion of the espM gene in M. marinum had only a subtle impact on ESX-1 activity, likely because several transcription factors regulate whiB6 gene expression (39, 54–56). Moreover, the M. tuberculosis EspM ortholog Rv3863 was previously reported to

**FIG 5** EspM fine-tunes ESX-1 function. (A) Western blot analysis of EsxA and EsxB secretion *in vitro*. 10 μg of protein was loaded per lane and resolved on a 4% to 20% gel. RpoB was used as the lysis control; Mpt-32 is a Sec-dependent secreted protein that served as a loading control. The image shown is representative of three biological replicates. (B) Hemolysis assay of *M. marinum* strains. The image shown represents at least three biological replicates, each performed in technical triplicate. Error bars represent the propagated errors. A one-way ordinary ANOVA (*P* < 0.0001) followed by a Tukey’s multiple-comparison test was performed. **,** *P* < 0.0001 (relative to the WT strain). OD<sub>405</sub>, optical density at 405 nm. (C) Cytolysis assay of RAW 264.7 cells following 24 h of infection with *M. marinum* at an MOI of 7. Black bars indicate median and quartiles. UI, uninfected. Statistical analysis was performed using a one-way ordinary ANOVA (*P* < 0.0001) followed by a Tukey’s multiple-comparison test. **,** *P* < 0.0001 (compared to the WT strain). Each dot represents the number of EthD-1-stained cells in a single field. A total of 10 fields were counted using ImageJ for each well. Processing of 3 wells was performed for each biological replicate. A total of 90 fields were counted for each strain.

**DISCUSSION**

Collectively, our findings identify EspM as a conserved transcription factor required for the ESX-1-dependent transcriptional response in pathogenic mycobacteria. Although the espM gene is adjacent to the esx-1 locus, EspM has not been previously characterized. The espM gene may not have been linked to the ESX-1 system previously because deletion of the espM gene in *M. marinum* had only a subtle impact on ESX-1 activity, likely because several transcription factors regulate whiB6 gene expression (39, 54–56). Moreover, the *M. tuberculosis* EspM ortholog Rv3863 was previously reported to
be essential for growth in vitro in some genome-wide studies (57, 58) and nonessential in others (59), which may complicate study in M. tuberculosis.

The identification of EspM further expands our understanding of the feedback control mechanism that links the levels of ESX-1 substrates, and other genes, to the assembly of the secretory apparatus (35). We found that deletion of the espM gene resulted in levels of whiB6 expression that were higher than those seen with the WT strain (Fig. 2B and C). We propose that whiB6 expression is repressed by EspM in the WT strain and that whiB6 gene expression is further repressed by EspM in the absence of the ESX-1 system (Fig. 6). Therefore, regulation by EspM is relevant in WT bacteria and not simply when the ESX-1 system is absent, which may or may not be physiologically relevant.

We do not yet understand how the ESX-1 membrane complex controls the magnitude of whiB6 repression by EspM. We do not think that the ESX-1 system transcriptionally regulates the espM gene. We observed no ESX-1-dependent change in espM transcript levels either here (Fig. 2A) or in our prior work (35). These findings contrast those of Abdallah et al. (36), which indicated that the Rv3863 transcript (espMMT) is regulated by the ESX-1 system in M. tuberculosis, similarly to the whiB6 gene. This may be an example of differential regulation between M. marinum and M. tuberculosis. The presence or absence of an assembled ESX-1 membrane complex likely posttranscriptionally controls the levels of EspM in M. marinum. We recapitulated the levels of whiB6 gene expression in the ΔeccCb1 strain by overexpressing espM in the WT strain (Fig. 3D). However, our published proteomic data indicate that EspM_{MMT} protein levels are reduced 2-fold in the absence of EccCb1, when repression of whiB6 expression is strongest (35). Regulation of the EspM transcription factor may be similar to the control of gene expression by type III secretion systems (T3SS) in Gram-negative bacteria (60–63). The injectisome T3SS uses cytoplasmic substrates and/or chaperones to posttranscriptionally modulate the levels or activity of transcription factors that regulate secretion-associated genes (62, 64–69). ESX-1 substrates or chaperones may posttranscriptionally regulate the activity of EspM in response to the presence or absence of the ESX-1 membrane complex.

Posttranscriptional regulation of EspM could occur through the predicted N-terminal forkhead-associated (FHA) domain. FHA domain-containing proteins posttranscriptionally regulate Gram-negative type III and type VI protein secretion systems (70–72). Staphylococcus aureus has an Ess-type VII secretion system similar to the ESX-1
The EccC-related protein EssC$_{5A}$ (75, 76) includes a twin-FHA domain that is essential for secretion (77). FHA domains also mediate oligomerization (78–80). We observed a second shift in mobility of the whiB6-espM probe by EMSA with increasing concentrations of EspM$_{MM}$ protein (Fig. 1C and F). We did not observe this supershifted product when using the C-terminal half of EspM$_{MM}$ alone (Fig. 1F), suggesting that the N-terminal half of the protein is important for this observation. The FHA domain may directly or indirectly control oligomerization of EspM in response to the ESX-1 membrane complex.

