

The Fur-like regulator Mur controls the expression of the gene encoding the manganese transporter MntH in *Brucella abortus* 2308 in response to manganese availability

by

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Brucella abortus is a Gram-negative, α -proteobacterium that causes bovine brucellosis, a prevalent zoonotic disease in many areas of the world. *B. abortus* is an intracellular pathogen that survives and replicates in the phagosomal compartment of host macrophages, a property that is critical for virulence. In this environment, *B. abortus* requires manganese, but the availability of this essential micronutrient is restricted in the phagosomal compartment. Host cells resist bacterial infection by limiting the availability of metals in the phagosome employing what is known as the metal withholding defense, a process mediated by Nramp1 (Natural resistance associated macrophage protein). To counter this, pathogenic bacteria produce high-affinity manganese transporters, and these transporters have been shown to be necessary for virulence.

MntH is the bacterial homolog of the eukaryotic Nramp and is the only identified high affinity manganese transporter in *Brucella* strains. In this study, the function and the metal-specificity of this protein was examined. Studies employing a genetically defined *B. abortus* *mntH* mutant show that MntH-mediated manganese acquisition is critical for normal *in vitro* growth and the establishment of infection in mice. The α -proteobacteria employ a structural

homolog of the ferric uptake regulator Fur which is Mn-responsive (and thus designated Mur for manganese uptake regulator) to control the expression of their manganese acquisition genes. Phenotypic analysis of a *mur* mutant showed that Mur is responsible for the Mn-responsive regulation of *mntH* expression in *B. abortus* 2308 and biochemical studies defined the Mur binding site in the *mntH* promoter.

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CHAPTER I: Introduction

The *Brucella* spp. are Gram-negative bacteria that belong to the α -proteobacteria and are phylogenetic relatives of endosymbionts, plant pathogens and other animal pathogens (Moreno et al., 1990; Ugalde 1999). The *Brucella* spp. infect both wild and domestic animals and are the causative agent of brucellosis, a disease that causes sterility in males and abortion in pregnant females (Roop et al., 2004). The disease can also be transmitted to humans via lab exposure or animal handling; however, naturally occurring disease in humans is always associated with an animal reservoir. In the human host, brucellosis causes a severe febrile illness that can be treated by extensive antibiotic therapy, but no safe, effective vaccine is available for humans (Roop et al., 2004). Because in its naturally occurring form it is a strictly zoonotic infection, the most efficacious method of preventing human brucellosis is by controlling the disease in food animals (Mantur, 2007).

Historical perspective

Brucellosis is a world-wide zoonosis that has been of public health concern since ancient times (D'Anastasio et al., 2009). The history of brucellosis is extensive; more modern translations of the book *Epidemics* by Hippocrates (*circa* 450 B. C.) described symptoms experienced by infected individuals that are believed to have had brucellosis. During the Crimean War (1854-1856) there were six recognized forms of fever: intermittent fever, remittent fever, simple continued fever, relapsing fever, typhus fever, and typhoid fever experienced by British soldiers stationed in Malta (Dossey, 1998). J. A. Marston, a British officer, documented the symptoms of soldiers who had fallen ill with varying symptoms on the island and published these records in a report entitled "Report on Fever (Malta)" in 1861 (Marston, 1861). In 1887, David Bruce, a medic in the British army, isolated the bacterium that he identified as

“*Micrococcus melitensis*” from the spleens of deceased soldiers that had fallen ill with remittent fever (Bruce, 1887). In 1897, Surgeon Captain M. Louis Hughes published his monograph entitled *Mediterranean, Malta or Undulant Fever*, and displayed a photograph of the organism isolated by Bruce and listed detailed symptoms of the disease. At the time of Bruce’s discovery, the Danish scientist Bernard Bang (1897) had isolated an organism that caused contagious abortion in cattle (Nicoletti, 2002) and in 1918 Alice Evans, an American bacteriologist, compared the bacteria isolated by Bruce and Bang and discovered they were the same (Evans, 1918). The bacterium isolated by Bruce that he termed, “*Micrococcus melitensis*” was later renamed *Brucella melitensis* in his honor.

Presently, animal and human brucellosis are endemic in the Middle East, Asia and the Mediterranean (Pappas et al., 2006). Annually, the disease affects approximately 500,000 people worldwide (Pappas et al., 2006) and the spread of disease in these regions of the world is dependent on custom practices and the consumption of unpasteurized dairy products from infected animals (Mantur, 2007). *Brucella* spp. are significant agents of agricultural disease and are considered Class B Select Agents by the Centers for Disease Control and Prevention (Valderas and Roop, 2006). *Brucella* can easily be disseminated by aerosol and a small inoculum of 10-100 bacteria is sufficient to cause disease. For reasons such as these, *Brucella* has been considered a potential biological warfare agent (Purcell et al., 1997). Increased awareness and education contribute greatly to the control of disease in animals and ultimately in humans; controlling disease in animals is the main reason for low incidence in humans. Human acquired *Brucella* infections are primarily occupational and those most at risk are animal handlers and laboratory workers (Mantur, 2007).

Taxonomy

In nature there are ten *Brucella* species recognized that are differentiated by their host specificity - *B. melitensis* (sheep and goats), *B. abortus* (cattle), *B. suis* (pigs), *B. ovis* (sheep), *B. canis* (dogs), *B. neotomae* (desert wood rats), *B. pinnipedialis* and *B. cetaceae* (seals, porpoises, and dolphins), *B. microti* (voles) and *B. inopinata* (humans) (Table 1) . All *Brucella* spp. share a high degree of conservation in genomic content (Ugalde, 1999), and nucleotide identity as shown by DNA-DNA hybridization (Verger et al., 1985) suggests *Brucella* be considered a monospecific genus. Of the ten currently recognized *Brucella* species, *B. melitensis*, *B. abortus* and *B. suis* are major concerns for causing human disease (Mantur, 2007).

Establishing infection

A number of pathogenic bacteria, including *Mycobacterium*, *Salmonella*, *Shigella*, and *Listeria*, reside within macrophages where they are capable of avoiding host immune factors (Scott et al., 2003). Macrophages are important cells involved in innate immunity and they engulf foreign particles and pathogenic organisms by a process known as phagocytosis (Gagnon et al., 2002). Bacteria are internalized within macrophages during this process and reside in a membrane bound phagosome that undergoes fusion events with different organelles in the endocytic pathway (Figure 1). During these fusion events, the phagosomal compartment becomes acidic, highly oxidizing and acquires hydrolytic enzymes that promote bacterial killing (Scott et al., 2003). Some bacteria, however, can manipulate trafficking through the endocytic pathway and divert fusion events that occur during phagocytosis (Scott et al., 2003). For instance, the pathogen *Leishmania* uses its lipophosphoglycan (LPG) to inhibit phagolysosomal fusion events whereas *Legionella* resides in specialized phagosomes that inhibit acidification (Duclos and Desjardins, 2000). Bacteria may also escape from phagosomes or vacuoles as

observed for *Shigella*, *Rickettsia* and *Listeria* (Scott et al., 2003) where bacteria produce proteins that interfere with phagosome formation and membrane stability; or bacteria may halt phagosomal maturation altogether as observed for *Salmonella*, *Neisseria* and *Mycobacterium* (Scott et al., 2003)

Brucella intracellular survival

Brucella spp. are engulfed by macrophages and reside within a *Brucella*-containing vacuole (BCV) that has been shown to briefly interact with lysosomes (Starr et al., 2009) before the BCV ultimately fuses with the endoplasmic reticulum. This course of trafficking of the BCV allows for the establishment of a niche suitable for replication (Gorvel and Moreno, 2002; Celli and Gorvel, 2004) (Figure 2). During the short fusion event between the BCV and lysosomes, the BCV acquires vacuolar ATPases that acidify the phagosomal lumen which is a stimulus for brucellae to express critical genes necessary for survival, such as those encoding the Type IV secretion system (Boschiroli et al., 2001) which will be discussed in more detail below.

Brucella spp. do not express classical virulence factors such as exotoxins, flagella, or capsules (Moreno and Moriyon, 2002; Seleem et al., 2008), and the brucellae stealthily avoid immune detection and minimize the host inflammatory response by inhibiting the biological activity of TNF- α and other major cytokines (Baldwin and Parent, 2002; Dornand et al., 2002). This stealthy nature is attributed to the low endotoxicity of the lipopolysaccharide, which is 1000 \times less active than the LPS of *E. coli* in eliciting the host inflammatory response (Barquero-Calvo et al., 2007). Brucellae also depend on the Type IV secretion machinery, which is encoded by the *virB* operon. The proteins expressed by the 12 genes in this operon assemble together and form an apparatus that secretes effector molecules that dictate the trafficking of the BCV to the endoplasmic reticulum (Boschiroli et al., 2002; Celli and Gorvel, 2004). Studies employing

targeted mutagenesis of genes in the *virB* operon demonstrate the necessity of this secretion system for virulence as these mutants are targeted for lysosomal degradation (den Hartigh et al., 2008); however, the effector proteins translocated by this system have not been well characterized (de Jong et al., 2009).

The ensuing disease

Brucella infects a variety of tissues and organs in the human host and these infections can give rise to a variety of clinical manifestations (Purcell et al., 1997). Macrophages inhabited by brucellae can travel to lymph nodes and be disseminated throughout the body. The ensuing illness can lay dormant for extended periods of time without the patient displaying any clinical symptoms, or immediately cause the patient to experience flu-like symptoms, myalgia, and weight-loss. Clinical symptoms of infected patients are wide ranging and the function of various organ systems can be affected (Purcell et al., 1997). More serious complications may also arise including orchitis, osteoarthritis, spondylitis, and endocarditis (Rajashekara et al., 2006; Mantur, 2007; Enright, 1990; Dornand et al., 2002; Corbel, 1997). Currently, no vaccine exists for human brucellosis that is safe or effective (Pappas et al., 2006). The World Health Organization (WHO) has recommended that a combination of antibiotics be taken, including doxycycline, rifampicin, streptomycin and gentamicin, as an effective treatment for brucellosis, yet, nearly 10% of all recovering patients relapse (Pappas et al., 2006; Ariza et al., 2007).

Metals in the system

Metal ions are an absolute requirement for the biological activity of one-third of all proteins (Pennella and Geidroc, 2005). All living cells require trace amounts of metals, and in order to obtain these, prokaryotes and eukaryotes express genes responsible for metal uptake. The physiology of many organisms requires magnesium, manganese, copper, zinc and iron

(Agranoff and Krishna, 1998; Andreini et al., 2008) and these micronutrients have also been shown to be essential for the *Brucella* spp. (Gerhardt et al., 1950; Evenson and Gerhardt, 1955). Pathogens that reside within mammalian hosts face conditions where many micronutrients, specifically metals, are not found free but complexed with host proteins and macromolecules (Thomas, 1970). Consequently, free metal ions are not available to pathogenic bacteria and to overcome this challenge bacteria utilize high affinity metal acquisition proteins to sequester metals from host proteins. However, regulatory mechanisms must be in place to control the expression of metal uptake genes in bacteria because the accumulation of metal ions beyond their physiologic concentrations is toxic (discussed below).

Iron

Iron dependent proteins are among the most common metalloproteins in cells and constitute $\sim 3.9\% \pm 1.6\%$ of bacterial proteins. It is hypothesized that more than 50% of these iron-containing proteins are utilized in electron transfer and for oxidoreductase activity (Andreini et al., 2009). Iron is an absolute requirement for virtually all cells and both prokaryotes and eukaryotes require this important micronutrient for basic cell function (Doherty, 2007). Iron homeostasis is intricately linked to bacterial pathogenesis because the mammalian host is an iron restricted environment where available iron is limited to 10^{-24} M (Doherty, 2007). Because of this, bacteria encode proteins responsible for iron uptake and utilization. However, iron has a high redox potential which means that in the ferrous form (Fe^{2+}), this ion is eager to donate an electron. Therefore, living cells must control total iron concentrations in the cell to prevent toxicity and Fenton reactions [$\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}\cdot$] which damage DNA and iron-sulfur clusters in proteins (Doherty, 2007; Griffiths, 1999; Kehres and Maguire, 2003).

Manganese

In the context of transition metals, iron has been highly studied and is considered the most important transition element in biological systems. However, in the past decade, much attention has been focused on manganese and the physiologic role it plays in the cell. Such focus on manganese emerged after it was observed that some pathogenic bacteria do not require iron but require manganese instead as observed for the Lyme disease bacterium *Borrelia burgdorferi* (Posey and Gherardini, 2000) and *Streptococcus suis* (Niven et al., 1999).

All living cells utilize manganese as an important cofactor for proteins and enzymes involved in basic metabolic and cellular functions (Kehres and Maguire, 2003). Some of these proteins and enzymes include arginase, glutamine synthetase, phosphoglyceromutase, enolases, pyruvate kinase, PEP carboxylase, PEP carboxykinase, and phosphatases (Kehres and Maguire, 2003; Papp-Wallace and Maguire, 2006; Zaharik and Finlay, 2004). However, one of the most important biological roles manganese has in the cell is to serve as a cofactor for SodA. SodA is the most phylogenetically widespread manganese-containing enzyme in bacteria (Kehres and Maguire, 2003) and is a primary anti-oxidant enzyme that facilitates the breakdown of toxic oxygen species, specifically superoxide radicals (O_2^-) (Whittaker, 2009).

Early studies by Fridovich and colleagues showed that Mn^{2+} can directly scavenge superoxide radicals *in vitro* (Archibald and Fridovich, 1982); but, the rate of this reaction is too slow in comparison to the enzymatic breakdown of superoxide facilitated by SodA, and is not a plausible mechanism for rapid detoxification in biological systems (Anjem et al., 2009). However, it has been demonstrated in bacteria that exogenous manganese supplementation increases resistance to oxidative damage independent of SodA (Horsburgh et al., 2002; Tseng et al., 2001). The Imlay group has shown that the increased resistance observed in bacteria upon

manganese supplementation is because Mn^{2+} can replace Fe^{2+} in mono-nuclear enzymes and not because Mn^{2+} facilitates the catalytic breakdown of reactive oxygen species (Anjem et al, 2009; Martin and Imlay, 2011). The ability of Mn^{2+} to substitute for Fe^{2+} abolishes the potential for Fenton reactions from occurring because Mn^{2+} does not possess the same redox potential as Fe^{2+} (Kehres and Maguire, 2003). In examining the electron chemistry of both these metals, Mn^{2+} has a lesser reducing potential than Fe^{2+} and is stable because of its electron configuration ($3d^5$) compared to that of Fe^{2+} ($3d^6$) (Kehres and Maguire, 2003). The Fe^{2+} ion, therefore, is more likely to donate an electron and participate in Fenton reactions to stabilize its *d* orbitals and consequently switch oxidation states to the more stable Fe^{3+} ($3d^5$).

