The Manganese Transporter MntH Is a Critical Virulence Determinant for *Brucella abortus* 2308 in Experimentally Infected Mice\(^\text{†}\)

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The gene designated BAB1_1460 in the *Brucella abortus* 2308 genome sequence is predicted to encode the manganese transporter MntH. Phenotypic analysis of an isogenic mntH mutant indicates that MntH is the sole high-affinity manganese transporter in this bacterium but that MntH does not play a detectable role in the transport of Fe\(^{2+}\), Zn\(^{2+}\), Co\(^{2+}\), or Ni\(^{2+}\). Consistent with the apparent selectivity of the corresponding gene product, the expression of the mntH gene in *B. abortus* 2308 is repressed by Mn\(^{2+}\), but not Fe\(^{2+}\), and this Mn-responsive expression is mediated by a Mur-like repressor. The *B. abortus* mntH mutant MWV15 exhibits increased susceptibility to oxidative killing in vitro compared to strain 2308, and a comparative analysis of the superoxide dismutase activities present in these two strains indicates that the parental strain requires MntH in order to make wild-type levels of its manganese superoxide dismutase SodA. The *B. abortus* mntH mutant also exhibits extreme attenuation in both cultured murine macrophages and experimentally infected C57BL/6 mice. These experimental findings indicate that Mn\(^{2+}\) transport mediated by MntH plays an important role in the physiology of *B. abortus* 2308, particularly during its intracellular survival and replication in the host.

*Bruceilla abortus* is a gram-negative bacterium that is responsible for the zoonotic disease brucellosis. Brucellosis causes spontaneous abortion and sterility in ruminants (27) and a debilitating febrile illness in humans known as undulant fever (17). The ability of brucellae to cause disease is directly related to their capacity to establish and maintain intracellular infection in host macrophages (63). Within the phagosomal compartment in these host cells, brucellae must cope with oxidative stress, low pH, and nutrient deprivation. The availability of metal ions is restricted within this environment due in part to the activity of the host natural resistance-associated macrophage protein (NRAMP-1), which transports divalent cations out of the phagosome (40). Mn\(^{2+}\) serves as an important cofactor for a variety of bacterial enzymes, including those involved in carbon metabolism, induction of the stringent response, and detoxification of reactive oxygen species (ROS) (55). Consequently, the inability of brucellae to acquire sufficient levels of this divalent cation may compromise their ability to successfully adapt to the environmental conditions encountered during residence in their intracellular niche.

Manganese uptake by bacteria is typically accomplished through the activity of either ABC-type transporters such as the SitABC complex (4, 42, 59, 65) or H\(^+-\)-dependent manganese transporters such as MntH (37, 41, 52, 60). Many bacteria possess both types of Mn\(^{2+}\) transporters (55), but a survey of the publicly available *Brucella* genome sequences (14, 20, 36, 57) suggests that these bacteria do not produce a SitABC-type transporter and rely solely on an MntH homolog for the high-affinity transport of Mn\(^{2+}\). *Escherichia coli* MntH was originally described as being able to transport both Mn\(^{2+}\) and Fe\(^{2+}\) (52), but subsequent studies indicated that this and other bacterial MntH proteins are highly selective Mn\(^{2+}\) transporters that play a minor, if any, role in Fe\(^{2+}\) transport under physiologically relevant conditions (41). To examine the role of *Brucella* MntH in Mn\(^{2+}\) transport and virulence, the gene annotated as BAB1_1460 in the *B. abortus* 2308 genome sequence was disrupted in this strain by gene replacement and the phenotype of the resulting mutant (MWV15) was examined. The results of these studies indicate that MntH plays a critical role in Mn\(^{2+}\) transport in *B. abortus* 2308 and that the presence of this manganese transporter is essential for the wild-type resistance of this strain to oxidative killing in vitro and its virulence in the mouse model.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** *Brucella abortus* 2308 and derivatives of this strain were cultivated on Schaedler agar supplemented with 5% defibrinated bovine blood (SBA) at 37°C with 5% CO\(_2\) or in brucella broth at 37°C with shaking unless otherwise noted. *Escherichia coli* strain DH5\(\alpha\) was used as the host strain for recombinant DNA procedures, and this strain was cultivated on tryptic soy agar at 37°C or in LB broth at 37°C with shaking. Growth media were supplemented with ampicillin (25 \(\mu\)g/ml for *B. abortus* and 100 \(\mu\)g/ml for *E. coli* DH5\(\alpha\)), chloramphenicol (5 \(\mu\)g/ml for *B. abortus* and 30 \(\mu\)g/ml for *E. coli* DH5\(\alpha\)), and/or kanamycin (45 \(\mu\)g/ml) as necessary. *Brucella* stock cultures were

