MavN is a *Legionella pneumophila* vacuole-associated protein required for efficient iron acquisition during intracellular growth

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Iron is essential for the growth and virulence of most intravacuolar pathogens. The mechanisms by which microbes bypass host iron restriction to gain access to this metal across the host vacuolar membrane are poorly characterized. In this work, we identify a unique intracellular iron acquisition strategy used by *Legionella pneumophila*. The bacterial Icm/Dot (intracellular multiplication/defect in organelle trafficking) type IV secretion system targets the bacterial-derived MavN (more regions allowing vacuolar colocalization N) protein to the surface of the *Legionella*-containing vacuole where this putative transmembrane protein facilitates intravacuolar iron acquisition. The $\Delta mavN$ mutant exhibits a transcriptional iron-starvation signature before its growth is arrested during the very early stages of macrophage infection. This intracellular growth defect is rescued only by the addition of excess exogenous iron to the culture medium and not a variety of other metals. Consistent with MavN being a translocated substrate that plays an exclusive role during intracellular growth, the mutant shows no defect for growth in broth culture, even under severe iron-limiting conditions. Putative iron-binding residues within the MavN protein were identified, and point mutations in these residues resulted in defects specific for intracellular growth that are indistinguishable from the $\Delta mavN$ mutant. This model of a bacterial protein inserting into host membranes to mediate iron transport provides a paradigm for how intravacuolar pathogens can use virulence-associated secretion systems to manipulate and acquire host iron.

Legionella | iron | vacuole | MavN | Icm/Dot

The etiological agent of Legionnaires' disease pneumonia is *Legionella pneumophila*. The environmental reservoir for this intracellular pathogen is a diverse array of protozoan species that inhabit natural and man-made water sources (1). Human disease is initiated after inhalation of contaminated water droplets followed by *Legionella* replication within alveolar macrophages (2). Within host cells, *Legionella* establishes a membrane-bound, vacuolar compartment that closely associates with the endoplasmic reticulum (ER) (3). This compartment allows the pathogen to evade host antimicrobial defenses and replicate. The Icm/Dot (intracellular multiplication/defect in organelle trafficking) type IV secretion system (T4SS) of *Legionella* is essential for establishing this *Legionella*-containing vacuole (LCV), and mutants lacking this secretion system fail to replicate intracellularly (4–6). This system delivers ~300 bacterial proteins into host cells, with a subset allowing *Legionella* to hijack host vesicle trafficking pathways, diverting the LCV toward interactions with mitochondria and the ER (3, 7–10). Other T4SS substrates play critical roles in hijacking host cell lipid metabolism, translation, and survival (11, 12). Although mutations within the Icm/Dot secretion apparatus abrogate intracellular replication, loss of any individual substrate typically has little or no effect on the outcome of infection. These results highlight the considerable functional redundancy among the Icm/Dot translocated substrates (IDTSs) (13).

Nutrient deprivation is a key host antimicrobial defense, of which iron restriction is a hallmark (14). Iron within mammalian

cells is present in two forms. In its insoluble ferric [Fe(III)] form, the metal is solubilized by association with high-affinity iron-binding proteins such as ferritin or accessed via endocytosis of transferrin and lactoferrin from extracellular sources. In its soluble, bioavailable, and highly reactive form, ferrous ion [Fe(II)] is found within cytosol in the tightly regulated labile iron pool (LIP) (15). During intracellular growth, microbes must either release ferric iron from host proteins and reduce it to a readily bioavailable form or gain access to the cytosolic LIP (14). Intravacuolar pathogens, such as *Legionella*, have the further problem of needing a strategy for accessing either of these sources across the membrane barrier in which the microorganism resides.

Strikingly, the molecular mechanisms that allow *Legionella* to acquire iron across the vacuolar membrane remain a mystery. Intracellular compartments harboring *Mycobacterium*, *Salmonella*, and *Leishmania* species interact with the endocytic pathway, potentially allowing access to transferrin-bound iron (16–23). The evasion of the endosomal pathway by the LCV, however, results in a compartment that lacks transferrin, indicating that *Legionella* must use previously unidentified mechanisms to access iron intracellularly (24).

Although the mechanisms of iron transport across the host membrane of the LCV are unknown, mechanisms of iron transport across the bacterial membrane during in vitro growth in broth culture have been elucidated. Ferrous iron import is mediated by the inner membrane transporter FeoB (25), whereas ferric iron acquisition is mediated by siderophores, low-molecular-weight iron scavengers. These siderophores are synthesized by LbtA

Significance

Limiting access of intracellular iron to intracellular microbes is a critical arm of the nutritional immune response. Little is known about the mechanisms used by intravacuolar pathogens to overcome this attempted starvation. We show here that *Legionella pneumophila* employs its major virulence-associated secretion system to deliver a unique transmembrane protein into host cells during infection. Utilizing microscopic, biochemical, and transcriptional analysis of intracellular bacteria, we demonstrate that the MavN protein integrates into the host-derived vacuolar membrane and facilitates transport of essential iron into the lumen of the vacuole to promote bacterial growth. These findings shed light on what is likely to be a myriad of strategies used by bacteria to overcome potent host nutrient restrictions.

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(legiobactin A) and FrgA [iron (Fe)-repressed gene A] and are imported and exported via membrane transporters (26–29). These proteins, however, are not required for intracellular replication, leaving unanswered the question of how *Legionella* gains access to essential iron within the LCV (25, 26, 28). Given the extensive role that the Icm/Dot T4SS plays in manipulating host biology during infection, it is possible that a secreted substrate could mediate the essential function of bringing iron into the vacuole. To date, however, there is no evidence that any of *Legionella*'s translocated substrates support iron acquisition across the vacuolar membrane.

A forward genetic screen identified two translocated proteins required for intracellular replication in macrophages, SdhA and MavN (previously called DimB) (30–32). Loss of either protein displays a unique phenotype: a severe growth defect approaching levels observed for *icm/dot* mutants. The SdhA protein plays an important role in maintaining the integrity of the LCV and preventing inflammasome-mediated host cell death (32, 33). In this work, we demonstrate that the IDTS MavN is translocated into host cells and integrated within the host-derived LCV membrane, where it specifically plays an essential role in the vacuolar acquisition of iron by *Legionella* during infection. Our findings here provide evidence of a bacterial translocated protein that can facilitate iron transport across host membranes.