Manganese in *Brucella*

The preferred niche of the *Brucella* spp. in human hosts is within macrophages of the reticuloendothelial system (Purcell et al., 1997), which are cells that deliberately restrict available metals ions from bacteria employing the metal withdrawal defense (discussed below). The total metal content of phagosomes inhabited by *Mycobacterium tuberculosis* (Wagner et al., 1995) and *Salmonella typhimurium* (Rishi et al., 2010) has been analyzed and shown to be manganese restricted, however, the bacterial cell is still capable of acquiring manganese from this restricted environment. In these bacteria, it has been shown that the expression of manganese acquisition systems is greatly increased in this environment (Wagner et al., 1995; Rishi et al., 2010).

Past studies on the nutritional requirements of *Brucella* strains show that manganese is required for proper growth (Gerhardt et al., 1950). *Brucella* not only requires manganese for growth under laboratory conditions, but manganese acquisition is also necessary for virulence (Anderson et al., 2009). Although the exact nature of *Brucella*'s manganese dependence has not

been elucidated, a few Mn^{2+} -dependent enzymes have been shown to be important for wild-type growth of *Brucella* *in vivo* and *in vitro*. As previously mentioned, SodA is an anti-oxidant enzyme required for the detoxification of superoxide radicals. Studies in *Brucella* show that this enzyme is required to detoxify oxygen species generated by aerobic metabolism (Baumgartner et al., 2010). More importantly, SodA activity is necessary for the establishment of infection in mice (Baumgartner et al., 2010). The activity of SodA in *Brucella* is dependent on manganese and this can be observed by measuring the activity of native SodA in wild-type *B. abortus* 2308 and the isogenic *mntH* mutant, MWV15, which is defective in manganese transport (see Chapter II) (Anderson et al., 2009). Mn^{2+} is also an important cofactor for Rsh which is a (p)ppGpp synthetase/hydrolase (Papp-Wallace and Maguire, 2006). This enzyme is necessary for induction of the stringent response and expression of the *virB* operon (Dozot et al., 2006) which is necessary for proper BCV trafficking in *Brucella* strains (Dozot et al., 2006). The stringent response enables bacteria to rapidly modulate gene expression mechanistically by the alarmone (p)ppGpp binding to RNAP and changing its preference for target promoters (Toulokxonov et al., 2001). By doing this, RNAP actively transcribes genes necessary for survival during nutrient deprived states. Also, the stringent response limits ribosome production preventing protein synthesis (Potrykus and Cashel, 2008). More recently, Imlay's group discovered that the proteins encoded by the *nrdEF* operon in *E. coli* are manganese-dependent ribonucleotide reductases (Martin and Imlay, 2011). Ribonucleotide reductases are absolutely necessary in living cells because they catalyze the formation of dNTP's (Elledge et al., 1992). This operon is conserved in *Brucella* spp., however, the metal requirements of the proteins encoded by the *nrdEF* operon in *Brucella* have not been determined.

Transcription of the manganese acquisition gene, *mntH*, in the enterics is highly induced when bacteria are exposed to oxidative stress (Kehres et al., 2000) and this induction of *mntH* transcription is observed in *B. abortus* 2308 when the bacterium is exposed to H₂O₂ (Evan Menscher, unpublished data). This observation suggests that *Brucella* facilitate Mn²⁺ uptake under environmental stress conditions similar to what is observed for the enterics to substitute for Fe²⁺ in proteins to confer greater protection from oxidative damage.

Nutritional immunity

The intimate link between the acquisition of metals and virulence has been demonstrated in a broad range of Gram-positive and Gram-negative bacteria (Agranoff and Krishna, 1998). Host cells restrict bacteria from acquiring metals by expressing specific metal-binding proteins such as transferrin, lactoferrin (Griffiths, 1999), lipocalin (Flo et al., 2004) and calprotectin (Corbin et al., 2008; Skaar et al., 2010). Manganese restriction by the host is also beneficial because it limits manganese concentrations between 3 and 20 μM in human adult tissues (Roth and Garrick, 2003). At values greater than 20 μM the potential for manganese toxicity occurring increases which is linked to the development of neurogenic and other severe diseases (Roth and Garrick, 2003).

As just mentioned, the acquisition of metals is linked to bacterial pathogenesis and therefore an effective anti-microbial host defense is to limit available metals in the environment where pathogens reside. Because some pathogens have evolved to reside inside macrophages, these pathogens have also evolved mechanisms to withstand the macrophage's harsh intracellular environment which includes low pH, exposure to reactive oxygen species, and restricted nutrient availability (Hassett and Cohen, 1989). After phagocytosis begins, bacteria are contained within phagosomes where the protein Nramp1 (natural resistance associated macrophage protein) is

expressed and inserted in the phagosomal membrane. Nramp1 is expressed exclusively in macrophages where it is responsible for pumping metal ions, specifically Fe^{2+} and Mn^{2+} , from the phagosomal compartment into the cytosol of the macrophage (Forbes and Gros, 2001). This defense tactic is known as the metal withholding defense and is a major component of human resistance to bacterial infection caused by *Mycobacterium* spp., *Salmonella enterica*, and *Leishmania donovani* (Gruenheid et al., 1997; Wyllie et al., 2002; Barton et al., 1999; Cellier et al., 2007). A second Nramp protein is also expressed in humans, Nramp2, which is a functional homolog of Nramp1 but this protein is expressed ubiquitously in cells and demonstrates a broad substrate specificity for metals including Fe^{2+} , Mn^{2+} , Co^{2+} , Cu^{2+} , Ni^{2+} , Pb^{2+} and Zn^{2+} (Haemig and Brooker, 2004). Nramp proteins are found among cells of plants, animals and yeasts with conserved function (Makui et al., 2000; Cellier et al., 2001) and play important roles in defense against invading pathogens in eukaryotic cells.

Prokaryotic MntH

Microbial Nramp-like proteins have been identified and are known as MntH (H^+ -dependent manganese transporter). MntH proteins have been identified and characterized in several bacteria, including *Escherichia coli* (Kehres et al., 2000), *Salmonella enterica* (Kehres et al., 2000), *Bradyrhizobium japonicum* (Hohle and O'Brien, 2009), *Mycobacterium tuberculosis* (Domenech et al., 2002) and more recently *Brucella abortus* (Anderson et al., 2009). MntH proteins are well-conserved between bacterial species and typically demonstrate greater than 30% amino acid sequence identity (Figure 3) (Kehres et al., 2000). MntH spans the cytoplasmic membrane of bacteria and translocates manganese from the external environment into the bacterial cytoplasm. The protein consists of 11-12 transmembrane domains and contains conserved acidic residues (Figure 4) that are critical for manganese transport (Haemig and

Brooker, 2004). MntH is a proton-symporter that functions best at low pH (Papp-Wallace and Maguire, 2006). For Gram-negative bacteria, manganese enters the periplasmic space through large porin proteins in the outer membrane but requires an inner membrane transporter to enter the cytoplasmic place because it is a charged ion (Figure 4).

ABC transporters

Another class of Mn^{2+} transporters are the ABC-type permeases identified in a large number of bacteria including *Neisseria gonorrhoeae* (Lim et al., 2008), *Streptococcus pneumoniae* (Novak et al., 1998), *Escherichia coli* (Sabri et al., 2006), and *Salmonella enterica* (Kehres et al., 2002). Genes encoding for Mn^{2+} specific ABC-type permeases have been shown to be important for virulence in *Yersinia* spp., *Streptococcus pneumoniae* and *Staphylococcus aureus* (Basavanna et al. 2009). These Mn^{2+} permeases are ATP-dependent and unlike MntH, function best at slightly alkaline pH (Davidson et al., 2008). Mechanistically ABC transporters rely on their cognate periplasmic binding protein, SitA, to bind Mn^{2+} and place the ion in position for transport through the protein products of *sitBC* (Figure 4). Genes encoding ABC proteins have been implicated in the import and export of a variety of substrates and constitute up to 5% of the *E. coli* and *B. subtilis* genomes (Davidson et al., 2008). However, not all bacteria possess Mn^{2+} specific ABC transporters. Genomic analysis of the *Brucella* spp. does not identify any homologs of the known Mn^{2+} specific ABC permeases that have been described in bacteria, and suggests *Brucella* relies solely on MntH for its high affinity manganese acquisition (Anderson et al., 2009).

Regulation of Mn transport

The beneficial transition elements Mg^{2+} , Ca^{2+} , Mn^{2+} , Fe^{2+} , Co^{2+} , Ni^{2+} , Cu^{2+} and Zn^{2+} are required for all living cells, however, an over abundance of any of these elements can be toxic to

the cell (Waldron and Robinson, 2009). Metals are competitive in their binding to proteins in cells and this can be explained by the Irving-Williams series (Mg^{2+} and Ca^{2+} (weakest binding) $< Mn^{2+} < Fe^{2+} < Co^{2+} < Ni^{2+} < Cu^{2+} > Zn^{2+}$). The Irving-Williams series lists, in order, the affinity each metal possesses for binding to proteins based on the presenting ligand environments (Kehres and Maguire, 2003; Waldron and Robinson, 2009). Because of this, cells must regulate metal acquisition in order to limit metals from wrongly binding active sites and perturbing biological function.

Because Mn^{2+} does not participate in Fenton chemistry (Kehres and Maguire, 2003) and ranks low in terms of its propensity to incorrectly bind with cellular proteins (Waldron and Robins, 2010), manganese is generally considered to be a metal of low toxicity (Waldron and Robinson, 2010). Nevertheless, many bacteria will not grow in the presence of elevated levels of this metal *in vitro* (Silver et al., 1972). Moreover, manganese-responsive transcriptional regulators that tightly control the expression of genes involved in manganese acquisition have been described in *Streptococcus pneumoniae* (Jakubovics and Valentine, 2009) and *Deinococcus radiodurans* (Sun et al., 2010). In cases where it has been examined, it appears that the basis for manganese toxicity is indirect. For instance, elevated intracellular levels of manganese in bacteria can result in the binding of Mn^{2+} instead of Fe^{2+} to the transcriptional regulators Fur (Hantke, 1987; Lam et al., 1994; Funahashi et al., 2000; Loprasert et al., 2000; Benson et al., 2004), Irr (Puri et al., 2010) and PerR (Fuangthong et al., 2002), which interferes with the capacity of these proteins to control the expression of genes involved in iron homeostasis and resistance to oxidative stress.

Mechanisms of Mn²⁺ responsive regulation

Bacteria concentrate Mn²⁺ between 10 μM and 100 μM in the cytoplasm (Finney and O'Halloran, 2003) by regulating the expression of metal transport proteins. Many metal-binding transcriptional regulators exert control at the genetic level by binding directly to DNA elements in target promoters via helix-turn-helix domains (Que and Helmann, 2000). The two most prevalent metal-responsive regulators in bacteria are the Fur (ferric uptake regulator) proteins identified in *E. coli* (Hantke, 1981) and DtxR (diphtheria toxin repressor) from *Corynebacterium diphtheriae* (Schmitt and Holmes, 1991). Fur and DtxR are prototypical iron-sensing regulators and both undergo conformational changes in the presence of iron and repress transcription of target genes.

MntR

MntR is a manganese-sensing regulator first identified in *Bacillus subtilis* (Que and Helmann, 2000) and is a functional homolog of DtxR. In *B. subtilis*, *E. coli* and *Salmonella*, MntR is responsible for controlling manganese homeostasis (Que and Helmann, 2000; Patzer and Hantke, 2001; Kehres et al., 2002). In these bacteria, MntR has been shown to repress transcription of the manganese acquisition genes *mntH* and *sitABC* by binding to a 20 base pair DNA sequence, 5'- AACATAGCnnnnGCTATGTT-3' known as the MntR box in the promoter regions of these genes. Genes encoding for MntRs have been identified in the α-proteobacteria *Rhodobacter capsulatus* and *Mesorhizobium loti*, but the phenotypes of *mntR* mutants of these strains have not been examined. A MntR-like binding site upstream of the *mntH* paralog in *Bradyrhizobium japonicum* has also been detected, but manganese responsive regulation of this gene (see below) has been shown to be under control of another metalloregulator (Rodionov et al., 2006). Consequently, MntR has not been linked to manganese homeostasis in the α-

proteobacteria and review of the *B. abortus* 2308 genome sequence does not reveal any genes that encode a homolog of MntR. This suggests that manganese homeostasis is under control of another manganese-sensing regulator in this strain.

Mur

The transcriptional regulator Mur belongs to the Fur family of metalloregulators (Lee and Helmann, 2007). Fur was first identified in *E. coli* as a transcriptional repressor involved in controlling the expression of iron transport genes (Hantke, 1981). Experimental evidence shows that Fur proteins repress their target genes under metal-replete conditions and that a metal cofactor is essential for DNA binding activity (Bagg and Neilands, 1987). Fur and its homologs are ~17-kDa polypeptides that share high conservation in amino acid sequence (Figure 5) and contain an N-terminal domain involved in DNA binding and a C-terminal domain that participates in multimerization of the protein (Escolar et al., 1999). The standing model of Fur-mediated repression is that in the presence of iron Fur dimerizes, and recognizes a 19-21 base pair palindrome GATAATGATAATCATTATC (DeLorenzo et al., 1988) known as a Fur box in target promoters (Lee and Helmann, 2007).

In the α -proteobacteria, Fur-like proteins have been identified but they are not involved in controlling iron metabolism genes in an iron-responsive manner. Rather, these Fur-like proteins are specific for manganese. Because of this differential preference for manganese, these proteins were renamed Mur (manganese uptake regulator) (Díaz-Mireles et al., 2004). Mur has been characterized in the *Rhizobaceae* and *Agrobacterium tumefaciens*, and is responsible for the manganese-responsive control of *mntH* and *sitABC* (Díaz-Mireles et al., 2004; Hohle and O'Brian, 2009; Kitphati et al., 2007; Platero et al., 2007) (Figure 6). Analysis of the nucleotide sequences critical for Mur binding upstream of manganese acquisition genes in *R.*

leguminosarum (Díaz-Mireles et al., 2005), *S. meliloti* (Platero et al., 2007) and *B. japonicum* (Hohle and O'Brian., 2009) by DNase I footprint analysis shows Mur binds a nucleotide sequence degenerate from the canonical Fur box found in *E. coli*. This binding site has been renamed the MRS (Mur-responsive site) (Díaz-Mireles et al., 2005) (Figure 7).

Statement of the Problem

The studies that form the basis for this thesis were performed to test two hypotheses: a) that *B. abortus* 2308 relies on MntH as its sole high affinity manganese transporter; and b) that the transcriptional repressor Mur regulates *mntH* expression in this strain in accordance with the availability of Mn^{2+} in the external environment.

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Table 1.1 *Brucella* spp. and their natural hosts

Taxonomic Classification of the *Brucella* species

<u>Species</u>	<u>Natural host</u>
<i>B. melitensis</i>	Goats, sheep, camels
<i>B. abortus</i>	Cows, camels, yaks, buffalo
<i>B. suis</i>	Pigs, wild hares, caribou, reindeer, wild rodents
<i>B. canis</i>	Canines
<i>B. ovis</i>	Sheep
<i>B. neotomae</i>	Rodents
<i>B. pinnipedialis</i> and <i>B. cetaceae</i>	Mink whales, dolphins, porpoises, seals
<i>B. microti</i>	Voles
<i>B. inopinata</i>	Humans(?)