Although WhiB6 directly binds the whiB6-espM promoter region, we did not identify WhiB6 in the DNA pulldown (Fig. 1; see also Table S1 in the supplemental material). We have not routinely identified WhiB6 from M. marinum lysates using mass spectrometry. We also did not identify the PhoP response regulator, which regulates whiB6 expression in M. tuberculosis. Under the conditions of our experiments, EspM may bind the intergenic region preferentially to other regulators, including WhiB6 and PhoP. This idea is supported by the finding that WhiB6 activates espM gene expression only in the absence of EspM (Fig. 4A). Also, it is possible that no single technique can identify all proteins that bind and regulate a specific region. For example, chromatin immunoprecipitation sequencing (ChIP-seq) experiments in strains overexpressing WhiB6 in M. tuberculosis did not identify direct binding of WhiB6 to the whiB6-espM intergenic region. And yet, overexpression of WhiB6 resulted in a significant upregulation of whiB6 gene expression in the same study (55, 56). Likewise, although PhoP bound the WhiB6 promoter directly in M. tuberculosis, overexpression of PhoP failed to significantly impact whiB6 gene expression (55, 56). Therefore, the absence of enrichment of regulators in our study does not preclude the possibility of a role for them in the regulation of whiB6 and espM expression. Finally, regulation of the whiB6 and espM genes may not be conserved between M. marinum and M. tuberculosis. In the case of whiB6 expression, it has already been established that there is variability in how PhoP regulates whiB6 in M. tuberculosis strains (39). It has not yet been established if whiB6 regulates esx-1 in M. marinum as part of the PhoPR regulon.

The back-to-back divergent arrangement of two regulators is a common theme in microorganisms (81), the best described of which are the cl and Cro regulators of bacteriophage $\lambda$ (82). Divergence in organization allows tight coordination of the expression of both transcription factors and of their regulons from a single genetic locus. The intergenic region between the espM and whiB6 genes likely contains binding sites for both WhiB6 and EspM. Indeed, we demonstrated using EMSAs that both EspM and WhiB6 bind this region in vitro (Fig. 1; see also Fig. S6 in the supplemental material) and that both contribute to regulating the whiB6 and espM genes (Fig. 4) (38, 39). Therefore, the genes regulated by WhiB6, the ESX-1 system, and EspM may be coordinated to fine-tune the ESX-1 secretion and for additional biological purposes. Moreover, whereas approximately half of the genes induced or repressed in the $\Delta$espM strain versus the complemented strain are associated with the ESX-1 system, other EspM-regulated genes may have a currently unrecognized role in ESX-1-associated functions.

Our data clearly demonstrate that EspM impacts the expression of esx-1-associated genes and is associated with corresponding changes in ESX-1 protein levels, supporting the idea that EspM functions to fine-tune ESX-1 function. Consistent with these findings, we observed reduced hemolytic activity upon overexpression of espM and increased cytolytic activity in the absence of EspM. Although hemolysis and macrophage cytolysis are both measures of ESX-1 function, our prior work indicated that the results of the two assays do not always align, especially when using strains with intermediate ESX-1 production or secretion levels (45). It is possible that there are additional roles for EspM in ex vivo infection that differ from those seen in our studies in vitro. Alternatively, EspM could impact the expression of additional genes required for phagosomal lysis or macrophage cytolysis. For example, phthiocerol dimycocerosate (PDIM) has been implicated in both phagosomal lysis and macrophage cytolysis (83, 84). However, we did not see changes in the expression of genes required for PDIM.
Regulation of Gene Expression by the ESX-1 System

Unlike most examples of T3SS-dependent gene expression, the genes regulated by EspM and the ESX-1 membrane complex are not restricted to the ESX-1-associated genes (35, 36). The C. trachomatis T3SS, which impacts global gene expression, may represent a temporal cue for regulating gene expression during infection (85). Likewise, the assembly of the ESX-1 system may serve as a temporal cue to regulate mycobacterial gene expression. While pathogenic mycobacteria elicit a transcriptional response essential for survival in the phagosome (1, 4), there has been no report of a transcriptional response to interaction with the macrophage cytosol. The cytoplasm is considered restrictive for bacterial survival and growth unless the pathogen adapts (86). Listeria monocytogenes, a pathogen that lyses the phagosomal membrane and accesses the cytoplasm (87), adapts by altering metabolism and inducing stress response pathways (86). We propose that the assembly of the ESX-1 membrane complex elicits gene expression pathways to link ESX-1-mediated phagosomal lysis and cytoplasmic adaptation. Indeed, several of the genes regulated by EspM and by the ESX-1 system are predicted to be associated with metabolism (Table S3). This is most notable in genes that are downregulated in the ΔespM strain or upregulated in the ΔwhiB6 and eccCb1 mutant strains. For example, the genes in the mce6 locus and surrounding genes were significantly downregulated in the ΔespM strain but were upregulated in the ΔwhiB6 and eccCb1, mutant strains (Fig. S4C), although, due to variability in the data, the results representing the gene induction in the ΔwhiB6 and eccCb1, mutant strains were not statistically significant. These data are supportive of the conclusion that the mce6 genes are repressed in a whiB6-dependent manner, although further characterization studies will be required to support this hypothesis. mce genes have been associated with carbon nutrient uptake, including mce1, promoting uptake of fatty acids (88), and mce4, promoting uptake of cholesterol (89). mce6 is absent in the M. tuberculosis genome but is present in the genomes of many nontuberculous mycobacterial species (90) and could play a role in controlling metabolite import to promote survival in the phagosome or cytosol. The mce6 locus may be important for the cytosolic lifestyle of M. marinum, which polymerizes host actin and exhibits cytosolic motility (5), which is not conserved in M. tuberculosis.