Results

MavN Is Required for Intracellular Replication of *Legionella*. To assess the role of MavN in *L. pneumophila* infection, we generated an unmarked $\Delta mavN$ deletion strain using allelic exchange and measured its ability to replicate within permissive host cells (Fig. 1). The intracellular growth of the $\Delta mavN$ strain was severely impaired in both amoeba, *Dictyostelium discoideum*, and primary A/J bone marrow-derived macrophages (BMDMs), resulting in 130-fold and 1,200-fold less growth than the wild-type *Legionella* at 72 h postinfection (hpi), respectively (Fig. 1A and B, compare $\Delta mavN$ to Lp02). The distinct lack of replication closely resembled the intracellular growth profile of the avirulent *dotA*[−] strain (Lp03) that lacks a functional T4SS apparatus. Previous work had shown that the intracellular growth defect associated with a *mavN* insertion mutation is partially complemented *in trans* (34). We show here, however, that the growth defect of the $\Delta mavN$ mutant is rescued completely by plasmid-encoded *mavN* (*pmavN*), confirming that the intracellular growth defect we observe is due to the absence of MavN (Fig. 1A and B). Unlike SdhA, which shows its distinctive growth defect only in murine macrophages, MavN is required for growth in both amoeba and primary macrophages, indicating that this protein is required for *Legionella* infection across a broad range of host cells.

To visualize intracellular growth within individual LCVs, we performed a microscopic analysis of primary BMDMs infected with *Legionella*, using staining with an anti-*Legionella* antibody. At 12 hpi, there was robust replication of the wild-type strain within macrophages, with 32% of the vacuoles harboring between five and nine bacteria and another 32% containing 10 or more bacteria (Fig. 1C, Lp02). In contrast, 92% of vacuoles generated by the $\Delta mavN$ mutant contained four or fewer bacteria (Fig. 1C). Although there was a profound growth defect, the intracellular accumulation of the $\Delta mavN$ mutant differed from that of the *dotA*[−] mutant, which was completely incapable of intracellular replication (Fig. 1C, Lp03). This difference is consistent with the $\Delta mavN$ mutant undergoing 1–2 rounds of replication before growth arrest, because vacuoles generated by the mutant contained bacterial numbers similar to those containing wild-type *Legionella* at 6 hpi (Fig. 1D). Introduction of a wild-type copy of *mavN* completely restored intracellular replication of the $\Delta mavN$ mutant, with yields of bacteria at least as large as that observed for the wild-type strain after 12 h of infection (Fig. 1C).

Fig. 1. *mavN* is required for intracellular replication in multiple hosts. *D. discoideum* (A) and primary A/J BMDMs (B) were challenged with Lp02 (*icm/dot*⁺), Lp03 (*dotA*³), $\Delta mavN$, or the complemented $\Delta mavN$ strain ($\Delta mavN + pmavN$) at an MOI of 0.05. Intracellular growth was monitored over 72 h by enumeration of cfus. Data shown are representative of three independent infections. Mean $\pm$ SEM is plotted. (C and D) Primary BMDMs were challenged with the indicated *Legionella* strains at an MOI of 0.5. At 12 hpi (C) and 6 hpi (D), the macrophage monolayers were fixed and stained with anti-*L. pneumophila* antibody to distinguish between intracellular and extracellular bacteria (Materials and Methods). The number of bacteria per vacuole was counted, and the percentage of vacuoles that contained the indicated number of bacterial cells is shown. One hundred vacuoles for two replicate infections were counted. Graph shows data from three independent bacterial cultures. Mean $\pm$ SEM is shown.

Legionella manipulates host vesicle trafficking to form a replication-competent LCV that evades fusion with the lysosome and associates with the ER (3, 9, 10, 35). Mutants lacking Icm/Dot components fail to establish this association. Unlike the *dotA*[−] mutant Lp03, the $\Delta mavN$ strain initially appeared to grow intracellularly before its replication was aborted (Fig. 1C and D), consistent with successful establishment of an ER-associated vacuolar compartment. To determine if the LCV of the $\Delta mavN$ strain is associated with ER, postnuclear supernatants containing intact LCVs from infected cells were prepared and localization of the ER membrane protein calnexin around the LCV was analyzed microscopically. Similar to wild-type *Legionella*, calnexin

colocalized with $\Delta mavN$ bacteria, suggesting that MavN does not play a role in LCV biogenesis (*SI Appendix, Fig. S1*).

MavN Is an Icm/Dot Substrate That Localizes to the Membrane of the LCV. MavN is a hypothetical protein of unknown function that shares no sequence homology with characterized proteins in other organisms. BLAST analysis indicates that *mavN* is present only in the genomes of *Legionella* species and that of the closely related arthropod pathogen *Rickettsiella grylli* (36, 37). Hydropathy analysis predicts that MavN is a highly hydrophobic protein with several transmembrane domains, consistent with this protein functioning within cellular membranes (38).

Two independent studies demonstrated that the MavN protein has a carboxyl terminal translocation signal recognized by the Icm/Dot system and that it is likely translocated into host cells during infection (8, 39, 40). This indicates that MavN may directly interface with the host cell cytoplasm during intracellular growth. To demonstrate that full-length MavN translocates across the LCV during macrophage infection, we expressed plasmid-encoded 3xFLAG-tagged MavN (*pmavN*) in the wild-type (Lp02) and *dotA*[−] (Lp03) strain backgrounds and assayed for translocation within BMDMs using immunofluorescence microscopy (Fig. 2*A*). The 3xFLAG-tagged MavN protein was functional and rescued the intracellular growth defect of the $\Delta mavN$ mutant, indicating that the tag did not interfere with intracellular growth (Fig. 1, $\Delta mavN$ + *pmavN*). After challenge of BMDMs, 3xFLAG-MavN could be detected on ~57% of vacuoles harboring the wild-type strain as early as 4 hpi (Fig. 2*B*, Lp02/*pmavN*). By 10 hpi, localization of MavN increased to 84% of the vacuoles. The FLAG staining had a morphology that was consistent with vacuolar membrane localization, as large LCVs at 10 hpi showed peripheral staining around bacterial clusters (Fig. 2*A*). In contrast, no FLAG staining was observed in association with vacuoles containing Lp03/*pmavN* at these time points (Fig. 2*A* and *B*), despite robust expression of the tagged protein in both strain backgrounds (Fig. 2*C*). This indicates that localization of MavN in host cells requires a functional T4SS. Therefore, MavN is an Icm/Dot translocated protein that localizes and accumulates on the vacuolar membrane as replication proceeds.