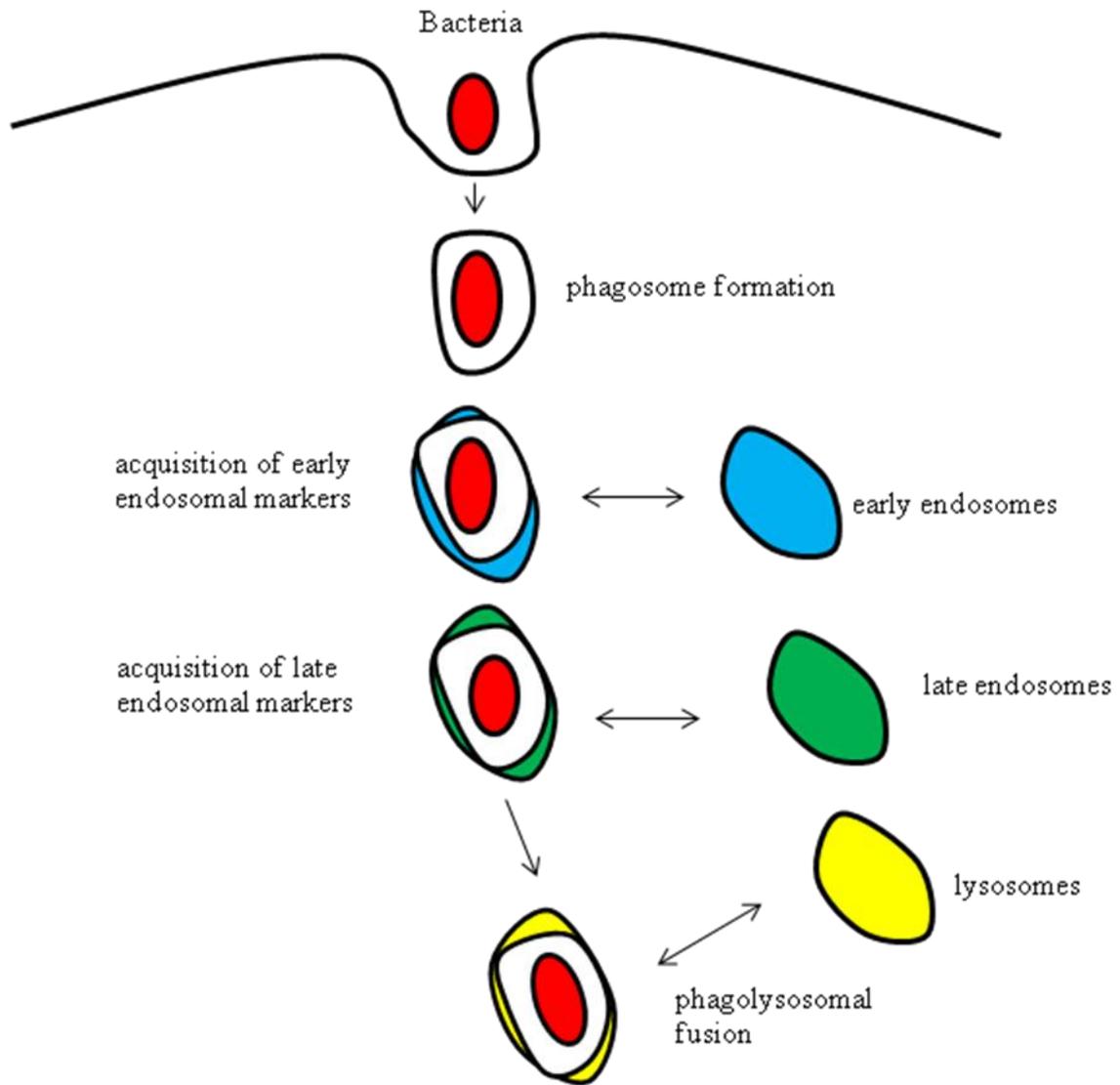


Figure 1.1 Macrophages internalize bacteria by phagocytosis. Bacteria are engulfed and trafficked normally through the endocytic pathway and interact with early and late endosomes before vacuoles containing bacteria fuse with lysosomes (Scott et al., 2003).

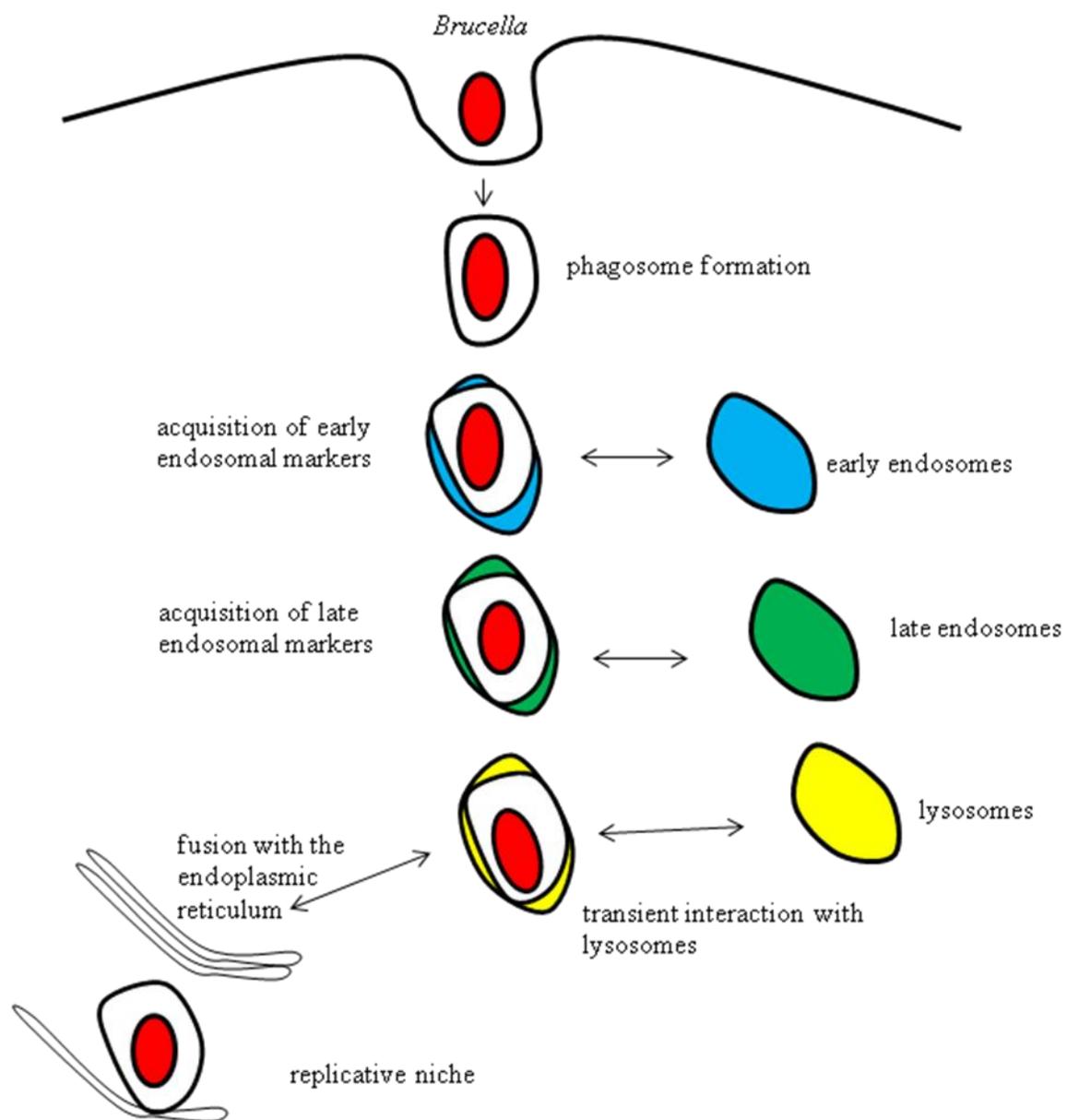


Figure 1.2 Development of the *Brucella*-containing vacuole (BCV). *Brucella* entry into macrophages via phagocytosis precedes trafficking through the endocytic pathway. The BCV interacts with both early and late endosomes and then briefly with lysosomes prior to fusion with the ER (Starr et al., 2008). Following the transient interaction with lysosomes the BCV interacts with the ER where it is suitable for bacterial replication (Starr et al., 2008).

<i>Brucella abortus</i> MntH	(1)	MVPHGSCPSGAREVT FEGWRRER-GEASMSDVHRTIRVNRSGSKFRRLSFFGPGYLAV
<i>Bradyrhizobium japonicum</i> MntH	(1)	----MDARSPDLTTDAAGWRDTPPTTKSLAEVNASVALPTAGVWVRRRLAFVGPGLVSV
<i>Escherichia coli</i> MntH	(1)	-----MTNYRVESSSGRAARKMR LALMGPAFIAAI
<i>Salmonella typhimurium</i> MntH	(1)	-----MTDNRVENS SGRAARKLR LALMGPAFIAAI
Consensus	(1)	S GWR S MVN RVE SSGRAARRRRLALMGPGYLAV
<i>Brucella abortus</i> MntH	(60)	* * GYMDPGNWATSLAGGSRFGYALLSVLLSNLMAVLLQALCTRLAVATGRDLAQACRDAYP
<i>Bradyrhizobium japonicum</i> MntH	(57)	GYMDPGNWATDLAGGSKFGYTLLSVLLSNLMAILLQSLAARLGI VTDRLAQACRATYS
<i>Escherichia coli</i> MntH	(31)	GYIDPGNFATNIQAGASFGYQLLWVVWANLMAMLIQILS AKLGIATGKNLAEQIRDHYP
<i>Salmonella typhimurium</i> MntH	(31)	GYIDPGNFATNIQAGASFGYQLLWVVWANLMAMLIQILS AKLGIATGKNLAEQIRDHYP
Consensus	(61)	GYMDPGNWATNLQGGSSFGYQLLWVVWNSLMAMLLQILSARLGIATGRNLAQQIRDHYP
<i>Brucella abortus</i> MntH	(120)	RFLAWPLWLLA* LAICAT* DLAEVIGTAIGLNLLFGIPLEIGVILTAVDVLVLYLQNKGF
<i>Bradyrhizobium japonicum</i> MntH	(117)	PAVNFLLWLACEAAI IACDLAEVIGTAIALKLLFGIPLIGALIAALDAFLLL LMNRGF
<i>Escherichia coli</i> MntH	(91)	RPVVWFYVWQAEI IAMATDLAEFIGAAIGFKLILGVSLLQGA VLTGIATFLI LMLQRRGQ
<i>Salmonella typhimurium</i> MntH	(91)	RPVVWFYVWQAEI IAMATDLAEFIGAAIGFKLILGVSLLQGA VLTGIATFLI LMLQRRGQ
Consensus	(121)	RPVVWFYVWQAEI I IAMATDLAEVIGTAIGLKLILGVSLLQGA VLTGIDTFLI LMLQRRGQ
<i>Brucella abortus</i> MntH	(180)	RRVEALIITLLGVIALCFLTQIIMAPQWGEVIKGFAPTEIVSNPDMLYIALG IIGATV
<i>Bradyrhizobium japonicum</i> MntH	(177)	RFLAEFVIALLAVIAVCFVAVQIVAAAPPVAEVLHGFMPKSEIFTNPEMLYIAIG IIGATV
<i>Escherichia coli</i> MntH	(151)	KPLEKVIIGLLLFVAAAYIVELIFSQPNLAQLGKGMVIPS--LPTSEAVFLAAGVLGATI
<i>Salmonella typhimurium</i> MntH	(151)	KPLEKVIIGLLLFVAAAYIVELFFSQPDMAQLGKGMVIPA--LPNPEAVFLAAGVLGATI
Consensus	(181)	RPLEKVIIGLLLVAAACYIVQLIFSQP AOVGKGMVPPSEILPNPEMVYLAAGVLGATV
<i>Brucella abortus</i> MntH	(240)	* MPHNLVYLGHSIVQTRDYGHHTAEKREAIRYATLDSTIALTFALVINASILILAAASFHAT
<i>Bradyrhizobium japonicum</i> MntH	(237)	MPHNLVYHSSIVQTRAYERNETGRREAIKWATTDSTIALMLALFINAAILVVAATFHKS
<i>Escherichia coli</i> MntH	(209)	MPHVIYHSSLTQHHLGG----SRQQRYSATKWDAIAMTIAGFVNLAMMATAAAFHFS
<i>Salmonella typhimurium</i> MntH	(209)	MPHVIYHSSLTQHHLGG----TRQQRYSATKWDAIAMTIAGFVNLAMMATAAAFHFS
Consensus	(241)	MPHVLYHSSLVQTRHYG RRQQRYSATTWVVTIAMTIALFVNLAMMATAAAFHFS
<i>Brucella abortus</i> MntH	(300)	GHTGVEDLDKHAHLLNPLLSAIPALFAIALCCGLNSTITATMAGQIVMEGFIDIRLK
<i>Bradyrhizobium japonicum</i> MntH	(297)	GHSDVAEIGQAFELLSPLLGIASTLFAIALLASGLNSTVTATLAGQIVMEGFLDLRLP
<i>Escherichia coli</i> MntH	(265)	GHTGVADLDEAYLTIQPLLSHAAA-TVFGLSLVAAGLSSTVVGTLAGQVVMQGFIRFHIP
<i>Salmonella typhimurium</i> MntH	(265)	GHTGIADLDQAYLTLEPLLSHAAA-TVFGLSLVAAGLSSTVVGTLAGQVVMQGFVRFHIP
Consensus	(301)	GHTGVADLDQAYLTL PLLSHAIA TVFGLSLVAAGLSSTVVGTLAGQVVMQGFIRFRLP
<i>Brucella abortus</i> MntH	(360)	PWIRRAITRFVAIVPAAIVTILYGSQGTTELLILSQVVLSQLPFAVIPLVIFTAQKKKM
<i>Bradyrhizobium japonicum</i> MntH	(357)	SWARRLLTRGIAIIPVIIVTAIYGERGTADLLVFSQVVLMSQLPFAVIPLVRFVSDRRKM
<i>Escherichia coli</i> MntH	(324)	LWVRRTVT----MLPSFIVIMLG--LDPTRILVMSQVLSFGIALALVPLLI FTSDSKLM
<i>Salmonella typhimurium</i> MntH	(324)	LWVRRTIT----MLPSFIVIMLG--LDPTRILVMSQVLSFGIALALVPLLI FTSNATLM
Consensus	(361)	LWVRRTITR AMLPSFIVTLMYG LGTTRLLVMSQVLSFQLPLAVVPLVIFTSD KLM
<i>Brucella abortus</i> MntH	(420)	* GSLAAPWVTFLLAAITAAIIVVNLKLIYDFFTGAPI-
<i>Bradyrhizobium japonicum</i> MntH	(417)	GKFAISPYVAIAIAWIVAGVI VVNLKLLADTLLG---
<i>Escherichia coli</i> MntH	(378)	GDLVNSKRVKQTGWVIVVLVVALNIWLLVGTALGL---
<i>Salmonella typhimurium</i> MntH	(378)	GELVNTRRVKQVGWIVVLVVALNIWLLVGTVMGLS---
Consensus	(421)	G LVNSPRVKQ GWIVVLVVLNLWLLVGT LGL