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maintained in brucella broth supplemented with 25% glycerol, and *E. coli* stock cultures were maintained in LB supplemented with 25% glycerol at −80°C.

**Construction of an mntH-lox2 transcriptional fusion.** Oligonucleotide primers (forward, 5'-GAGGGGGGCGATCCTTTCGAA-3'; reverse, 5'-GTGTCGGGAGGTTCCATAAC-3') and PCR were used to amplify a 1,031-bp fragment of genomic DNA from *B. abortus* 2308 containing portions of the *mntH* (BAB1_1458) and *mntH* (BAB1_1460) genes and intervening regions. The resulting PCR fragment was digested with HindIII and KpnI, and a 239-bp HindIII-KpnI fragment containing upstream sequences and extending 8 bp into the *mntH* coding region was directionally cloned into the *lacZ* transcriptional fusion vector pMR15 (32). The authenticity of the *mntH-lox2* fusion in the resulting plasmid, pEAM1, was verified by restriction mapping and nucleotide sequence analysis. Transcriptional activity of the β-galactosidase reporter fusion was determined using the methods described by Miller (53).

**Construction of the *B. abortus* mur mutant Fur2.** Plasmid pDS1 contains an 867-bp fragment of genomic DNA from *B. abortus* 2308 containing the mur homolog designated BAB1_1668 cloned into pUC9 (35). An inverse PCR strategy (23) was used to generate a linear derivative of pDS1 that lacks a 338-bp region internal to the 426-bp coding region of *Burkella mur* by using the primers 3'mur (5'-GGCTCAATCTGATGCTCATA-3') and 5'mur (5'-CAATCCGCGTTCAAAATTCC-3'). The chloramphenicol resistance gene from pBCSK (NEB) was ligated to the linear 3'mur derivative of pDS1, and the resulting plasmid was used to introduce a mur mutation into the genome of *B. abortus* 2308 by gene replacement using the methods described by Elzer et al. (25). The genotype of the *B. abortus* mur mutant (Fur2) constructed in this fashion was confirmed by Southern blot analysis with cat- and mur-specific probes (25).

**Construction and genetic characterization of the *B. abortus* mntH mutant MWV15.** A 7-mm-diameter Whatman filter paper disk was placed in the center of each plate and impregnated with 10 μl of one of the following solutions: 30% *H2O2*, 0.5 M paraquat, or 10 mM menadione. After 72 h of incubation at 37°C with 5% CO2, the diameter of the zone of inhibition surrounding each disk on the plates was measured to the nearest millimeter. The diameters of the zones of inhibition from five separate plates were measured for each bacterial strain examined.

**Determination of superoxide dismutase (SOD) activity in *Burkella* strains.** *Burkella* strains were grown to mid-log phase in brucella broth, and the cultures were adjusted to an optical density at 600 nm of 0.15 (approximately 10^9 CFU/ml). One hundred microliters of each cell suspension was mixed with 3 ml of tryptic soy broth containing 0.7% agar and spread evenly over the surface of either a tryptic soy agar plate (for the *H2O2* sensitivity assays) or a tryptic soy agar plate supplemented with 1,000 U/ml of bovine liver catalase (Sigma) (for the assays measuring sensitivity to the superoxide generators paraquat and menadione). A 7-mm-diameter Whatman filter paper disk was placed in the center of each plate and impregnated with 10 μl of one of the following solutions: 30% *H2O2*, 0.5 M paraquat, or 10 mM menadione. After 72 h of incubation at 37°C with 5% CO2, the diameter of the zone of inhibition surrounding each disk on the plates was measured to the nearest millimeter. The diameters of the zones of inhibition from five separate plates were measured for each bacterial strain examined.