To determine if MavN localizes to the surface of the LCV, we prepared postnuclear supernatants containing intact LCVs from human macrophages challenged with *Legionella*. Intact vacuoles were stained, in the absence of permeabilization, with an anti-FLAG antibody. At 6 hpi, vacuoles isolated from macrophages infected with wild-type *Legionella* expressing 3xFLAG-MavN showed robust staining around the vacuolar periphery (Fig. 2*D*, Lp02/*pmavN*). In contrast, macrophages infected with *Legionella* harboring only the 3xFLAG epitope contained vacuoles devoid of FLAG staining, indicating that the FLAG staining pattern was a consequence of MavN localization. These data also showed that MavN was exposed on the cytosolic surface of the LCV because no staining of luminal *Legionella* with anti-*Legionella* antibody could be observed until after membrane permeabilization with methanol [Fig. 2*D*, *Legionella* (outside) vs. *Legionella* (inside)]. Thus, the N-terminal 3xFLAG epitope on MavN was detected on the surface of intact LCVs. We also confirmed the host membrane localization of MavN biochemically by mechanically separating the cytosolic and membrane fractions after bacterial challenge of human macrophages, selectively solubilizing proteins from host membranes using digitonin extraction, and probing for 3xFLAG-MavN. MavN was present in the digitonin-soluble membrane fraction, indicating that it was extracted from cholesterol-containing host membranes (Fig. 2*E*). Calnexin, an ER transmembrane protein that localizes to the LCV membrane, was also detected in the digitonin-soluble fraction, demonstrating the effectiveness of this technique. Consistent with MavN being an Icm/Dot T4SS substrate, it was not detected in the membranes of macrophages challenged

with Lp03/*pmavN*, a T4SS-deficient *Legionella* strain. As a control for bacterial contamination, the abundant *Legionella* protein DotF was not observed in the digitonin-soluble membrane fraction. Taken together, the microscopic and biochemical analysis indicates that MavN integrates into the host LCV membrane during intracellular replication.

The $\Delta mavN$ Mutant Is Iron-Starved During Macrophage Infection. The intracellular growth defect of the $\Delta mavN$ mutant is not the result of aberrant vacuolar trafficking (*SI Appendix, Fig. S1*), and the mutant appeared to initiate intracellular replication with normal kinetics before its growth was arrested at ~6 hpi (Fig. 1 *C* and *D*). Replication that aborts in this fashion could be due to the host immune response, a general stress response of the mutant, or starvation of a critical nutrient required for intracellular growth. In previous work, we showed that the host transcriptional response to the $\Delta mavN$ mutant appears similar to that of the wild-type strain, arguing that this dramatic growth arrest is not due to hyperstimulation of host innate immune factors (41) (*dimB* mutant). Therefore, we determined if increased intracellular stress or an inability to acquire a critical nutrient caused the defect exhibited by the absence of MavN.

Bacteria undergoing a stress response will transcriptionally activate regulons appropriate for alleviating that stress, whereas bacteria starved of an essential nutrient will increase production of proteins necessary for acquisition of that nutrient (42). To determine if the $\Delta mavN$ strain shows a hyperresponse diagnostic of either stress or starvation, transcriptional up-regulation of indicated transcripts was measured using total RNA harvested from infected macrophages at 3, 6, and 9 hpi, followed by quantitative RT-PCR (qRT-PCR). The transcription of the global stress response gene *gspA* (43), which is induced during intracellular growth (44), was indistinguishable in the mutant and wild-type strains, with each showing ~200-fold induction of *gspA* by 9 hpi (Fig. 3*A*). Similarly, we found no evidence for increased exposure of the mutant to reactive oxygen species based on equivalent transcriptional patterns of the superoxide dismutase *sodC* during residence of the wild-type and the mutant strains within host cells (Fig. 3*A*) (45). Finally, in response to the intracellular environment, both wild-type and $\Delta mavN$ mutant showed identical induction kinetics of *dpsL*, the gene for the *Legionella* paralog of the Dps protein that is involved in oxidative stress resistance (Fig. 3*A*) (46). Consequently, we found no evidence of enhanced expression of stress response-associated genes after challenge with the $\Delta mavN$ strain relative to wild-type *Legionella*.

The potential for starvation of a specific nutrient was next investigated. During conditions in which amino acids are limiting, *Legionella* growth is arrested and the bacteria transitions into a nonreplicative, transmissive phase that results in increased flagellin expression (47). When the flagellin *fliC* gene was analyzed, no significant differences were observed between the $\Delta mavN$ mutant and wild-type *Legionella*, indicating that the mutant is unlikely to be starved for amino acids (Fig. 3*A*).