Figure 1.3 Amino acid alignment of selected MntH proteins identified in bacteria. The asterisk depicts amino acid residues shown by site-directed mutagenesis to be critical for Mn²⁺ transport by the *E. coli* MntH and residues in red are conserved among MntH proteins (Chaloupke et al., 2005; Haemig and Brooker, 2004)

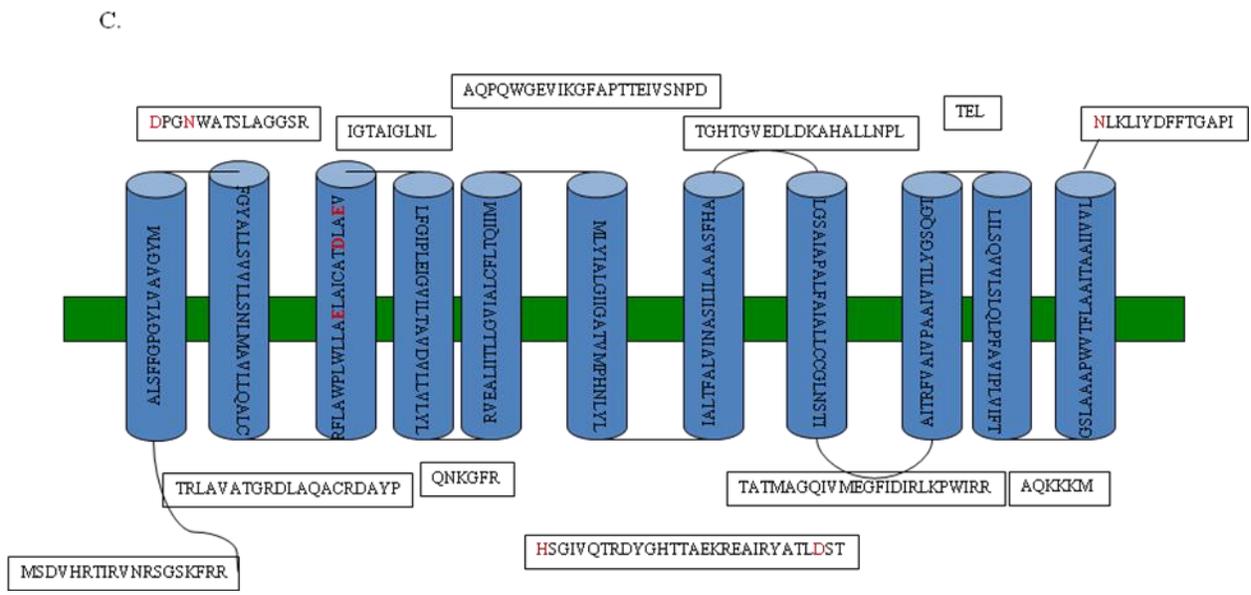
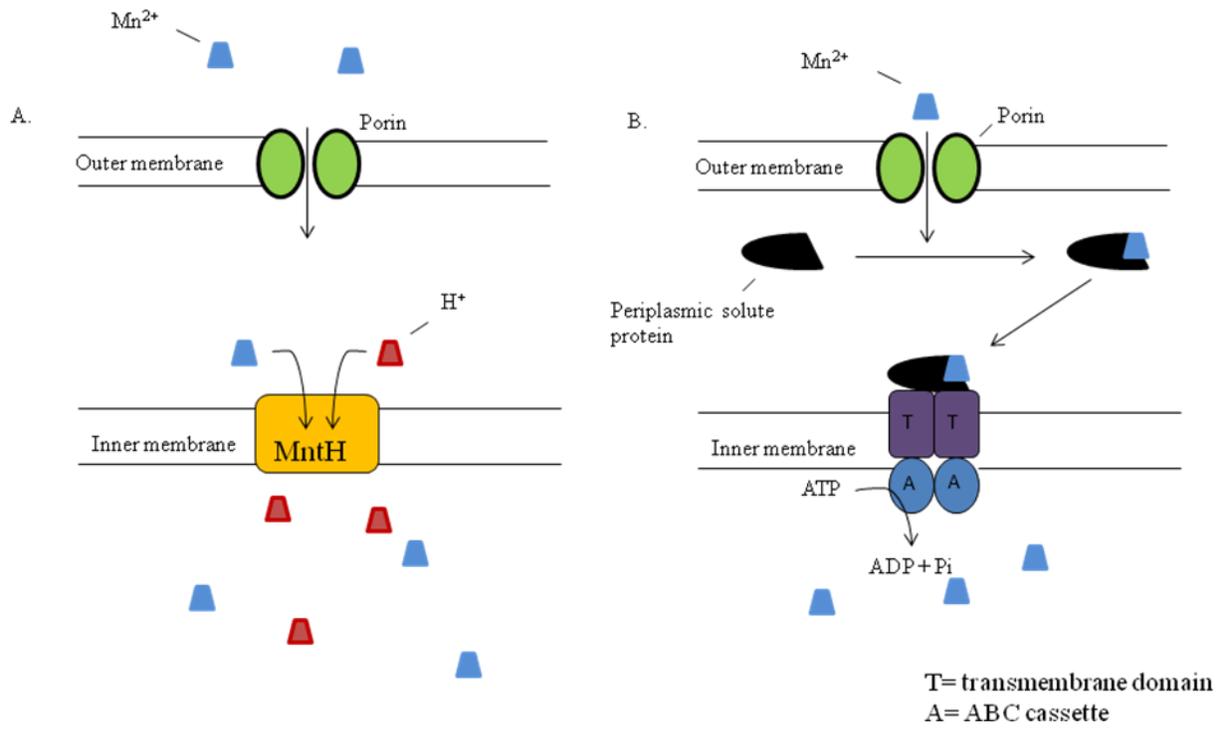


Figure 1.4 Schematic representation of the different methods of Mn^{2+} transport in bacterial cells. A) MntH utilizes a proton gradient to co-transport Mn^{2+} and H^+ across the cytoplasmic membrane and functions best at acidic pH. B) SitABC is an efficient transporter in alkaline conditions and requires a periplasmic binding protein to bind Mn^{2+} and bring this metal within proximity to the permease for transport through a transmembrane domain that requires the hydrolysis of ATP. C) Schematic representation of the MntH transmembrane domains. Residues that have been shown to be important for Mn^{2+} transport are highlighted in red.

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      Brucella abortus Mur (1) -----MNKPYTKPDYEQE*LR*RAGVRI*TR*PRRI*LNILNETED*-HPDALEI
      Sinorhizobium meliloti Mur (1) ----MSQSKNRIELEGILREGGVRVTRQRAAILKILAEAEED-HPDASEL
      Rhizobium leguminosarum Mur (1) -----MTDVAKTLEELCTERGM*RMTE*Q*RRVIARILEDS*ED-HPDVEEL
      Bradyrhizobium japonicum Mur (1) M*TALKPSSASKASGIEARCAATG*MRMTE*Q*RRVIARVLA*EAVD-HPDVEEL
      Escherichia coli Fur (1) -----MTDNNTALKKAGLKVTL*PR*LKI*LEVL*QEPDNHHVSAEDL
      Pseudomonas aeruginosa Fur (1) -----MVENSELRKAGLKVTL*PR*VKI*LQMLDSAEQRHMSAEDV
      Consensus (1) DLE L*RKAGLRVT Q*RRVILRIL EAED HPDAEEL

      DNA-binding-helix
      Brucella abortus Mur (45) FRRAVEEDDSISLSTVYRTMKLLE*ERGA*IHRHAFAGG*PSRFEQASGAH*H*HD
      Sinorhizobium meliloti Mur (46) HRRAKEIDATVSLSTVYRTLSALEQQGVVQRHAFENATARFETADAPH*H*HD
      Rhizobium leguminosarum Mur (43) YRRSVKVDAKISISTVYRTVKLFEDAGI*IARHDFRDGRSRYETVPEEH*H*HD
      Bradyrhizobium japonicum Mur (50) YRRCAVAVDDKISISTVYRTVKLFEDAGI*IERHDFREGRRARYETMRD*SH*HD
      Escherichia coli Fur (40) YKRLIDMGEEIGLATVYRVLNQFDDAGI*VTRHNFEGGKSVFELTQQH*H*HD
      Pseudomonas aeruginosa Fur (39) YKALMEAGEDVGLATVYRVLTQFEAGLVVRHNF*DGGHAVFELADSG*H*HD
      Consensus (51) YRRAVEVDD ISLSTVYRTLKLFEDAGII RH FEGGRARFETA HHD

      Brucella abortus Mur (95) *HIIDMSGDVVE*EFHSDK*IEKLQ*EIARS*LGFEIV*HHRLELYC*KKLKS---
      Sinorhizobium meliloti Mur (96) HLIDIETGAVIEFRSDKIEQLQAEIAAELGYDLVRRHRELYC*RKRK*D---
      Rhizobium leguminosarum Mur (93) HLIDLKTGTVIEFRSPEIEALQ*ERIAREHG*FRLVDH*RLELYG*VP*LKKEDL
      Bradyrhizobium japonicum Mur (100) HLI*NLRDGK*VIEFTSEIEKLQ*EIARK*LG*YKLV*DH*RLELYC*VP*LDDDKP
      Escherichia coli Fur (90) HLI*CLDCGK*VIEFSDD*SEARQ*EIAKHG*IRLTN*HS*LYLYG*HCAEGDCR
      Pseudomonas aeruginosa Fur (89) HMVCVDTGEVIEFMDA*EIEKRQ*EIVRE*RGFELVD*HNLV*LYVR*KKK---
      Consensus (101) HLIDLDTG VIEF SDEIEKLQ EIARELGFRLVDHRELYC*RKLK D

      Brucella abortus Mur (142) -----
      Sinorhizobium meliloti Mur (143) -----
      Rhizobium leguminosarum Mur (143) -----
      Bradyrhizobium japonicum Mur (150) TS-----
      Escherichia coli Fur (140) EDEHAHEGK
      Pseudomonas aeruginosa Fur (135) -----
      Consensus (151)

```

Figure 1.5 Amino acid alignment of Mur proteins identified in the α -proteobacteria and Fur proteins identified in *E. coli* and *P. aeruginosa*. Asterices above indicated residues show identified metal bindings sites of the *P. aeruginosa* Fur protein (Bellini and Hemmings, 2006).

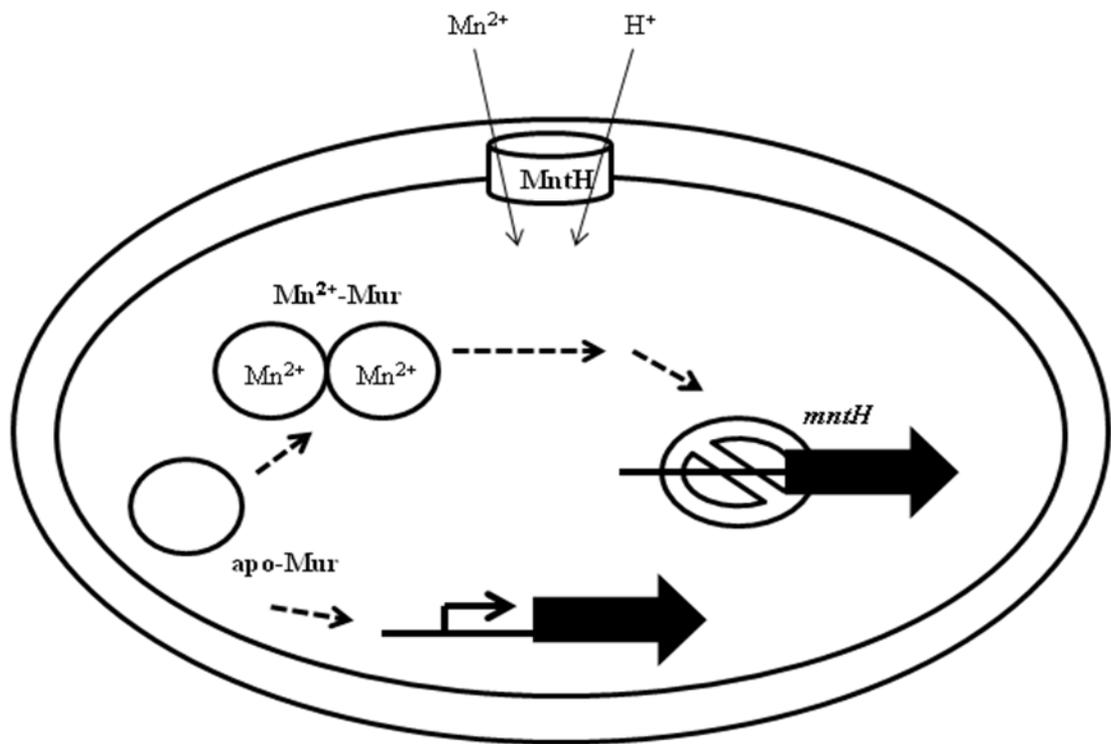


Figure 1.6 Model depicting Mur regulation in the α -proteobacteria. Mur negatively regulates the expression of *mntH* and *sitABC* in response to manganese availability. In the model shown, in the presence of Mn^{2+} , Mur dimerizes and binds to its cognate Mur box upstream of target genes to repress transcription. In the absence of metal, Mur can be found in the apo-form and does not possess DNA binding capabilities enabling active transcription.

A) Identified Mur binding sites in the α -proteobacteria

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Rhizobium leguminosarum MRS1(1) ----ATCATTGTTGCAATCATTCTTAATTGCACAATAGTCGAAAACA
Rhizobium leguminosarum MRS2(1) TCAAGCATTTTTGGCAAATCATTCTGAATGCAACTTTGTTC-----
Sinorhizobium meliloti MRS(1) -----CTAGTTGCAAATGCTTCTCA-TTGCA---TTGAC-----
Consensus (1) TTGTTGCAAATCATTCTCAATTGCAC T GTC

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B) Predicted Mur binding sites in *Brucella*

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 $\alpha$ -proteobacteria MRS consensus (1) -----TTGTTGCAAATCATTCTCAATTGCACTGTC-----
B. abortus MRS upstream mntH (1) -----TTAATGCAAAT-ATTGCAACTGCA-----
B. abortus MRS upstream perR (1) -----ATTGCAAATGGTTGCAATTGCA-----
Consensus (1) TTATTGCAAAT ATTGCAATTGCA

```

c)



Figure 1.7 Nucleotide alignment of the identified Mur binding sites in the α -proteobacteria

A) Nucleotide alignments of Mur binding sites in *Rhizobium leguminosarum* and *Sinorhizobium meliloti* (Díaz-Mireles et al., 2004; Platero et al., 2007) B) Nucleotide alignment comparing the consensus Mur binding site from the α -proteobacteria to potential Mur binding sites identified in *B. abortus* 2308. C) Schematic of the Mur binding sites upstream of *mntH* and *perR* in *B. abortus* 2308.