**BRUCELLA MntH IS REQUIRED FOR VIRULENCE**
number of intracellular brucellae was determined by serial dilution of the lysates in PBS and plating on SBA, followed by incubation of the SBA plates at 37°C with 5% CO₂.

Experimental infection of C57BL/6 mice. Six-week-old female C57BL/6 mice were infected via the peritoneal route with 5 × 10⁷ CFU of B. abortus 2308, MWV15 (2308 ∆mntH), or MWV15.C [MWV15(pEA31)], and the spleen colonization profiles of these strains in the mice were determined using previously described methods (30).

Statistical analysis. All statistical analyses were performed using the two-tailed Student t test (64). P values of ≤ 0.05 were considered significant.

RESULTS

BAB1_1460 is predicted to encode an MntH homolog, and the corresponding gene exhibits Mn-responsive repression in B. abortus 2308. The gene designated BAB1_1460 in the B. abortus 2308 genome sequence is predicted to encode a 456-amino-acid protein that shares 35.9% identity with the Escherichia coli manganese transporter MntH (52). Analysis of the amino acid sequence of the putative Brucella MntH homolog with the TMpred algorithm (http://www.ch.embnet.org/software/TMPRED_form.html) predicts that this protein, like its E. coli counterpart, is an integral membrane protein with multiple membrane-spanning regions. All eight of the amino acid residues (Asp34, Asn37, Glu102, Asp109, Glu112, His211, Asp238, and Asn401) that have been shown by site-directed mutagenesis to be critical for Mn²⁺ transport by E. coli MntH (15, 35) are conserved in Brucella MntH. Based on the annotation of the B. abortus 2308 genome sequence (14), the Brucella mntH homolog is located downstream of a gene (BAB1_1459) encoding a hypothetical protein. Whether or not BAB1_1459 is expressed, or mntH is cotranscribed in an operon with this gene, has not been experimentally determined.

A bioinformatics-based study described by Rodionov et al. (62) predicts the presence of a “Mur” box upstream of the Brucella melitensis mntH homolog BMEI0569. This conserved sequence (AATGCAAATAGTTTGCAAC) is also centered 35 nucleotides upstream of the putative mntH coding region (BAB1_1460) in the B. abortus 2308 genome sequence. Mur is a structural homolog of the ferric uptake regulator Fur, which controls the expression of manganese transport genes in Rhizobium leguminosarum (21) and Sinorhizobium meliloti (16), two alphaproteobacteria that are close phylogenetic relatives of Brucella spp., in a manganese-responsive manner. As shown in Fig. 1A, the level of β-galactosidase production from an mntH-lacZ transcriptional fusion in B. abortus 2308 during growth in a nutritionally complete growth medium (brucella broth) suggests that mntH exhibits a considerable degree of basal expression in this bacterium even when sufficient levels of Mn²⁺ are present. The addition of increasing amounts of MnCl₂ to the culture medium ranging from 50 μM to 1 mM represses β-galactosidase production in B. abortus 2308 (Fig. 1A) but not in the isogenic mur mutant Fur2 (Fig. 1B). In contrast, the addition of up to 500 μM FeCl₃ fails to repress the expression of the mntH-lacZ fusion in either B. abortus 2308 or the mur mutant (Fig. 1B). This pattern of manganese-responsive expression is consistent with the proposed function of MntH and further indicates that Mur plays an active role in regulating the expression of the corresponding gene.

The B. abortus mntH mutant MWV15 exhibits a manganese-selective defect in metal acquisition in vitro. The B. abortus mntH mutant MWV15 exhibits slower growth in brucella broth, a complex growth medium, than does the parental 2308 strain or a derivative of MWV15 carrying a plasmid-borne copy of mntH (Fig. 2A). Consistent with the predicted function of Brucella MntH as a manganese transporter, supplementation of brucella broth with 50 μM MnCl₂ allows MWV15 to grow with the same vigor in brucella broth as does strain 2308. Supplementation of this medium with 50 μM FeCl₃, in contrast, will not rescue the growth defect exhibited by MWV15 in brucella broth (Fig. 2A).