Iron starvation as a consequence of the $\Delta mavN$ lesion was next investigated by monitoring the expression of *lbtA*, the first gene in the siderophore synthesis operon (26). By 3 hpi, there was ~10-fold more *lbtA* transcript in the $\Delta mavN$ mutant compared with wild-type Lp02, although both strains expressed equivalent levels of transcript during growth in vitro before macrophage challenge (Fig. 3*B* and *SI Appendix, Fig. S2A*). Even at 6 hpi, when there was a clear increase in *lbtA* expression in Lp02, *lbtA* expression in the $\Delta mavN$ strain was still more than 10-fold higher. This hyperinduction of *lbtA* in the $\Delta mavN$ strain persisted at 9 hpi (Fig. 3*B*). A similar pattern of premature induction in the $\Delta mavN$ strain could be observed for the iron-regulated, putative siderophore synthase gene *frgA* (48) (Fig. 3*B*). Addition of a wild-type copy of the *mavN* gene to the $\Delta mavN$ mutant

Fig. 2. MavN is a translocated Icm/Dot substrate that localizes to the membrane of the LCV. (A) Primary A/J BMDMs were challenged with either Lp02 (WT)/pmavN or Lp03 (dotA3)/pmavN. At 4 and 10 hpi, macrophage monolayers were fixed and stained with anti-*L. pneumophila* (Lp) (red) and anti-FLAG (green). Insets represent 2 $\times$ magnification of selected area. (Scale bars, 5 μ m.) (B) Quantification of *L. pneumophila*/FLAG colocalization. nd, not detected. Data are displayed as the percentage of *L. pneumophila* vacuoles positively staining for 3 $\times$ FLAG-MavN. One hundred vacuoles from two replicate infections were counted. Graph shows data from three independent bacterial cultures. Mean $\pm$ SEM is shown. (C) Crude lysates were prepared from Lp02/pmavN and Lp03/pmavN grown overnight in AYE + 0.5 mM IPTG before bacterial challenge. Western blot analysis was used to determine the expression of FLAG-MavN in both strains using an anti-FLAG antibody. Anti-DotF was used as a loading control. (D) U937 macrophages were challenged with either Lp02/pFLAG or Lp02/pmavN. Postnuclear supernatants were prepared from cells at 6 hpi, adhered to poly-lysine-coated coverslips, and stained with

abolished the premature induction of both *lbtA* and *frgA*, indicating that the observed induction was due to the absence of *mavN* (Fig. 3B and *SI Appendix*, Fig. S2B). To determine if all known iron acquisition genes are prematurely induced, we monitored the expression of the ferrous iron transporter *feoB* and observed no differences in the pattern of expression between wild-type *Legionella* and the Δ *mavN* mutant during infection (Fig. 3B, panel 3). Thus, a subset of iron starvation-responsive genes was prematurely hyperinduced in the Δ *mavN* strain during infection. This indicates that the LCV established by the Δ *mavN* mutant is an iron-deficient vacuolar compartment.

The basis for the lack of response of *feoB* (part of the *feoAB* operon) was not clear. *lbtA*, *frgA*, and *feoB* all contain putative ferric uptake regulator (Fur)-binding elements (fur boxes) within their promoters ((25, 28, 48). Fur is a transcription factor that binds fur boxes and represses gene expression when cellular iron levels are high. Upon iron depletion, this repression is relieved and the expression of iron acquisition genes is induced (49). The *feoAB* fur box in *L. pneumophila* serotype Philadelphia-I contains five mismatches compared with the *Escherichia coli* consensus fur box sequence. To determine why this operon showed little regulation in the Δ *mavN* strain, we measured the *feoB* transcript levels of wild-type *Legionella* grown in broth culture containing the iron chelator deferoxamine (DFX). After an 8-h incubation in this iron-poor medium, there was a 12-fold induction of *feoB* compared with growth in unsupplemented AYE (*SI Appendix*, Fig. S2C). In contrast, *frgA*, which contains two overlapping fur boxes in its promoter, exhibited a robust 102-fold induction. The gene *lpg2814* located immediately upstream of *mavN* showed no such induction ($\sim$ 1.7-fold), consistent with the lack of a fur box within its promoter. Therefore, the lack of intracellular induction of *feoB* is a reflection of its lower sensitivity to iron starvation. Taken together, these results indicate that the Δ *mavN* mutant is iron-starved within the LCV.

MavN Is an Iron-Regulated Gene. The transcriptional profile of the *mavN* gene was next analyzed to determine whether it is iron-regulated. The transcriptional start site of *mavN*, mapped using rapid amplification of 5' complementary cDNA ends (5' RACE), revealed that the ORF was incorrectly annotated in the published *L. pneumophila* Philadelphia-1 genome (50). Instead, we found that the start site initiated 42 base pairs downstream of the annotated site (Fig. 4A). A search for iron regulatory elements within this newly defined promoter region identified a putative fur box that aligns with the canonical *E. coli* fur box at 16 of 19 base pair positions (Fig. 4B). A similar fur box had been identified upstream of the *mavN* ortholog annotated in the *L. pneumophila* strain Paris isolate (34). Using this experimentally determined start site, *mavN* transcript levels were measured during growth in broth culture to determine if it was regulated by iron. Exponentially growing wild-type *Legionella* was grown in AYE, AYE supplemented with ferric nitrate (FN), or AYE containing the iron chelator DFX. After an 8-h incubation in iron-poor media, there was a $\sim$ 45-fold induction of *mavN* compared with growth in

indicated antibodies. Before membrane permeabilization with methanol, coverslips were stained with anti-FLAG antibody (outside FLAG) (green) and rat anti-*L. pneumophila* antibody (blue). After permeabilization, cells were stained with rabbit anti-*L. pneumophila* antibody (inside Lp) (red) (*Materials and Methods*). (Scale bars, 5 μ m.) (E) Icm/Dot-dependent localization of MavN to host cell membranes during infection. U937 macrophages were infected with the indicated strain at an MOI of 5. At 6 hpi, infected macrophages were mechanically lysed, and the cytosolic fraction was collected. Proteins were selectively extracted and solubilized from the host membrane fraction using digitonin. Fractions were analyzed by immunoblotting with indicated antibodies.

Fig. 3. The $\Delta mavN$ mutant prematurely induces the expression of a subset of iron acquisition genes. Total RNA was harvested from RAW264.7 macrophages at 3, 6, and 9 hpi. qRT-PCR was performed to determine the relative transcription levels of selected genes in both the Lp02 (WT)- and $\Delta mavN$ -challenged monolayers. Non-iron-regulated (A) and iron-regulated (B) genes were examined. Relative transcript levels are normalized to Lp02 at 3 hpi (#). Significance, determined by Student's *t* test, was as follows: *lbtA*, 3 hpi, Lp02 vs. $\Delta mavN$ ($P < 0.01$); *lbtA*, 6 h, Lp02 vs. $\Delta mavN$ ($P < 0.005$); *frgA*, 3 h, Lp02 vs. $\Delta mavN$ ($P < 0.05$); and *frgA*, 6 h, Lp02 vs. $\Delta mavN$ ($P < 0.05$).

unsupplemented AYE (Fig. 4C). These results paralleled those observed for the iron-regulated genes *frgA* and *lbtA* (Fig. 4C).