Chapter II: The manganese transporter MntH is a critical virulence determinant for *Brucella abortus* 2308 in experimentally infected mice

Abstract

The gene designated as 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⁺⁺, Zn⁺⁺, Co⁺⁺ or Ni⁺⁺. Consistent with the apparent selectivity of the corresponding gene product, expression of the *mntH* gene in *B. abortus* 2308 is repressed by Mn⁺⁺, but not Fe⁺⁺, 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 2308, and a comparative analysis of the superoxide dismutase activity 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⁺⁺ 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.

Introduction

Brucella abortus is a Gram-negative bacterium that is responsible for the zoonotic disease brucellosis. Brucellosis causes spontaneous abortion and sterility in ruminants (Enright, 1990) and a debilitating febrile illness in humans known as undulant fever (Corbel, 1997). The ability of the brucellae to cause disease is directly related to their capacity to establish and maintain intracellular infection in host macrophages (Roop et al., 2004). Within the phagosomal compartment in these host cells, the 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 (Jabado et al., 2000). Mn^{++} serves as an important co-factor for a variety of bacterial enzymes including those involved in carbon metabolism, induction of the stringent response and detoxification of reactive oxygen species (ROS) (Papp-Wallace and Maguire, 2006). Consequently, the inability of the 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 like the SitABC complex (Bearden and Perry, 1999; Kehres et al., 2002; Platero et al., 2003; Runyen-Janecky et al., 2006) or H^+ -dependent manganese transporters such as MntH (Kehres et al., 2000; Makui et al., 2000; Que and Helmann, 2000; Hohle et al., 2009). Many bacteria possess both types of Mn^{++} transporters (Papp-Wallace and Maguire, 2006), but a survey of the publicly available *Brucella* genome sequences (del Vecchio et al., 2002; Paulsen et al., 2002; Chain et al., 2005; Halling et al., 2005) 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^{++} . The *E. coli* MntH was originally described as being able to transport both Mn^{++} and Fe^{++} (Makui et al., 2000), but subsequent studies indicated that this and other bacterial MntH proteins are highly selective Mn^{++} transporters that play a minor, if any, role in Fe^{++} transport under physiologically relevant conditions (Kehres et al., 2000). To examine the role of the *Brucella mntH* in Mn^{++} 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) examined. The results of these studies indicate that MntH plays a critical role in Mn^{++} 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% CO₂ or in brucella broth at 37°C with shaking unless otherwise noted. *Escherichia coli* strain DH5- α 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 μ g/ml for *B. abortus* and 100 μ g/ml for *E. coli* DH5- α), chloramphenicol (5 μ g/ml for *B. abortus* and 30 μ g/ml for *E. coli* DH5- α) and/or kanamycin (45 μ g/ml) as necessary. *Brucella* stock cultures were 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-lacZ* transcriptional fusion.

Oligonucleotide primers (forward 5'-GAGCGGGCCATCCTTCTGAA-3'; reverse 5'-GTTGCCGGGATCCATATAACC-3') and PCR were used to amplify a 1031 bp fragment of genomic DNA from *B. abortus* 2308 containing portions of the *mraW* (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 (Gober and Shapiro, 1992). The authenticity of the *mntH-lacZ* 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 (Miller, 1972).

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 (Bellaire, 2001). An inverse PCR strategy (Dorrell et al., 1996) was used to amplify a linear derivative of pDS1 that lacks 338 bp internal to the 426 bp coding region of the *Brucella mur* using the primers $\Delta fur1$ (5'-GCCTCAGCTCCTGCTCATAAT3') and $\Delta fur2$ (CATCACCGGCTTGAACCTTA-3'). The chloramphenicol resistance gene from pBC-SK (NEB) was ligated to the linear Δmur 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. (Elzer et al., 1994). 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 (Bellaire, 2001).

Construction and genetic complementation of the *B. abortus mntH* mutant MWV15.

A 1566 bp region encompassing the putative *mntH* gene (BAB1_1460) (forward primer – 5'- TTCCCCTATTCCCTTAACAT-3', reverse primer – 5'-GATCGGCGTTCTATTTCTTT-3') was amplified from *B. abortus* 2308 genomic DNA by PCR using Pfx polymerase (Invitrogen) and cloned into pGEM[®]-T Easy (Promega). The resulting plasmid was digested with BamHI and HindIII to remove a 696 bp internal to the predicted *mntH* coding region, treated with the Klenow fragment of DNA polymerase I and ligated with a 987 bp SmaI/HincII fragment containing the chloramphenicol acetyltransferase (*cat*) gene from pBlueCM2 (Robertson et al., 2000). This plasmid was then used to construct a *mntH* mutant from *B. abortus* 2308 via gene replacement using previously described procedures (Elzer et al., 1994). The genotype of the *B. abortus mntH* mutant (designated MWV15) was confirmed by PCR analysis of genomic DNA from this strain with *mntH*-, *cat*- and pGEM[®]-T Easy specific primer sets.

For genetic complementation of the *mntH* mutation in MWV15, a 1595 bp DNA fragment containing the *mntH* coding region was amplified by PCR (forward primer 5'-AACATACTCCCCCTACTCCCTTATTC-3', reverse primer 5'-GTATCAGATCGGCGTTCTATTTCTT-3') from *B. abortus* 2308 genomic DNA using Pfu polymerase (Stratagene) and cloned into pGEM[®]-T Easy after A-tagging of the PCR fragments following the instructions provided by the manufacturer. The cloned DNA fragment was then excised from this plasmid as an ApaI/SacI fragment and cloned into the corresponding restriction sites in pMR10 (Gee et al., 2005). This *mntH* containing plasmid, pEA31, was introduced into *B. abortus* MWV15 by electroporation and the resulting strain was given the designation MWV15.C.

Capacity of MnCl₂ to stimulate the growth of *B. abortus* strains in the presence of EDDA.

B. abortus strains were grown on SBA for 48 h at 37°C with 5% CO₂ and harvested into PBS. The optical density of the bacterial cell suspensions was adjusted to 0.15 at 600 nm (10⁹ CFU/ml), 100 µl of each cell suspension was mixed with 3 ml of tryptic soy broth containing 0.7% agar and spread evenly over the surface of tryptic soy broth solidified with 1.5% agar containing 300 µM EDDA in 100 × 15 mm Petri plates. A 7-mm-diameter Whatman filter paper disk was placed in the center of each plate and impregnated with 10 µl of a solution containing 7.95 µM, 79.5 µM, 795 µM or 7.95 mM MnCl₂. After 72 h incubation at 37°C with 5% CO₂, the diameter of the zone of bacterial growth surrounding each disk was measured to the nearest millimeter. The diameters of the zones of growth from five separate plates were measured for each bacterial strain examined.

Growth of the *B. abortus* strains in manganese- and iron-restricted media.

Low manganese minimal medium was prepared using a modification of the protocols originally described by López-Goñi et al. (López-Goñi et al., 1992). Briefly, a minimal medium based on Gerhardt's minimal medium (Gerhardt, 1958) but supplemented with 0.5 g/L yeast extract was treated twice with the chelator 8-hydroxyquinolone. After each treatment, the metal-chelate complexes were removed by chloroform extraction and the residual chloroform removed from the medium by flash evaporation. Following chelator treatment, 50 μM FeCl_3 and MgCl_2 (final concentrations) were added to the culture medium to ensure iron- and magnesium-replete growth conditions. Analysis of representative samples of this medium by atomic absorption spectrophotometry indicates that it contains $< 3 \mu\text{M}$ manganese. Low iron minimal medium was prepared in the same manner with the exception that following treatment with 8-hydroxyquinolone, 50 μM MgCl_2 and 50 μM MnCl_2 were added to the medium. Analysis of representative samples of the low iron minimal medium prepared in this manner indicates that it contains $< 3 \mu\text{M}$ iron.

B. abortus strains were grown on SBA at 37°C with 5% CO_2 for 72 h, harvested into the low manganese minimal medium and the optical density of the cell suspensions adjusted to 0.15 at 600 nm (10^9 CFU/ml). One hundred (100) μl of each suspension was used to inoculate 100 ml of low manganese medium or low manganese medium supplemented with 1, 5, 10 or 50 μM MnCl_2 , 5 μM $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 20 μM $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ or 100 μM $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$, ZnCl_2 , or CuCl_2 . *Brucella* strains grown as above were also harvested and inoculated into low iron minimal medium at a cell density of 10^6 CFU/ml. Bacterial cultures were incubated at 37°C in 500 ml flasks with shaking at 175 rpm. Bacterial growth in these cultures was evaluated by serial dilution and plating on SBA followed by incubation of the SBA plates at 37°C with 5% CO_2 .

Resistance of the *B. abortus* strains to H₂O₂ and the superoxide generators paraquat and menadione in disk sensitivity assays.

Brucella abortus 2308, MWV15, and MWV15.C 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 10⁹ CFU/ml). One hundred µl 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 (TSA) plate (for the H₂O₂ sensitivity assays) or a TSA 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 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 activity in *Brucella* strains.

Brucella strains were grown to mid-log phase in brucella broth. Twenty-five ml samples from each culture were harvested by centrifugation at 10,000 × *g* for 10 min at 4°C and re-suspended in 2 ml native gel buffer [40 mM glycine, 50mM Tris (pH 8.9)]. The cell suspensions were transferred to 2 ml screw cap polypropylene tubes (catalog no. 72.693, Sarstedt) containing 1 g of 0.1 mM Zirconia beads and the cells disrupted by subjecting them to 6 – 40 second cycles at 6 m/s in a Savant FastPrep 120 bead beater (Bio 101), with 1 minute on ice between each cycle. Cellular debris was harvested by centrifugation at 4°C for 20 minutes at 10,000 × *g*. Cleared supernatant was removed and the protein concentration determined using the Bradford

assay (Bradford, 1976). Fifteen μg of each supernatant was loaded onto a 12% native acrylamide gel and subjected to electrophoresis for 1.5 hours at 75 milliamps. Superoxide activity in the gels was determined using previously described methods (Langford et al., 2002). Briefly, the gel was soaked in 50 ml of Solution 1 [50 mM phosphate buffer (pH 7.5), 28 mM TEMED, 0.028 mM riboflavin] for 10 minutes in the dark with gentle rocking. Solution 1 was poured off and 50 ml of Solution 2 [50mM phosphate buffer (pH 7.5), 2.5 mM nitroblue tetrazolium] was added and the gel allowed to gently rock in this solution for 10 minutes in the dark. Solution 2 was then poured off and the gel exposed to a bench lamp for 15 minutes. Color development in the gel was stopped by the addition of a 7% solution of acetic acid and a flatbed scanner was used to capture images of the gels.

Infection of cultured murine resident peritoneal macrophages.

Following euthanasia, macrophages were harvested from the peritoneal cavities of 9-week-old C57BL/6 mice by lavage with Dulbecco's modified Eagle's medium (DMEM)-5% fetal calf serum (FCS) using previously described procedures (Elzer et al., 1996). Pooled macrophages in 200 μl of DMEM-5% FCS were cultivated in 96-well plates at a concentration of 1.6×10^5 per well at 37°C with 5% CO_2 . Cell cultures were enriched for macrophages by washing away non-adherent cells after overnight incubation. *B. abortus* cells were opsonized for 30 min with a sub-agglutinating dilution (1:2,000) of hyperimmune C57BL6 mouse serum in DMEM-5% FCS. Opsonized bacteria were added to macrophages at a ratio of approximately 100 brucellae per macrophage, and the mixture was incubated at 37 °C for 1.5 h to allow time for phagocytosis. The culture medium was then replaced with 200 μl of DMEM-5% FCS containing 50 $\mu\text{g}/\text{ml}$ gentamicin and incubated for 1 h to kill the extracellular brucellae. Macrophages were then washed three times in warm PBS-5% FCS and maintained in DMEM-5% FCS containing

12.5 µg/ml gentamicin for the remainder of the experiment. At 2, 24, and 48 h after infection, the macrophages were lysed with 0.1% deoxycholate and the number of intracellular brucellae 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 C57BL6 mice were infected via the peritoneal route with 5×10^4 of *B. abortus* 2308, MWV15 (2308 Δ *mntH*), or MWV15.C [MWV15(pEA31)] and the spleen colonization profiles of these strains in the mice determined using previously described methods (Gee et al., 2005).

Statistical analysis.

All statistical analyses were performed using the student two-tailed *t* test (Rosner et al., 2000). *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 (Makui et al., 2000). 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 Mn⁺⁺ transport by the *E. coli* MntH (Haemig et al., 2004;Chaloupka et al., 2005) are conserved in the *Brucella* MntH. Based on the annotation of the *B. abortus* 2308 genome sequence (Chain et al., 2005), 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 co-transcribed in an operon with this gene, has not been experimentally determined.

A bioinformatics based study described by Rodionov et al. (Rodionov et al., 2006) predicts the presence of a “Mur” box upstream of the *B. 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 that controls the expression of manganese transport genes in *Rhizobium leguminosarum* (Díaz-Mireles et al., 2004) and *Sinorhizobium meliloti* (Chao et al., 2004), two α -proteobacteria that are close

phylogenetic relatives of the *Brucella* spp., in a manganese-responsive manner. As shown in Figure 1A, the level of β -galactosidase production from an *mntH-lacZ* 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 Mn^{++} are present. The addition of increasing amounts of $MnCl_2$ to the culture medium ranging from 50 μM to 1 mM represses β -galactosidase production in *B. abortus* 2308 (Figure 1A), but not in the isogenic *mur* mutant Fur2 (Figure 1B). In contrast, the addition of up to 500 μM $FeCl_3$ fails to repress expression of the *mntH-lacZ* fusion in either *B. abortus* 2308 or the *mur* mutant (Figure 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 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 or a derivative of MWV15 carrying a plasmid-borne copy of *mntH* (Figure 2A). Consistent with the predicted function of the *Brucella* MntH as a manganese transporter, supplementation of brucella broth with 50 μM $MnCl_2$ allows MWV15 to grow with the same vigor in brucella broth as does strain 2308. Supplementation of this medium with 50 μM $FeCl_2$, in contrast, will not rescue the growth defect exhibited by MWV15 in brucella broth (Figure 2A).

The *B. abortus mntH* mutant MWV15 also displays a significantly reduced ability to use $MnCl_2$ as a manganese source compared to the parental 2308 strain on a solid growth medium containing the chelator ethylenediaminediacetic acid (EDDA) (Table 1). Good growth is observed

for the parental 2308 strain surrounding disks containing 79.5 μM MnCl_2 , but a comparable level of growth is only observed for the *mntH* mutant around disks containing 7.95 mM MnCl_2 in this assay. EDDA has an approximately hundred-fold greater affinity for Fe^{++} (equilibrium constant = 6.45×10^9) than it does for Mn^{++} (5.13×10^7) (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 (Cox, 1994). Thus, it is notable that although the *B. abortus mntH* mutant exhibits a reduced zone of growth around disks containing FeCl_3 or $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ on plates containing the same concentration of EDDA compared to 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 (Figure 2B). The addition of 1 μM MnCl_2 partially alleviates this growth defect and the addition of 10 μM MnCl_2 restores wild type growth of the *mntH* mutant in this medium. In contrast, the *B. abortus mntH* mutant exhibits the same growth profile in low iron minimal medium as the parental 2308 strain (data not shown).