The B. abortus mntH mutant MWV15 also displays a significantly reduced ability to use MnCl₂, as a manganese source compared to the parental 2308 strain on a solid growth medium containing the chelator EDDA (Table 1). Good growth is observed for the parental 2308 strain surrounding disks containing 7.95 mM MnCl₂, but a comparable level of growth is observed for the mntH mutant only around disks containing 7.95 mM MnCl₂ in this assay. EDDA has an approximately 100-fold-greater affinity for Fe²⁺ (equilibrium constant = 6.45 × 10⁹) than it does for Mn²⁺ (5.13 × 10⁷) (13). Because
of its high affinity for iron, EDDA is often the chelator of choice for in vitro experiments designed to detect defects in iron acquisition in bacterial mutants (18). Thus, it is notable that although the B. abortus mntH mutant exhibits a reduced zone of growth around disks containing FeCl₃ or Fe(NH₄)₂(SO₄)₂ on plates containing the same concentration of EDDA compared to strain 2308, this reduction in zone size cannot be rescued by adding increasing amounts of either iron source to the disks (data not shown).

Also consistent with its predicted function, the B. abortus mntH mutant exhibits delayed growth compared to 2308 when these strains are cultivated in a low-manganese minimal medium in broth culture (Fig. 2B). The addition of 50 μM MnCl₂ to the disks (data not shown).

Figure 3 illustrates the growth of B. abortus mntH mutant in low-manganese medium supplemented with various divalent cations, including Mn²⁺, Fe²⁺, Zn²⁺, Cu²⁺, Co²⁺, and Ni²⁺. Bacterial cell cultures were inoculated into low-manganese medium at cell densities of approximately 10⁵ CFU/ml, and the number of bacteria present in these cultures following 48 h of incubation was enumerated by serial dilution and plating. Shown are the B. abortus parental strain (2308), the mntH mutant (MWV15), and the mntH mutant grown in low-manganese minimal medium supplemented with MnCl₂ at concentrations of 1.5, 5, and 10 μM (designated Mn1, Mn5, and Mn10, respectively). MWV15 cultures supplemented with 100 μM Fe(NH₄)₂(SO₄)₂ (Fe 100), 100 μM ZnCl₂ (Zn 100), 5 μM CoCl₂ · 6H₂O (Co 5), and 20 μM NiCl₂ · 6H₂O (Ni 20) are shown as well.

Growth of the B. abortus mntH mutant MWV15 carrying a plasmid-borne copy of mntH (MWV15.C) in low-manganese minimal medium is also shown. The data presented are the means and standard deviations for triplicate determinations from single flasks for each strain and experimental condition in a single experiment. The data are representative of multiple (≥3) experiments from which equivalent results and statistical trends were obtained. *P < 0.05; **P < 0.005 (for comparisons of strain 2308 with MWV15 or MWV15.C).

**TABLE 1.** Capacity of B. abortus 2308, MWV15, and MWV15.C to use MnCl₂ as a manganese source.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth (mm) around disk containing a</th>
<th>79.5 μM MnCl₂</th>
<th>795 μM MnCl₂</th>
<th>7.95 mM MnCl₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>2308</td>
<td>NG</td>
<td>43.1 ± 0.6</td>
<td>52.3 ± 1</td>
<td>67.3 ± 0.6</td>
</tr>
<tr>
<td>MWV15</td>
<td>NG</td>
<td>43.4 ± 1.8</td>
<td>52.7 ± 0.3</td>
<td>69 ± 3.2</td>
</tr>
<tr>
<td>MWV15.C</td>
<td></td>
<td>43.4 ± 1.8</td>
<td>52.7 ± 0.3</td>
<td>69 ± 3.2</td>
</tr>
</tbody>
</table>

a The diameters of the zones of bacterial growth on a solid medium growth supplemented with 500 μM EDDA around filter disks impregnated with 10 μl of a 10-μg/ml, 100-μg/ml, or 1-mg/ml solution of MnCl₂ were measured after 72 h of incubation at 37°C with 5% CO₂. The values presented are the means ± standard deviations of the zone sizes obtained from three separate experiments, and three separate determinations of growth were obtained for each strain in each experiment. ***, P ≤ 0.005 (for comparisons of MWV15 with 2308 and MWV15.C).

b NG, no growth. No zone of growth was observed surrounding the disks impregnated with 10 or 100 μg/ml MnCl₂ for B. abortus 2308.
Genetic complementation of *B. abortus* MWV15 with a plasmid-borne copy of the parental *mntH* gene restored the ability of this strain to use MnCl$_2$ as a manganese source on the EDDA-containing plates with the same efficiency as the parental 2308 strain (Table 1) and restored its ability to replicate with the same growth kinetics as 2308 in the low-manganese minimal medium (Fig. 2A).