The presence of a putative fur box within the *mavN* promoter is consistent with its expression being controlled by the iron-sensing transcription factor Fur. To test this hypothesis, mutations were generated in the *mavN* fur box, preserving the -35 region that was predicted by 5' RACE mapping (*mavN* "fur mut") (Fig. 4B), and *mavN* transcript levels were measured during growth in either iron-replete or -depleted broth. In iron-replete broth (normally repressing conditions), the *mavN* fur box mutation resulted in a 15-fold increase in transcript levels compared with the wild-type fur box (Fig. 4C). Growth in iron-depleted medium resulted in transcriptional induction of *mavN* in the wild-type strain, whereas the fur box mutant strain showed no further increase (Fig. 4C), indicating that fur box mutation caused constitutive expression, typical of an inability to bind Fur (51–53). The behavior of the fur box mutant is consistent with MavN allowing access to host iron stores during intracellular growth as it is induced under iron-limiting conditions. The alternative model is that MavN is a regulatory element required for the expression of iron-regulated genes. To test this model, the transcription of *frgA* and *lbtA* was measured in *Legionella* strains possessing either a wild-type or mutant *mavN* fur box. As a further control, a fur box mutant was generated that was predicted to contain a defective -35 box (-35 mut), which would inhibit RNA polymerase-driven transcription of *mavN* (Fig. 4B). Accordingly, there was no *mavN* expression in the -35 mut, even under conditions of iron limitation (Fig. 4C). Consistent with an inability to transcribe *mavN*, the -35 fur box mutant phenocopied a $\Delta mavN$ mutant and was defective for intracellular growth in BMDMs (SI Appendix, Fig. S3). Transcription of *frgA* and *lbtA* was unaffected by any of the *mavN* fur box mutants during growth in broth culture, and both genes showed identical iron-responsiveness in all strains (Fig. 4C).

Therefore, transcription of other putative Fur-regulated genes is independent of *mavN* expression during in vitro growth in broth culture. This result is in striking contrast to the behavior of these genes during intracellular growth (Fig. 3B), arguing that MavN plays a specific role during infection.

Defective Intracellular Growth of the *mavN* Mutant Is Rescued by Excess Iron. Our data suggest that MavN is required for *Legionella* to obtain adequate iron across the membrane of the LCV. If this is correct, the requirement for MavN may be bypassed by increasing host iron levels during infection. To test this hypothesis, macrophages were challenged with either wild-type *Legionella* or the $\Delta mavN$ mutant, and at 2 hpi, the macrophage media was supplemented with varying amounts of either ferric ammonium citrate [Fe(III)-AC] or ferrous sulfate [Fe(II)SO₄]. At 96 hpi, there were significant dose-dependent increases in intracellular replication of the $\Delta mavN$ mutant. Supplementation with 100 μ M Fe(III)-AC or Fe(II)SO₄ resulted in a 72-fold or 160-fold increase in bacterial growth of the $\Delta mavN$, respectively, compared with growth in unsupplemented macrophage media (Fig. 5A). In contrast, intracellular growth of the wild-type Lp02 was unaffected by iron supplementation (Fig. 5A). This growth rescue was specific to iron supplementation because addition of zinc (ZnSO₄), manganese (MnSO₄), nickel (NiSO₄), or copper (CuSO₄) failed to increase the intracellular growth of the $\Delta mavN$ mutant. In fact, copper exacerbated the $\Delta mavN$ growth defect, with medium containing 100 μ M CuSO₄ causing a 12-fold decrease in intracellular growth of the mutant compared with the absence of

Fig. 4. *mavN* is an iron-regulated gene. (A) Schematic of *mavN* locus, highlighting the location of the fur box (yellow shading) and the predicted (first nucleotide) and experimentally determined (gray shading) transcriptional start sites. (B) Alignment of the consensus *E. coli* fur box with the *L. pneumophila mavN* fur box. The mutations of the *mavN* fur box generated in this study are also shown. The position of the putative -35 box of *mavN* is marked. (C) Fur box mutation results in constitutive expression of *mavN*. Total RNA was harvested from bacteria grown in AYE, AYE supplemented with 334 μ M FN (black bars), or AYE media treated with 7.5 μ M DFX (white bars). Transcript levels are displayed relative to expression level in bacteria grown in untreated AYE. Data displayed are from two independent experiments.

Fig. 5. Exogenous iron supplementation rescues the intracellular growth defect of the $\Delta mavN$ mutant. (A) Metal rescue is limited to iron. BMDMs were challenged with either Lp02 (WT) or the $\Delta mavN$ mutant. At 2 hpi, macrophage cultures were treated with 5, 50, or 100 μ M of the indicated metal. At 96 hpi, macrophage lysates were plated for cfus. Each bar represents mean $\pm$ SEM of triplicate samples. $*P < 0.0001$, based on Student's t test. (B) Iron rescue occurs throughout intracellular growth. BMDMs were pretreated with 0 or 100 μ M ferric ammonium citrate for 24 h before challenge with bacteria. At indicated time points, macrophages were lysed and cfus were determined. (C) MavN is not required for in vitro growth in broth culture. Lp02 (WT) or the $\Delta mavN$ mutant were cultured in AYE or AYE containing indicated concentrations of DFX (5, 7.5, 10 μ M). Mean $\pm$ SEM of three independent cultures is shown. (D) MavN is not required for in vitro growth on solid media. Lp02, the $\Delta mavN$ mutant, and the complemented $\Delta mavN+p\Delta mavN$ were grown to exponential phase in unsupplemented AYE broth, washed with PBS, and 10-fold serial dilutions were spotted onto AYE plates containing supplemental iron or the indicated concentration of DFX. Growth was monitored after a 4-d incubation at 37 $^{\circ}$ C.

metal supplementation (Fig. 5A, compare Lp02 to $\Delta mavN$). As copper treatment has been linked to decreased intracellular iron stores, the $\Delta mavN$ -specific defect could be the result of copper aggravating the iron starvation experienced by the $\Delta mavN$ mutant (54).