To further examine the selectivity of the metal acquisition defect exhibited by *B. abortus mntH* mutant MWV15, the capacity of increasing concentrations of MnCl_2 or other metals that have been reported to be transported by MntH homologs (e.g. Fe^{++} , Zn^{++} , Cu^{++} , Co^{++} and Ni^{++}) to alleviate the growth restriction exhibited by this strain in low manganese medium was evaluated. As shown in Figure 3, only MnCl_2 was able to restore wild-type growth of the *B. abortus mntH* mutant in this medium. CoCl_2 and NiCl_2 were used at lower levels than the other divalent cation

sources in these experiments because Co^{++} and Ni^{++} both inhibit the growth of *B. abortus* 2308 and MWV15 equally when used at higher concentrations.

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 or low iron minimal medium (Figure 2A).

The *B. abortus mntH* mutant MWV15 displays reduced Mn superoxide dismutase 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_2O_2 (Figure 4A) and O_2^- generated by the redox cyclers paraquat (Figure 4B) and menadione (Figure 4C) in disk sensitivity assays than the *mntH* mutant and introduction of a plasmid-borne copy of *mntH* into the mutant restores wild-type levels of resistance to H_2O_2 , paraquat and menadione in these assays (Figure 4A-C). In most bacteria that have been studied, there is a strong link between the ability to acquire Mn^{++} and resistance to oxidative stress (Horsburgh et al., 2002b). *B. abortus* 2308 produces a manganese containing superoxide dismutase (SodA) (Sriranganathan et al., 1991) and phenotypic analysis of a *B. abortus soda* mutant indicates that SodA is an important antioxidant in this bacterium (Gee, 2004). Consequently, it is possible that the *B. abortus mntH* mutant is unable to transport sufficient Mn^{++} to produce wild-type levels of Mn-SOD activity. The results shown in Figure 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. MW15 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 H₂O₂ is unknown, but hypersensitivity to H₂O₂ has been previously reported for other bacterial strains that are deficient in SOD activity (Capparelli et al., 2007).

MntH is required for wild-type virulence of *B. abortus* 2308 in the C57BL/6 mouse model.

The *B. abortus mntH* mutant MWV15 displays extreme attenuation compared to the parent strain in both cultured murine macrophages (Figure 6) and in experimentally infected mice (Figure 7). In both cases, the attenuation of the *B. abortus mntH* mutant is alleviated by introduction of a plasmid-borne copy of the parental *mntH* gene into this strain.

Discussion

The experimental findings presented in this report support the proposition that MntH serves as the sole high affinity manganese transporter in *B. abortus* 2308. A similar role for MntH has also recently been described in *Bradyrhizobium japonicum*, a close phylogenetic relative of the brucellae (Hohle et al., 2009). Many bacteria contain both proton-dependent (MntH-type) and ATP-dependent (SitABCD-type) Mn^{++} transporters, and mutation of both the *mntH* and *sitABCD* genes is often required before prominent defects in Mn^{++} utilization are observed (Boyer et al., 2002; Runyen-Janecky et al., 2006). This is clearly not the case with the *B. abortus mntH* mutant where loss of MntH produces prominent growth defects in both rich and minimal growth media that can only be relieved by supplementation of these media with elevated levels of Mn^{++} . It is important to note, however, that the *B. abortus mntH* mutant eventually attains the same cell density as the parental strain in a manganese-deprived minimal medium (Figure 1) and the addition of high levels of $MnCl_2$ (e.g. 7.95 mM) to disks will allow this strain to grow on plates containing EDDA (Table 1). These experimental findings demonstrate that *B. abortus* 2308 has an alternate means of acquiring Mn^{++} when MntH is not present, but this alternate mechanism is apparently much less efficient at Mn^{++} transport than MntH.

Like its counterparts in other bacteria (Kehres et al., 2000), the *Brucella* MntH appears to be a manganese-selective transporter. Although supplementation of the culture medium with Mn^{++} relieves the growth defects exhibited by the *B. abortus 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 homologs (e.g. Fe^{++} , Co^{++} , Zn^{++} , Ni^{++} , Cu^{++}) (Gunshin et al., 1997; Makui et al., 2000) does not. Particular attention was paid to the possibility that MntH might also be playing a role in iron transport in *B. abortus*

2308 because prokaryotic MntHs have the ability to transport Fe^{++} at least under laboratory conditions (Agranoff et al., 1999; Kehres et al., 2000; Makui et al., 2000) and efficient iron acquisition has been reported to be an important virulence determinant for this bacterium in both natural (Bellaire et al., 2003) and experimental hosts (Paulley et al., 2007). No experimental evidence linking MntH to iron transport in *B. abortus* 2308 was obtained, however.

Mur, rather than MntR, appears to be the major regulator of Mn^{++} -acquisition genes in most of the α -proteobacteria that have been examined (Chao et al., 2004; Díaz-Mireles et al., 2004; Platero et al., 2004; Rodionov et al., 2006; Kitphati et al., 2007; Hohle et al., 2009). Thus, the fact that *mntH* expression in *B. abortus* 2308 is regulated by manganese in a Mur-dependent manner, but is not responsive to iron, is also consistent with the role of MntH as a manganese-selective transporter. The observation that *mntH* apparently exhibits a relatively high level of basal expression during growth in a nutritionally replete medium may be a reflection of the critical role that MntH plays in providing this bacterium with sufficient levels of Mn^{++} to meet its physiologic needs and the low toxicity exhibited by Mn^{++} compared to other divalent cations (Kehres and Maguire, 2003). The precise nature of the regulatory link between *mntH*, Mur and cellular Mn levels in *B. abortus* 2308 is presently being examined. A computational analysis of the *B. melitensis* 16M genome sequence suggests that the *Brucella* Mur regulon may be limited to only a few genes other than *mntH* (Rodionov et al., 2006).

In most bacteria that have been studied, there is a strong link between the ability to acquire Mn^{++} and resistance to oxidative stress (Horsburgh et al., 2002b), and this same relationship is observed for *B. abortus* 2308 and the isogenic *mntH* mutant MWV15. *B. abortus* 2308 produces a manganese containing superoxide dismutase (SodA) (Sriranganathan et al., 1991) and phenotypic analysis of a *B. abortus* *sodA* mutant indicates that SodA is an important

antioxidant in this bacterium (Gee, 2004). 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 *B. abortus mntH* to the redox cyclers menadione and paraquat which generate O_2^- in the cytoplasmic compartment of bacterial cells. This link between inefficient Mn^{++} transport and suboptimal levels of SOD activity is also similar to the one described for *Sinorhizobium meliloti sitA* mutants (Davies and Walker, 2007). It is quite possible, however, that the increased sensitivity of the *B. abortus mntH* mutant to oxidative stress results from defects in addition to its inability to produce wild-type levels of SodA. Anjem et al. (Anjem and Imlay, 2009), for example, recently presented evidence suggesting that MntH-mediated Mn^{++} transport allows *E. coli* to metallate key metabolic enzymes with this divalent cation instead of Fe^{++} , possibly protecting these enzymes from oxidative damage via Fenton chemistry. These authors also showed that MntH-dependent Mn^{++} transport is an important component of this bacterium's OxyR-mediated response to H_2O_2 exposure. Intracellular levels of Mn^{++} can also influence the activity of transcriptional regulators such as PerR that control the expression of genes important for resistance to oxidative stress (Horsburgh et al., 2002a; Lee and Helmann, 2006; Wu et al., 2006). Preliminary studies suggest that the gene annotated as BAB1_0393 in the *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^{++} levels remains to be determined experimentally. Mn^{++} has also been shown to be able to directly detoxify H_2O_2 and O_2^- in *in vitro* assays (Archibald et al., 1982, Stadtman et al., 1990). It has been postulated, however, that only a few bacteria such as the lactobacilli have the capacity to accumulate sufficient levels of Mn^{++} (e.g. mM) to make direct intracellular detoxification of

ROS by this metal biologically significant (Horsburgh et al., 2002b). Previous studies have shown that wild-type *Brucella* strains require low levels of manganese for growth (Sanders et al., 1953; Evenson and Gerhardt, 1955) and those findings are supported by the growth properties exhibited by *B. abortus* 2308 in the studies reported here (Figure 1). Thus it seems unlikely that direct detoxification of ROS by intracellular Mn^{++} plays a major role in oxidative defense in *Brucella* strains.

Nramp1-mediated efflux of Mn^{++} 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 typhimurium* (Jabado et al., 2000; Cellier et al., 2007). Studies have shown, however, that Nramp1 does not play a prominent role in protecting experimentally infected mice from *Brucella* infections (Guilloteau et al., 2003). Studies in 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 (Capparelli et al., 2007, Paixão et al., 2007). Thus, the severe attenuation exhibited by the *B. abortus mntH* mutant in C57BL/6 mice (which lack a functional Nramp1) (Skamene et al., 1982) 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 (Boyer et al., 2002; Domenech et al., 2002), and significant attenuation of *S. typhimurium mntH sitABCD* double mutants (which lack both of their high affinity manganese transporters) is only seen in mice that have a functional Nramp1 locus (Zaharik et al., 2004). These findings suggest that Mn^{++} , like Mg^{++} and Zn^{++} (Kim et al., 2004; Lavigne et al., 2005; Yang et al., 2006), represents an important micronutrient for the brucellae during residence in the mammalian host. They also suggest that

the levels of Mn^{++} present in the tissues of the 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 loss of MntH has on virulence in *B. abortus* 2308, it is intriguing that the brucellae rely on a single high affinity divalent cation transporter to meet their physiologic needs for Mn^{++} during intracellular replication in the host. Indeed, having only one high affinity Mn^{++} 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 SitABCD-type Mn^{++} transporters (Papp-Wallace and Maguire, 2006). The bacterial SitABCD-type transporters that have been studied in detail, however, have been reported to be unable to transport Mn^{++} at acidic pH (Kehres et al., 2002; Papp-Wallace and Maguire, 2006) and thus this type of transporter might be of limited utility to the brucellae during the early stages of their intracellular residence in host macrophages when these bacteria occupy acidified compartments (Köhler et al., 2003; Bellaire et al., 2005). In contrast, if the *Brucella* MntH is similar to its *Salmonella* counterpart (Kehres et al., 2000) and exhibits optimum Mn^{++} transport at acidic pH, it would appear to be well suited to the intracellular lifestyle of the brucellae in their mammalian hosts.

There are multiple reasons why an insufficient level of intracellular Mn^{++} 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. The *Brucella* spp. also produce a single bifunctional (p)ppGpp synthetase/hydrolase that has been given the designation Rsh (RelA/SpoT hybrid) (Dozot et al., 2006), and the enzymatic activity of this class of bacterial proteins is

Mn⁺⁺-dependent (Papp-Wallace and Maguire, 2006). Because production of (p)ppGpp is required for the stringent response in bacteria (Magnusson et al., 2005), reduced Rsh activity due to inadequate Mn⁺⁺ 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 (Köhler et al., 2003; Roop et al., 2004). The presence of a functional Rsh has also been shown to be required for proper expression of the *virB* genes that encode the Type IV secretion machinery in *B. melitensis* 16M and *B. suis* 1330 (Dozot et al., 2006). 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 are indeed reduced in the *B. abortus mntH* mutant MWV15 compared to the parental 2308 strain (J. Gaines and E. Anderson, unpublished), but the nature of the link between MntH and *virB* expression remains to be experimentally determined.

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Table 2.1 Capacity of *B. abortus* 2308, MWV15 (2308 *mntH*) and MWV15.C (MWV15 [pEA31]) to use MnCl₂ as a manganese source.

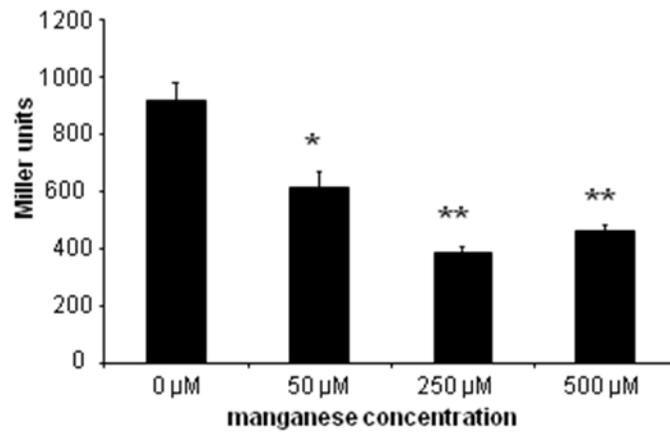
Strain	79.5 μM MnCl ₂	795 μM MnCl ₂	7.95 mM MnCl ₂
2308	43.1 ± 0.6 ^a	52.3 ± 1	67.3 ± 0.6
MWV15	NG ^b	NG	42.8 ± 1.4 ^{***}
MWV15.C	43.4 ± 1.8	52.7 ± 0.3	69 ± 3.2

^aThe diameters of the zones of bacterial growth on a solid growth medium supplemented with 300 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 incubation at 37°C with 5% CO₂ and recorded in mm. The values presented in this table are the means ± standard deviations of the average zone sizes obtained from three separate experiments and three separate determinations of growth were obtained for each strain in each experiment.

^bNG = no growth – no zone of growth was observed surrounding the disks impregnated with 10 or 100 μg/ml MnCl₂ for *B. abortus* MWV15.

Significance - ^{***} = $P \leq 0.005$ for comparisons of MWV15 vs. 2308 and MWV15.C.

A.



B.