The *B. abortus* mntH mutant MWV15 displays reduced Mn SOD activity compared to the parental 2308 strain. The presence of MntH is required for wild-type resistance of *B. abortus* 2308 to oxidative killing in in vitro assays. The parental 2308 strain is considerably more resistant to killing by both H$_2$O$_2$ (Fig. 4A) and O$_2^-$ generated by the redox cyclers paraquat (Fig. 4B) and menadione (Fig. 4C) in disk sensitivity assays than the *mntH* mutant, and the introduction of a plasmid-borne copy of *mntH* into the mutant restores wild-type levels of resistance to H$_2$O$_2$, paraquat, and menadione in these assays (Fig. 4A to C). In most bacteria that have been studied, there is a strong link between the ability to acquire Mn$^{2+}$ and resistance to oxidative stress (39). *B. abortus* 2308 produces a manganese-containing SOD (SodA) (68), and phenotypic analysis of a *B. abortus* sodA mutant indicates that SodA is an important antioxidant in this bacterium (29). Consequently, it is possible that the *B. abortus* mntH mutant is unable to transport sufficient Mn$^{2+}$ to produce wild-type levels of Mn SOD activity. The results shown in Fig. 5 support this proposition. Equivalent levels of Cu/Zn SOD activity are observed in native gels for cell lysates from *B. abortus* 2308 and MWV15, but the *mntH* mutant displays a greatly reduced level of Mn SOD activity compared to the parental 2308 strain. MWV15 carrying a plasmid-borne copy of the *mntH* gene, on the other hand, displays the same level of Mn SOD activity as 2308. The basis for the increased sensitivity of the *B. abortus* mntH mutant to H$_2$O$_2$ is unknown, but hypersensitivity to H$_2$O$_2$ has been previously reported for other bacterial strains that are deficient in SOD activity (11).

**FIG. 4.** Resistance of *B. abortus* 2308, MWV15 (2308 mntH), and MWV15.C (MWV15(pEA31)) to H$_2$O$_2$ (A), paraquat (B), and menadione (C) in disk sensitivity assays. The data presented are zones of inhibition around disks containing H$_2$O$_2$ (A), paraquat (B), or menadione (C). The values are the means ± standard deviations of the zone sizes obtained from three separate experiments, and three separate determinations of growth were obtained for each strain in each experiment. ***, $P \leq 0.005$; ****, $P \leq 0.001$ (for comparisons of MWV15 with strain 2308 or MWV15.C).

**FIG. 5.** SOD activity in *B. abortus* 2308, MWV15, and MWV15C. Cu/Zn SOD (SodC) (cyanide-sensitive) and Mn SOD (SodA) (cyanide- and H$_2$O$_2$-resistant) activities, detected in the parental 2308 strain (lane A), the *mntH* mutant MWV15 (lane B), and a derivative of MWV15 carrying a plasmid-borne copy of *mntH* (lane C). The *B. abortus* sodC mutant MEK2 (26) was included as a control (lane D). The gel presented is representative of multiple experiments (≥3) in which equivalent results were observed.