The rate of intracellular growth was also analyzed in macrophages preloaded with 100 μ M Fe(III)-AC for 24 h. The addition of iron had no effect on growth of either Lp02 or Lp03 (Fig. 5B). In contrast, the stimulation of $\Delta mavN$ growth by iron supplementation was obvious by 24 hpi, resulting in an ~ 3 -log increase in intracellular cfus by 72 hpi (Fig. 5B). Addition of exogenous iron to the macrophage media was accompanied by a concordant increase in host ferritin levels, a homeostatic response to maintain cytoplasmic iron levels (SI Appendix, Fig. S4A). The enhanced *Legionella* growth was not the result of bacterial growth in the macrophage medium because bacteria were unable to grow in this medium at any concentration of Fe(III)-AC addition (SI Appendix, Fig. S4B). Therefore, the increase in $\Delta mavN$ replication after iron supplementation represented increased growth within host macrophages.

To determine if MavN is required for growth under iron-limiting conditions in axenic cultures, we compared the growth of Lp02 and the $\Delta mavN$ mutant in broth containing increasing concentrations of the iron chelator DFX. Growth of the $\Delta mavN$ mutant was indistinguishable from that of wild-type *Legionella* when iron was limiting in liquid broth culture (Fig. 5C) or on solid media (Fig. 5D). In contrast to previous work that suggests MavN is involved in iron transport in bacteriological cultures (34), our data argue strongly against the hypothesis that MavN is involved in iron acquisition during extracellular growth. The fact that iron-regulated genes are induced in MavN-defective mutants during growth within macrophages, but not during growth in broth culture, indicates that the protein is only required for iron acquisition during intracellular growth.

Identification of a Putative MavN Iron-Binding Motif. To elucidate the mechanism of action of MavN protein, we scanned the amino acid sequence for motifs that may be involved in directly binding iron (Fig. 6A). We first searched for EXXE motifs, which are required for iron transport activity in numerous fungal iron transporters and are found in ferritin and a *Salmonella* iron sensor (55–58). Nine EXXE motifs were identified, and site-specific mutagenesis was performed to mutate each one to AXXE in the 3xFLAG-tagged MavN. Mutated *mavN* constructs were introduced into the $\Delta mavN$ mutant and their ability to complement the intracellular growth defect was investigated. One of the nine mutant constructs, pEXXE-3 (E439A), located in loop 7, failed to complement the $\Delta mavN$ mutant, indicating that it is critical for MavN activity (Fig. 6B and C).

Loop 7, which contains the critical glutamine residue of EXXE-3, is highly enriched in histidine residues (Fig. 6A and B). His-rich sequences can be found in diverse families of metal transporters (59). Additionally, histidines tend to be enriched in binding sites for iron (60). We generated three mutant constructions—a 3-His (H445A, H450A, H452A), a 4-His (H460A, H462A, H465A, H466A), and a composite 7-His mutant—by site-specific mutagenesis (Fig. 6B). Complementation of the $\Delta mavN$ mutant replication defect was only observed with the 4-His mutant construction, indicating that one or all of the first three histidines within that region are essential for MavN function (Fig. 6C). The lack of complementation is not due to a failure to produce these protein derivatives because there were equivalent steady-state levels of each one (SI Appendix, Fig. S5A). The lack of complementation is also not due to mislocalization of these mutant proteins, as they are translocated and localized to the LCV membrane similarly to wild-type MavN (SI Appendix, Fig. S5B). Given the identification of essential amino acids in loop 7, we created two additional mutant constructs that alter regions

Fig. 6. Identification of a putative *mavN* iron-binding region. (A) Schematic of the putative topology of MavN as determined by the topology prediction program TOPCONS (topcons.net). The His-rich loop is predicted to be in the lumen of the vacuole, based on accessibility of N-terminal FLAG tag to the antibody (Fig. 2D). EXXE motifs are numbered and shaded gray. The putative loop 7 is labeled with histidines shaded red. (B) Loop 7 mutations analyzed in this study. The sequence of the loop 7 region of MavN aligned with MavN mutations generated. (C) Selected loop 7 mutations disrupt intracellular growth. BMDMs were challenged (MOI, 0.05) with a $\Delta mavN/lux^+$ harboring indicated pJB908-derived plasmids. Intracellular replication was monitored by luciferase activity. Growth is reported as relative luminescence units (RLUs). Data represent triplicate macrophage cultures and displayed as the mean $\pm$ SEM.

rich in Glu and Asp residues in this loop, as acidic side chains are often involved in metal binding (60) (Fig. 6B, EXXE-4b and EXXE-5b). Both of these mutants complemented the $\Delta mavN$ strain for intracellular growth, indicating that these residues are not essential for the function of MavN (Fig. 6C). Thus, a single E439A mutant and the 3-His (H445, H450A, H452A) mutant caused unique defects in intracellular growth. Taken together, these data could indicate that residues thought to be associated with iron binding and transport are required for MavN function.

Discussion

The LCV is sequestered from host endocytic compartments (61, 62). As a consequence, it is likely that there is limited contact of

Legionella with the vesicular traffic of iron-containing proteins, requiring an alternative iron acquisition strategy to allow bacterial growth (24). This work proposes that the *Legionella* protein MavN is the central bacterial player in driving this strategy. Our data support the model that, after establishing the LCV, the bacterium experiences iron limitation, resulting in enhanced expression of *mavN*, which encodes an Icm/Dot translocated transmembrane protein that localizes to the host-derived vacuolar membrane. Given that the growth defect of *mavN* mutants is partially bypassed by flooding cells with iron and that the absence of the protein results in premature iron starvation, it is likely that MavN is a vacuolar iron transporter.