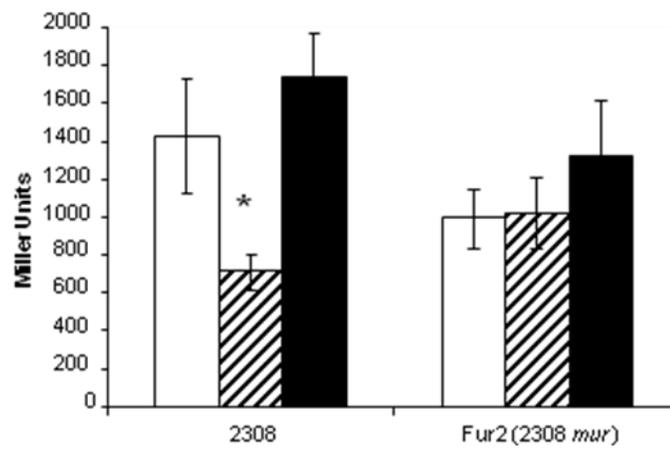
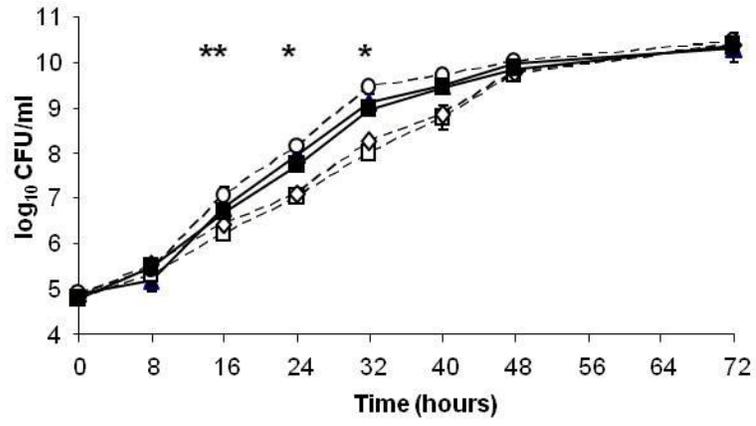


Figure 2.1 Manganese-responsive repression of an *mntH-lacZ* transcriptional fusion in *B. abortus* 2308 is mediated by Mur. (A). Expression of a *mntH-lacZ* fusion in *B. abortus* 2308 following 24 hours growth in brucella broth or brucella broth supplemented with 50, 250 and 500 μM MnCl_2 . (B). Activity of the *mntH-lacZ* fusion in *B. abortus* 2308 and the isogenic *mur* mutant (Fur2) following 24 hours growth in unsupplemented brucella broth (white bars), brucella broth supplemented with 500 μM MnCl_2 (hatched bars) or brucella broth supplemented with 500 μM FeCl_2 (black bars). β -galactosidase activity is presented on the y-axis in Miller units (53). The data presented are means and standard deviations for triplicate determinations from a single culture in a single experiment. The data presented are representative of multiple (≥ 3) experiments performed from which equivalent results and statistical trends were obtained. Significance - * = $P < 0.05$ and ** = $P < 0.005$ for comparisons of β -galactosidase production in unsupplemented medium vs. medium supplemented with MnCl_2 or FeCl_3 .

A.



B.

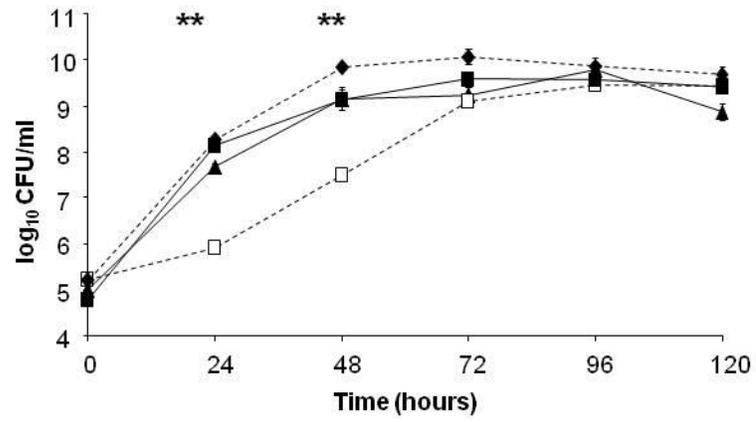


Figure 2.2 Supplemental MnCl₂ restores wild-type growth of the *B. abortus mntH* mutant in broth culture. (A) Growth of *B. abortus* 2308 (▲), MWV15 (2308 *mntH*) (□), and MWV15.C (MWV15 [pEA31]) (■) in brucella broth and MWV15 in brucella broth supplemented with 50 μM MnCl₂ (◆) or 50 μM FeCl₂ (◇). (B) Growth of *B. abortus* 2308 (▲), MWV15 (□), and MWV15.C (■) in low manganese minimal medium and MWV15 in low manganese medium supplemented with 50 μM MnCl₂ (◆). The data presented are the means and standard deviations for triplicate determinations from a single flask for each strain at each experimental time point in a single experiment. The data are representative of multiple (≥3) experiments from which equivalent results and statistical trends were obtained. Significance - * = $P < 0.05$ and ** = $P < 0.005$ for comparisons of 2308 vs. MWV15 or MWV15.C.

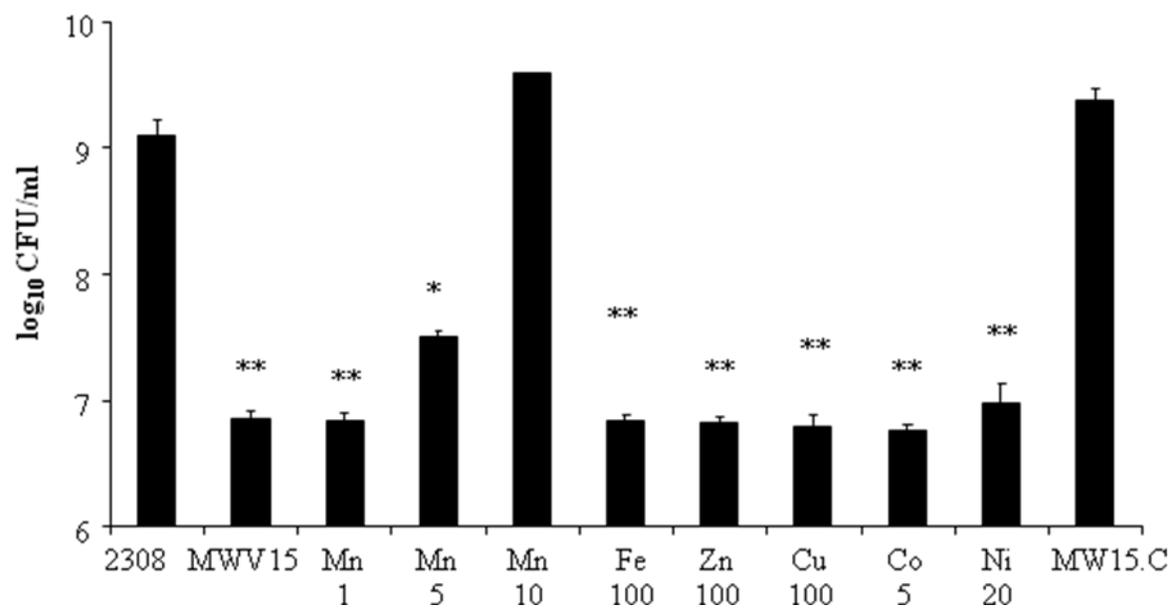
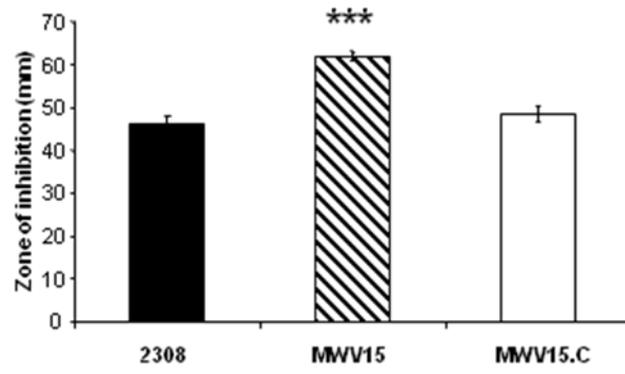
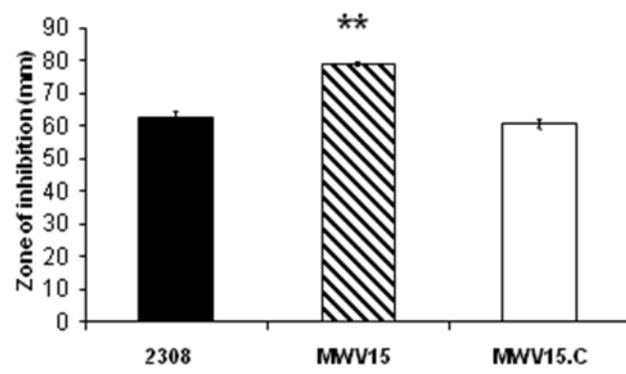


Figure 2.3 Growth of the *B. abortus mntH* mutant in low manganese medium supplemented Mn^{++} , Fe^{++} , Zn^{++} , Co^{++} , Ni^{++} and Cu^{++} . Bacterial cell cultures were inoculated into low manganese medium at cell densities of approximately 10^5 CFU/ml and the number of bacteria present in these cultures following 48 hours 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 MnCl_2 at concentrations of 1, 5 and 10 μM (designated as Mn1, Mn5 and Mn10, respectively). MWV15 cultures supplemented with 100 μM $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ (Fe 100), 100 μM ZnCl_2 (Zn 100), 5 μM $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (Co 5), and 20 μM $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ (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. Significance - * = $P < 0.05$ and ** = $P < 0.005$ for comparisons of 2308 vs. MWV15 or MVW15.C.

A.



B.



C.

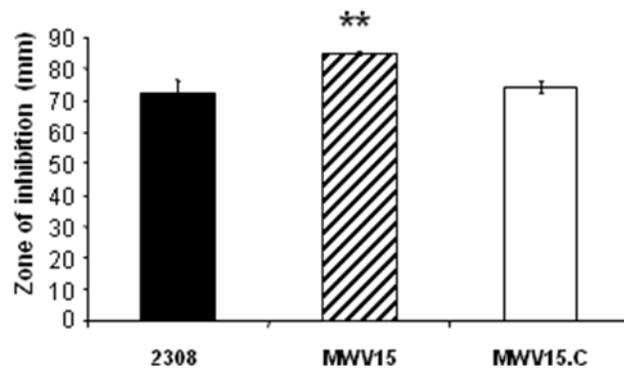


Figure 2.4 Resistance of *B. abortus* 2308, MWV15 (2308 *mntH*) and MWV15.C (MWV15 [pEA31]) to (A) H₂O₂, (B) paraquat, and (C) menadione in disk sensitivity assays. The data presented are zones of inhibition around disks containing (A) H₂O₂, (B) paraquat, or (C) menadione. The values are the means \pm standard deviations of the average zone sizes obtained from three separate experiments and three separate determinations of growth were obtained for each strain in each experiment. Significance - ** = $P \leq 0.005$ and *** = $P \leq 0.001$ for comparisons of MWV15 vs. 2308 or MWV15.C.

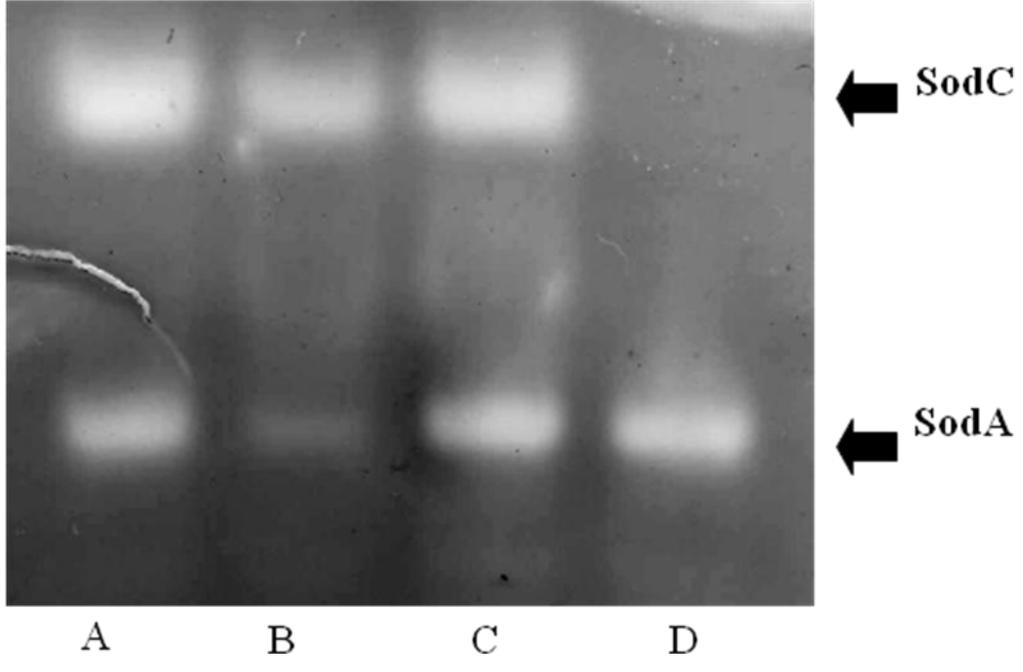


Figure 2.5 Superoxide dismutase activity in *B. abortus* 2308, MWV15 and MWV15C. Cu,Zn SOD (SodC) [cyanide-sensitive] and Mn SOD (SodA) activity [cyanide- and H₂O₂-resistant], detected in the parental 2308 strain (lane A), the *mntH* mutant MWV15 (lane B) and a derivative of MWV15 carrying a plasmid- borne copy of *mntH* (lane C) . The *B. abortus sodC* mutant MEK2 (Elzer et a., 1996) was included as a control (lane D). The gel presented is representative of multiple experiments (≥ 3) in which equivalent results were observed.

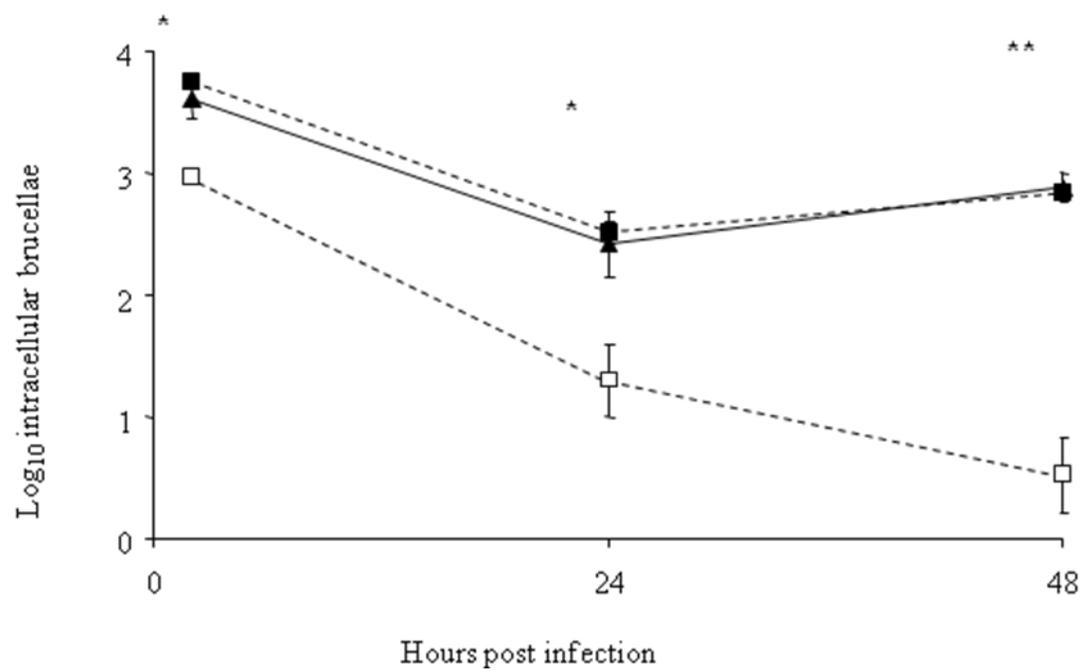


Figure 2.6 Survival and replication of *B. abortus* 2308 (▲), MWV15 (2308 *mntH*) (□) and MWV15C (MWV15 [pEA31]) (■) in cultured resident peritoneal macrophages from C57BL/6 mice. The data are means and standard deviations from the number of intracellular brucellae recovered for each strain from three separate wells of cultured macrophages at each experimental time point in a single experiment. The data are representative of multiple (≥ 3) experiments from which equivalent results and statistical trends were obtained. Significance - * = $P < 0.05$ and ** = $P < 0.005$ for comparisons of MWV15 vs. 2308 and MWV15.C.