**DISCUSSION**

The experimental findings presented in this paper support the proposition that MntH serves as the sole high-affinity manganese transporter in *B. abortus* 2308. A similar role for MntH in *Bradyrhizobium japonicum*, a close phylogenetic relative of brucellae, has also recently been described (37). Many bacteria contain both proton-dependent (MntH-type) and ATP-dependent (SitABCD-type) Mn$^{2+}$ transporters, and mutation of both the *mntH* and *sitABCD* genes is often required before prominent defects in Mn$^{2+}$ utilization are observed (8, 65).
This is clearly not the case with the \textit{B. abortus} \textit{mntH} mutant, where loss of MntH produces prominent growth defects in both rich and minimal growth media that can be relieved only by supplementation of these media with elevated levels of Mn\textsuperscript{2+}. It is important to note, however, that the \textit{B. abortus} \textit{mntH} mutant eventually attains the same cell density as the parental strain in a manganese-deprived minimal medium (Fig. 2), and the addition of high levels of MnCl\textsubscript{2} (e.g., 7.95 mM) to disks will allow this strain to grow on plates containing EDDA (Table 1). These experimental findings demonstrate that \textit{B. abortus} 2308 has an alternate means of acquiring Mn\textsuperscript{2+} when MntH is not present, but this alternate mechanism is apparently much less efficient at Mn\textsuperscript{2+} transport than MntH.

Like its counterparts in other bacteria (41), \textit{Brucella} MntH appears to be a manganese-selective transporter. Although supplementation of the culture medium with Mn\textsuperscript{2+} relieves the growth defects exhibited by the \textit{B. abortus} \textit{mntH} mutant when this strain is grown in a rich medium or low-manganese minimal medium, supplementation of these media with other divalent cations reported to be transported by Nramp/MntH homolog medium, supplementation of these media with other divalent cations (43). The precise nature of the regulatory link between \textit{mntH}, Mur, and cellular Mn levels in \textit{B. abortus} 2308 is presently being examined. A computational analysis of the \textit{Brucella melitensis} 16M genome sequence suggests that the \textit{Brucella} Mur regulon may be limited to only a few genes other than \textit{mntH} (62).

In most bacteria that have been studied, there is a strong link between the ability to acquire Mn\textsuperscript{2+} and resistance to oxidative stress (39), and this same relationship is observed for \textit{B. abortus} 2308 and the isogenic \textit{mntH} mutant MWV15. Activity gels indicate that MWV15 produces considerably lower levels of Mn SOD activity than the parental strain, and this deficit in SodA activity is consistent with the increased sensitivity of the \textit{B. abortus} \textit{mntH} mutant to the redox cyclers menadione and paraquat, which generate O\textsubscript{2}\textsuperscript{−} in the cytoplasmic compartment of bacterial cells. This link between inefficient Mn\textsuperscript{2+} transport and suboptimal levels of SOD activity is also similar to the one described for \textit{Sinorhizobium meliloti sitA} mutants (19). It is quite possible, however, that the increased sensitivity of the mutant \textit{B. abortus} \textit{mntH} mutant to oxidative stress results from defects in addition to its inability to produce wild-type levels of SodA. Anjem et al. (2), for example, recently presented evidence suggesting that MntH-mediated Mn\textsuperscript{2+} transport allows \textit{E. coli} to metatalte key metabolic enzymes with this divalent cation instead of Fe\textsuperscript{2+}, possibly protecting these enzymes from oxidative damage via Fenton chemistry. Those authors also showed that MntH-dependent Mn\textsuperscript{2+} transport is an important component of this bacterium’s OxyR-mediated response to H\textsubscript{2}O\textsubscript{2} exposure. Intracellular levels of Mn\textsuperscript{2+} can also influence the activity of transcriptional regulators such as PerR that control the expression of genes important for resistance to oxidative stress (38, 49, 70). Preliminary studies suggest that the gene annotated as BAB1_0393 in the \textit{B. abortus} 2308 genome sequence may be a PerR homolog (E. S. Anderson,
unpublished data), but the nature of the genes that are controlled by this putative regulator and whether or not the activity of the regulator is influenced by cellular Mn$^{2+}$ levels remain to be determined experimentally. Mn$^{2+}$ has also been shown to be able to directly detoxify H$_2$O$_2$ and O$_2^-$ in in vitro assays (3, 69). It has been postulated, however, that only a few bacteria such as lactobacilli have the capacity to accumulate sufficient levels of Mn$^{2+}$ (e.g., mM) to make direct intracellular detoxification of ROS by this metal biologically significant (39). Previous studies have shown that wild-type Brucella strains require low levels of manganese for growth (28, 66), and those findings are supported by the growth properties exhibited by B. abortus 2308 in the study reported here (Fig. 1). Thus, it seems unlikely that direct detoxification of ROS by intracellular Mn$^{2+}$ plays a major role in oxidative defense in Brucella strains.