Finally, because EspM regulates a subset of genes controlled by the ESX-1 system, there are likely additional transcription factors that make up an ESX-1-dependent transcriptional network. We focused on proteins that specifically bound the whiB6/espM intergenic region. Studies aimed at identifying proteins that bind additional ESX-1-responsive promoters would identify additional transcription factors in the ESX-1-responsive network.

In conclusion, we have identified a conserved transcription factor, EspM, which is encoded by a gene adjacent to the esx-1 locus that is required for the repression of whiB6 gene expression in the absence of the ESX-1 system. Our study results begin to define a transcriptional network that links the assembly of the ESX-1 system to widespread changes in gene expression, including the regulation of the ESX-1 apparatus and substrates.

MATERIALS AND METHODS

A fully detailed explanation of the methods used in this study can be found in Text S1 in the supplemental material. All M. marinum strains were derived from M. marinum strain M (BAA-535). Where indicated, the parental strain included a FLAG epitope tag at the C terminus of the whiB6 gene (35). Maintenance of the M. marinum strains and E. coli strains is described in Text S1. Enriched proteins were analyzed using quantitative nano-high-performance liquid chromatography–tandem mass spectrometry (nano-UHPLC-MS/MS) proteomics. All mycobacterial strains were generated using the allelic exchange protocol developed by Parish and Stoker (91) as described previously (35, 45, 52, 92). All strains, constructs, and primers (IDT, Coralville, IA) used in this study are listed in Table S2 in the supplemental material. All plasmids and genetic deletions were confirmed by targeted DNA sequencing performed at the Notre Dame Genomics and Bioinformatics Facility. All proteins were expressed in E. coli with 6×His affinity tags and purified using metal chelation affinity chromatography as described in Text S1. EMSAs were performed as reported previously (93–95), with modifications listed in Text S1. β-Galactosidase assays on M. marinum strains bearing the whiB6::lacZ+, attB::pwhiB6-lacZ+, or attB::pespM-lacZ-
reporter were performed as described previously (52). Hemolysis assays were performed as described previously (53). ESX-1 secretion assays were performed as described previously (35), except that 10 μg of protein was analyzed for all protein fractions. Western blot analysis was performed as described previously (35). Macrophage (RAW 264.7 cells) infections were performed as described previously (45) at an estimated multiplicity of infection (MOI) of 7 (2.5 × 10^6 cells/ml). Cells were imaged and ethidium-homodimer uptake by perforated cells was quantified using ImageJ (35, 52).

For transcriptional profiling, *M. marinum* strains were grown and RNA was extracted exactly as described previously (35). RNA sequencing was conducted as described previously (96), and the results were analyzed using SPARTA software (97). For analysis of differentially expressed genes (>2-fold; q value of <0.05), lists were filtered for genes with average counts greater than 4 (log₂ CPM), with full unfiltered data sets available in Table S3.

**Data availability.** The transcriptional profiling data are available at the NCBI GEO database (accession number GSE135072). All statistical analysis was performed as described in each figure legend, using PRISM v8.1.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

TEXT S1, PDF file, 0.2 MB.

FIG S1, PDF file, 0.4 MB.

FIG S2, PDF file, 0.1 MB.

FIG S3, PDF file, 2.6 MB.

FIG S4, PDF file, 0.8 MB.

FIG S5, PDF file, 0.2 MB.

FIG S6, PDF file, 0.7 MB.

TABLE S1, XLSX file, 1.9 MB.

TABLE S2, PDF file, 1.1 MB.

TABLE S3, XLSX file, 2 MB.

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We contributed as follows. K.G.S., M.J.F., and P.A.C. conceptualized the study. K.G.S., M.J.F., M.M.C., R.B.A., and P.A.C. developed the methodology. All of us contributed to the investigation, validation, data visualization, formal data analysis, writing, and editing of the manuscript. P.A.C. and R.B.A. acquired funding. P.A.C., M.M.C., and R.B.A. provided resources. P.A.C. administered and supervised the project.

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