Previous studies have identified two *Legionella* iron acquisition systems that play a role in bacteriological culture but have variable requirements for intracellular replication. The absence of *feoB* results in a 10-fold growth defect in the human U937 macrophage-like cell line, although it has no discernible effect on growth within the amoeba *Hartmannella vermiformis* (25). Mutants disrupted in genes involved in synthesis, export, and import of the *Legionella* siderophore legiobactin, on the other hand, have no complementable growth defects in any cell type tested (26, 28, 29). In contrast, our data indicate that there is no requirement for MavN during growth in any bacteriological culture condition tested, despite the stringent intracellular growth requirement for this protein. This conclusion conflicts with recent work that posits a role for MavN during growth in iron-limiting bacteriological cultures in the *Legionella* strains 130b and Paris (34). An explanation for these conflicting results could be that the function of MavN differs among different *Legionella* strains. Alternatively, in some strain backgrounds, MavN could function to facilitate iron transport across both prokaryotic and eukaryotic membranes. Finally, the *mavN* mutants described previously may harbor other genetic perturbations that affect growth in culture. Consistent with this explanation, the intracellular growth defect associated with these strains is not fully complemented by a wild-type copy of *mavN* (34).

Several lines of evidence support a specific role for MavN in facilitating intracellular iron acquisition. First, within host cells, *Legionella* $\Delta mavN$ mutants showed premature transcriptional activation of iron starvation-associated genes. Premature induction occurs as early as 3 hpi, including genes involved in siderophore biosynthesis, which are transcribed 10-fold higher than in wild-type cells at this time point. Second, we could partially rescue the intracellular growth defect of the $\Delta mavN$ mutant by supplementing the macrophage culture medium with exogenous iron. Iron supplementation resulted in a clear increase in intracellular iron stores within the host cell, with consequent up-regulation of cytoplasmic ferritin. The mechanism by which exogenous iron supplementation bypasses the requirement for MavN is unknown, although a similar phenomenon has been previously observed during *Candida glabrata* infection (63). It is possible that this bypass could result from aberrant interactions between the LCV and ferritin/iron-containing endosomes as the host cells try to maintain homeostatic levels of iron during ferric ammonium citrate-induced iron overload (64, 65). Rescue was specific to supplementation with iron, as manganese, copper, zinc, and nickel failed to reverse the growth arrest of the $\Delta mavN$ mutant. Third, environmental iron levels transcriptionally regulated *mavN* expression, controlled by a promoter that we demonstrated contains a functional iron-responsive fur box. Fur is a divalent metal ion-binding protein that recognizes a 19-bp fur box in response to abundant iron. As the fur box often overlaps with the -10 and -35 promoter elements, Fur binding occludes RNA polymerase binding to block transcription. A *Legionella* Fur homolog has been identified (66), and the promoters of *feoAB*, *lbtABC*, and *lbtU* contain putative fur boxes. A fur box participates in the iron regulation of *mavN* because mutation of this promoter

element resulted in constitutive, iron concentration-independent expression of *mavN*.

For *Legionella* growing in vitro, ferrous iron can be directly imported through the *feoB* inner membrane transporter, whereas siderophores can be used to scavenge ferric iron, importing it through inner and outer membrane transporters (25, 26, 28, 29). The bacterium also secretes HGA-melanin, which has a ferric reductase activity that could allow ferrous iron to be assimilated via the *feoB* transporter (67). Within host cells, the majority of iron is present either as ferric iron tightly bound to host glycoproteins that prevent easy access or is sequestered in the cytosol within the LIP. Vacuolar pathogens that interact with endosomes are able to scavenge iron from the host glycoproteins within these compartments and may not require specialized vacuolar transporters to acquire iron. *Mycobacterium tuberculosis* secretes siderophores that acquire iron from transferrin after fusion of endosomes with the replication compartment (18, 20, 68), and siderophore mutants are severely attenuated for growth in macrophages (69). *Salmonella* spp., which reside in an acidic compartment, similarly uses multiple siderophores to acquire iron intracellularly, possibly from transferrin as well (21). *Leishmania* parasites, which reside in a phagolysosome-like vacuole, express a membrane-bound ferric reductase LFR1, which generates ferrous iron that can be taken up by the LIT1 ferrous iron transporter, which is also present in the parasite membrane (22, 23). The absence of direct interaction of the LCV with transferrin receptor-rich endosomes (24) raises a problem for *Legionella* during intracellular growth that we propose is solved by the Icm/Dot substrate MavN. The unique incidence of this protein within *Legionella* species and *R. gryllii* supports this line of reasoning. *Coxiella burnetii* is a close evolutionary relative of *Legionella* and also possesses an Icm/Dot translocation system. *Coxiella*, however, does not have a homolog of MavN, which we propose is not needed because the bacterium resides in a vacuole that fuses with lysosomes, allowing access to the transferrin iron uptake pathway (70). Thus, MavN appears to be the key bacterial protein that allows the bacterium access to host iron, and its position on the vacuolar membrane could facilitate access to the cytosolic LIP.

MavN integrates into the host vacuolar membrane during infection, and our ability to detect an N-terminal FLAG epitope tag on the surface of intact, isolated LCVs indicates that the N terminus of this transmembrane protein is exposed to the cytoplasm during infection. Further experimental analysis is required to determine the exact topology of this unique protein, although hydropathy plots offer predictive models (Fig. 6A) (38). The hydrophobic nature of MavN argues that additional bacterial or host factors may chaperone its translocation and integration into the LCV membrane. A bioinformatic analysis of predicted hydrophobic regions in IDTSs identified 71 putative transmembrane proteins, including a *Legionella* Icm/Dot substrate shown to integrate into the inner mitochondrial membrane (71). The abundance of such hydrophobic bacterial factors indicates that *Legionella* must have mechanisms for integrating bacterial proteins such as MavN into host cell membranes.