Nramp1-mediated efflux of Mn$^{2+}$ and other divalent cations from the phagosomal compartments of macrophages has been proposed to be an important component of host defense against intracellular pathogens such as Mycobacterium tuberculosis and Salmonella enterica serovar Typhimurium (12, 40). Studies have shown, however, that Nramp1 does not play a prominent role in protecting experimentally infected mice from Brucella infections (33). Studies of ruminants have also shown that the contributions of this divalent cation transporter to host defense against Brucella infections vary depending on the species of ruminant being examined (10, 54). Thus, the severe attenuation exhibited by the B. abortus mntH mutant in C57BL/6 mice (which lack a functional Nramp1) (67) and cultivated macrophages obtained from these mice is particularly striking. This is especially true considering the fact that bacterial mntH mutants often exhibit subtle, if any, attenuation in experimental hosts (8, 22), and significant attenuation of S. Typhimurium mntH sitABCD double mutants (which lack both of their high-affinity manganese transporters) is seen only in mice that have a functional Nramp1 locus (72). These findings suggest that Mn$^{2+}$, like Mg$^{2+}$ and Zn$^{2+}$ (44, 48, 71), represents an important micronutrient for brucellae during residence in the mammalian host. They also suggest that the levels of Mn$^{2+}$ present in the tissues of experimentally infected C57BL/6 mice are insufficient to meet the physiologic requirements of the B. abortus mntH mutant for this divalent cation even in the absence of a functional Nramp1 in this mammalian host. Considering the dramatic effect that the loss of MntH has on virulence in B. abortus 2308, it is intriguing that brucellae rely on a single high-affinity divalent cation transporter to meet their physiologic needs for Mn$^{2+}$ during intracellular replication. Indeed, having only one high-affinity Mn$^{2+}$ transporter appears to be an “Achilles’ heel” for these bacteria during residence in the mammalian host. Most other bacterial pathogens that have been studied possess both MntH- and Sit-ABCD-type Mn$^{2+}$ transporters (55). The bacterial SitABCD-type transporters that have been studied in detail, however, have been reported to be unable to transport Mn$^{2+}$ at an acidic pH (42, 55), and thus, this type of transporter might be of limited utility to brucellae during the early stages of their intracellular residence in host macrophages when these bacteria occupy acidified compartments (7, 46). In contrast, if Brucella MntH is similar to its Salmonella counterpart (41) and exhibits optimum Mn$^{2+}$ transport at an acidic pH, it would appear to be well suited to the intracellular lifestyle of brucellae in their mammalian hosts.

There are multiple reasons that an insufficient level of intracellular Mn$^{2+}$ could lead to attenuation in the B. abortus mntH mutant. Based on its sensitivity to oxidative killing, the B. abortus mntH mutant may be compromised in its ability to withstand the oxidative stresses it encounters during its interactions with host phagocytes. Brucella spp. also produce a single bifunctional (pppGpp synthetase/hydrolase that has been given the designation Rsh (RelA/SpoT hybrid) (24), and the enzymatic activity of this class of bacterial proteins is Mn$^{2+}$-dependent (55). Because production of (pppGpp is required for a stringent response in bacteria (51), reduced Rsh activity due to inadequate Mn$^{2+}$ levels in the B. abortus mntH mutant may interfere with this strain’s ability to cope with the nutrient deprivation encountered during long-term residence in the phagosome of host macrophages (46, 63). The presence of a functional Rsh protein has also been shown to be required for the proper expression of the virR genes, which encode the type IV secretion machinery in B. melitensis 16M and Brucella suis 1330 (24). Consequently, inefficient expression of the virB genes due to reduced Rsh function could also be contributing to the attenuation exhibited by the B. abortus mntH mutant in macrophages and mice. Preliminary studies suggest that virB4 and virB5 expression levels are indeed reduced in the B. abortus mntH mutant MWV15 compared to those in the parental 2308 strain (J. Gaines and E. Anderson, unpublished data), but the nature of the link between MntH and virB expression remains to be experimentally determined.

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