We favor a model in which MavN acts as an iron transporter. Transmembrane loop 7 is of particular interest in this regard. The loop contains a 22-residue region, starting at His⁴⁴⁵, that contains seven histidine residues. Simultaneously mutating His⁴⁴⁵, His⁴⁵⁰, and His⁴⁵² to alanine abrogates the function of MavN without altering steady-state levels or localization of the protein, indicating that these residues are required for MavN activity. His-rich motifs have been identified in numerous metal ion transporters and are present in the cytoplasmic loops of the functionally characterized metal transporters Zrt1 (*Saccharomyces cerevisiae*) and Irt1 (*Arabidopsis thaliana*) (72). Although the function of His-rich regions in these proteins is not yet known, they have been implicated in metal binding (59). Consistent with the hypothesis that MavN is an iron transporter, loop

7 could define an iron-binding domain. As our current topological prediction places loop 7 within the lumen of the LCV, we predict that these critical residues may facilitate iron traffic through the membrane or coordinate with iron transport systems on the bacterial membrane. Similar localization of a histidine-rich motif on the face of the membrane opposite the source of the metal is quite common in metal transporters of the ZIP (Zrt- and Irt-like Proteins) and CDF (Cation Diffusion Family) families (72). It has been postulated that the histidines within this motif in the human zinc transporter hZip1 may coordinate with residues within the transmembrane domain to mediate zinc during transport (73). In addition, it is worth noting that topological prediction programs differ in the number of transmembrane domains predicted for MavN, so experimental validation of the displayed orientation is required. It is also possible that MavN may mediate vacuolar iron acquisition by another mechanism, such as recruiting and facilitating interactions with host or bacterial factors that transport iron into the LCV. Analysis of the interaction of purified derivatives of MavN and host proteins involved in cellular iron homeostasis should contribute significantly to understanding the molecular basis of iron acquisition across the replication vacuole membrane.

Materials and Methods

Bacterial Strains, Cell Culture, and Growth Media. *E. coli* strains were cultured as described (74). *L. pneumophila* strain Philadelphia-1, Lp02 (*thyA*⁺), served as the parental wild-type strain, and all Lp02 derivatives were cultured as described, except where noted (74). Details of the cloning strategy and strain construction used are described in *SI Appendix, SI Materials and Methods*.

D. discoideum strain Ax4 was cultured as described (75), as were primary BMDMs (3). U937 cells (ATCC CRL-1593.2) were maintained and differentiated as described previously (76). The RAW264.7 macrophage-like cell line (ATCC TIB-71) was obtained from the ATCC and was cultured in Dulbecco's modified Eagle medium (DMEM) (Gibco) supplemented with 10% (vol/vol) heat-inactivated FBS (Gibco), 1,000 U/mL penicillin (Gibco), and 1,000 U/mL streptomycin (Gibco).

Growth Assays. Details of *Legionella* growth in either liquid culture (measuring optical density at 600 nm) or on solid media (monitoring colony formation) are provided in *SI Appendix, SI Materials and Methods*. Quantitative and microscopic intracellular growth assays, in the presence and absence of transition metals, were performed as described previously (74), with modifications detailed in *SI Appendix, SI Materials and Methods*. Fluorescence microscopy was performed as previously described (32).

Translocation Assays. Icm/Dot-dependent translocation of MavN into differentiated U937 macrophages was assessed by immunofluorescence in intact cells and isolated vacuoles from postnuclear supernatants as described previously (30, 77).

Transcriptional Analyses. To analyze transcription during broth growth, overnight cultures of indicated strains were grown to exponential phase in liquid AYE. Bacterial cells were pelleted, resuspended in fresh AYE, and subcultured into either AYE, AYE + 334 μ M FN, or AYE + 7.5 μ M DFX. Cultures were then incubated at 37 °C on a rotating platform. After an 8-h incubation, 1 mL of each culture was harvested, RNA Protect Bacteria Reagent (Qiagen) was added, and total RNA was harvested using RNeasy Mini kit (Qiagen) and treated with Turbo DNase (Ambion). qRT-PCR samples were prepared using the Power SYBR RNA-to-CT 1-Step (Applied Biosystems), and the reaction was analyzed in an Applied Biosystems Step One-Plus. Results were analyzed using the Step One Software, v2.2. Relative expression, $\Delta\Delta$ Ct analysis, was performed using 16S rRNA as a reference transcript.

Transcription during intracellular growth was analyzed in RAW264.7 macrophage-like cells. The RAW264.7 cells were seeded in DMEM + 10% FBS (no antibiotics) and challenged with indicated *Legionella* strains at an MOI (multiplicity of infection) of 1–1.5 in two 10-cm Petri dishes. Plates were centrifuged at 200 $\times$ g for 5 min and incubated at 37 °C, 5% CO₂ for 2 h. Medium was removed and replaced with medium containing 50 μ g/mL gentamicin for 1 h to kill extracellular bacteria. Macrophage monolayers were then washed three times with DMEM alone, before being incubated in DMEM + 10% FBS (no antibiotics). At indicated time points, macrophages were lysed

with TRIzol Reagent (Ambion) and RNA was prepared according to the manufacturer's instructions. Briefly, culture supernatants were decanted, and 4 mL TRIzol Reagent was quickly added before cell lysis with vigorous pipetting. Lysates from two plates were combined, flash frozen in liquid nitrogen, and stored at -80°C until RNA harvesting. The lysates were thawed at 37°C , incubated at 95°C for 10–15 min, and incubated on ice for 5 min. We added 1.6 mL chloroform to the lysate and shook it vigorously. After incubation of 2–3 min at room temperature, the samples were transferred to a prespun 15 mL phase lock tube (5-Prime) and centrifuged at $1,500 \times g$ for 4 min at 4°C . The aqueous phase was collected, and 4 mL isopropanol was added to precipitate the RNA. After 10 min of incubation at room temperature, the sample was centrifuged at $12,000 \times g$ for 15 min at 4°C . The pellet was washed with 70% ethanol, allowed to air dry, resuspended in 200 μL ultrapure water (Invitrogen), and RNA was purified using the RNeasy Midi (Qiagen), removing contaminating DNA with Turbo DNA-free (Ambion).

qRT-PCR analysis was performed as described for bacteria grown in bacteriological culture.

The transcriptional start sites were determined using the 5' RACE System for Rapid Amplification of cDNA Ends, version 2.0 (Invitrogen). The products of the nested PCR were sequenced to determine the transcriptional start site (Genewiz).

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