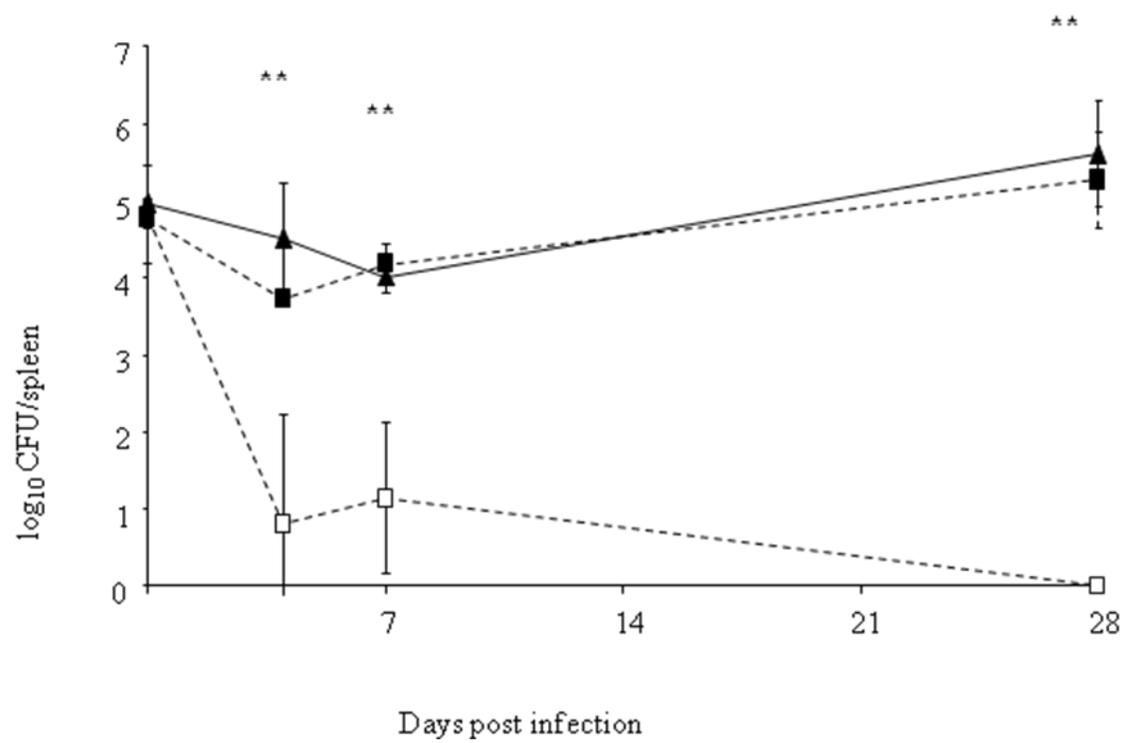


Figure 2.7 Spleen colonization profiles of *B. abortus* 2308 (▲), MWV15 (2308 *mntH*) (□) and MWV15.C (MWV15 [pEA31]) (■) in C57BL/6 mice. The data presented are means and standard deviations for the number of brucellae detected in the spleens of five mice infected with each strain at each experimental time point in a single experiment. Significance - * = $P < 0.05$ and *** = $P < 0.001$ for comparisons of MWV15 vs. 2308 and MWV15.C.

Chapter III: Mur regulates the gene encoding the manganese transporter MntH in *Brucella abortus* 2308

Abstract

MntH is the only high affinity manganese transporter identified in *Brucella*. A previous study showed that MntH is required for the wild-type virulence of *Brucella abortus* 2308 in mice (E. S. Anderson, J. T. Paulley, J. M. Gaines, M. W. Valderas, D. W. Martin, E. M. Menscher, T. D. Brown, C. S. Burns, and R. M. Roop II, *Infection and Immunity* 77:3466-3474, 2009), and indicated that the *mntH* gene is regulated in a manganese-responsive manner in this strain by a Mur homolog. In the study presented here, the transcriptional start site for *mntH* in *B. abortus* 2308 was determined by primer extension analysis, specific interactions between Mur and the *mntH* promoter region were demonstrated in an electrophoretic mobility shift assay, and a Mur-binding site was identified in the -55 to -24 region of the *mntH* promoter by DNase I footprint analysis. The specificity of the interaction of Mur with the putative “Mur box” was further evaluated in EMSA employing oligonucleotides in which the consensus nucleotides in this region were substituted. These studies not only confirm a direct role for Mur in the Mn-responsive regulation of *mntH* expression in *Brucella abortus* 2308, but also identify the *cis*-acting elements upstream of *mntH* responsible for this regulation.

Introduction

A substantial number of bacterial proteins require metal ions for their activity and proper function. However, the accumulation of metals beyond the level at which they are needed can be toxic due to incorrect metal-protein interactions (Waldron and Robinson 2010) and the capacity of certain metals such as iron and copper to participate in the production of toxic oxygen radicals (Summers, 2009). To ensure that they only accumulate the levels of metals they need to meet their physiologic requirements, bacteria produce transporters that mediate both the influx and efflux of specific metal ions (Waldron and Robinson 2010). The genes encoding these transport systems are typically tightly regulated by transcriptional regulators whose activities respond to the levels of specific metal ions in the bacterial cell (Geidroc and Arunkumar, 2007). This specific and differential regulation of metal transport genes enables bacteria to actively adapt to different and sometimes rapidly changing levels of available metals in the external environment (Waldron and Robinson, 2010).

Manganese is an essential co-factor for a variety of bacterial proteins (Papp-Wallace and Maguire, 2006) but excess accumulation of manganese can be toxic (Silver et al., 1972), and bacterial genes encoding manganese transporters are typically regulated by MntR- or Mur-type transcriptional regulators (Guedon and Helmann, 2003; Rodionov et al., 2006). Mur is a structural homolog of the iron-responsive ferric uptake regulator (Fur) (Hantke 1981; 1984) which controls the expression of iron uptake genes in many bacteria (Lee and Helmann, 2007). Mur has only been described in the α -proteobacteria (Rodionov et al., 2006) and it has been shown that the *mur* gene from *R. leguminosarum* will restore iron-responsive gene expression in an *E. coli fur* mutant by genetic complementation (Wexler et al., 2003). However, genetic and biochemical studies have clearly shown that Mur functions a manganese-responsive

transcriptional regulator of manganese uptake genes in this organism (Díaz-Mireles, et al. 2004) and other α -proteobacteria (Chao et al., 2004; Platero et al., 2004). The iron-responsive transcriptional regulators Irr and RirA, instead of Fur, appear to be the major regulators of the iron metabolism genes in the α -proteobacteria (Rodionov et al., 2006; Rudolph et al., 2006; Johnston et al., 2007).

The *Brucella* spp. are members of the α -proteobacteria and the causative agent of brucellosis (Roop et al., 2009). Brucellosis causes sterility and abortion in wild and domestic animals and a severe febrile illness in humans (Nicoletti, 1989). *Brucella* strains rely upon MntH as their sole high affinity manganese transporter and MntH plays a critical role in the virulence of *B. abortus* 2308 in experimentally infected mice (Anderson et al., 2009). Expression of the *mntH* gene is regulated in a manganese-responsive manner in this strain, and genetic studies have implicated Mur in this regulation (Anderson et al., 2009). The purpose of the studies described in this report was to determine whether or not Mur plays a direct role in the manganese-responsive regulation of *mntH* expression in *B. abortus* 2308, and if so to identify the nucleotide sequences to which Mur binds in the *mntH* promoter region.

Materials and methods

Bacterial strains and media –

B. abortus 2308 and derivatives of this strain were cultivated on Schaedler agar supplemented with 5% defibrinated bovine blood (SBA) at 37°C with 5% CO₂ or in brucella broth at 37°C with shaking. Low-manganese minimal medium was prepared as previously described (Anderson et al., 2009). Ampicillin (25 µg/ml) and kanamycin (45 µg/ml) were included in these growth media as appropriate for selection of *Brucella* strains carrying antibiotic resistance markers. *Brucella* stock cultures were stored at -80°C in brucella broth supplemented with 25% glycerol.

Escherichia coli DH5α and BL-21 were used as hosts for recombinant DNA procedures, and these strains were grown at 37°C on LB agar or in LB broth or these media supplemented with 100 µg/ml ampicillin or 45 µg/ml kanamycin as needed. *E. coli* stock cultures were maintained in LB supplemented with 25% glycerol at -80°C.

Construction and evaluation of an *mntH-lacZ* transcriptional fusion in *B. abortus* 2308 and an isogenic *mur* mutant

The construction of pEAM1, a low copy number pMR15-based plasmid carrying an *mntH-lacZ* transcriptional fusion, and the *B. abortus mur* mutant Fur2 have been described previously (Anderson et al., 2009). To facilitate genetic complementation of the *B. abortus mur* mutant, an 826 bp fragment encompassing the *mur* gene (BAB1_1668) was amplified by PCR from *B. abortus* 2308 genomic DNA using Pfx polymerase and the oligonucleotide primers mur-Forward and mur-Reverse (Table 1) and cloned into the SmaI site of pBBR-1MCS4 (Kovach et al., 1995). The resulting plasmid, designated pEAM2, was introduced into *B. abortus* Fur2 by electroporation (Elzer et al., 1994).

B. abortus strains were grown on SBA at 37°C with 5% CO₂ for 72 h and harvested into low manganese minimal medium, and the optical density of the cell suspensions at 600 nm adjusted to 0.15 (approximately 10⁹ CFU/ml). Fifty microliters of each suspension was used to inoculate 50 ml of low manganese medium or low manganese medium supplemented with 50 μM MnCl₂ in 250 ml flasks. These flasks were incubated at 37°C with shaking at 175 rpm. At selected times after inoculation, portions of the cultures were removed and assayed for β-galactosidase production employing the methods described by Miller (1972).

Production of recombinant *Brucella* Mur

The oligonucleotide primers rMur Forward and Reverse (Table 1) which encode *Bsa*I restriction sites were used with Pfx polymerase to amplify a 426 bp DNA fragment containing the *mur* gene from *B. abortus* 2308 genomic DNA by PCR, and this fragment was cloned into the expression vector pASK-IBA6 (IBA GmbH; Göttingen, Germany). The resulting plasmid, which contains the *mur* coding region fused to the Strept-tag II at the N-terminus of the gene was introduced into *E. coli* DH5α by chemical transformation for screening purposes and propagation of the plasmid. Once the authenticity of the gene fusion was confirmed by nucleotide sequence analysis, this plasmid was electroporated into *E. coli* strain BL21 (DE3). For recombinant protein production, *E. coli* cultures were grown overnight at 37°C in LB broth and 10 ml of these cultures used to inoculate 1 L of LB in a 4 L flask. These cultures were grown at 37°C with shaking at 250 rpm to an OD_{600nm} of approximately 0.5, at which time 0.2 mg/ml anhydrotetracycline (AHTC) was added to the cultures and incubation continued. Three to four h after addition of the AHTC, bacterial cells were harvested by centrifugation at 2700 × g for 15 minutes at 4°C, and lysed with CellLytic™ B Cell Lysis Reagent (Sigma) in the presence of the protease inhibitor PMSF on ice for 30 minutes. The cell lysates were subjected to centrifugation

at $17,510 \times g$ for 15 min at 4° C and the supernatant was collected and passed through a Strep-Tactin gravity flow column (IBA GmbH; Göttingen, Germany) following the manufacturer's instructions. Protein fractions were pooled and dialyzed in EMSA binding buffer (10 mM Tris-HCl, 40mM KCl, 10 mM MgCl₂, 1 mM dithiothreitol and 5% glycerol, [pH 7.5]) and concentrated by centrifugation through a 15 ml Amicon Ultra-15 centrifugal filter with a 3,000 molecular weight cut-off (Millipore, Billerica, MA, USA). Total protein was quantified using the Bradford assay (Bradford, 1976), and the degree of purity was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Aliquots of recombinant Mur protein were maintained at - 80°C in the presence of 10% glycerol.

Identification of the *mntH* transcriptional start site by primer extension analysis

Total RNA was isolated from *B. abortus* 2308 grown in low manganese medium and this medium supplemented with 50 µM MnCl₂ employing methods described elsewhere (Mohanety et al., 2008). The primer *mntH* – PE (Table 1) was annealed to 50 µg of total RNA and the primer extension reaction was carried using a modification of the protocol described by Sambrook et al. (1989). Briefly, the reaction was performed using Superscript™ II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) in 5× first strand buffer [250 mM Tris-HCl, pH 8.3, 375 mM KCl; 15 mM MgCl₂, 0.1 M DTT] plus 0.5 mM dNTP. The 5' end of the *mntH* transcript was identified by electrophoresis in a denaturing 6% (w/v) acrylamide and 7 M urea sequencing gel in glycerol tolerant buffer (0.089M Tris-base, 0.0285M taurine, and 0.5mM Na₂EDTA) electrophoresed along-side a DNA sequence ladder. The PCR product used to generate the sequencing ladder was a 1 kb DNA fragment encompassing the coding region of *mntH* and upstream sequence with the primers *mntH*-Forward and Reverse (Table 1). DNA sequence

analysis was performed using the SequiTherm EXCEL™ II DNA Sequencing kit following the instructions provided by the manufacturer (Epicentre, Madison, WI, USA).

Electrophoretic mobility shift assay (EMSA)

The methods described by Platero et al. (2007) were employed for the EMSAs, with the exception that the non-ionic detergent NP-40 was excluded from the reaction. The DNA fragments representing the *mntH* promoter region were amplified by PCR from *B. abortus* 2308 genomic DNA using Pfx polymerase and the primers designated *mntH*-MurboxF and *mntH*-MurboxR (Table 1) and end-labeled with [γ -³²P] ATP (Perkin Elmer, Waltham, MA, USA) using T₄ polynucleotide kinase (Promega, Madison, WI, USA).

EMSA analysis was performed with synthetic and complimentary oligonucleotides (Murbox control and Murbox mutations 1-5; Table 1) 35 nucleotides in length within which selected nucleotides within the MRS in the *mntH* promoter region were substituted and EcoRI and BamHI sites were incorporated on the ends for cloning purposes. Complementary oligonucleotides were annealed, phosphorylated and ligated into BamHI-EcoRI digested pUC19 as described previously (Stojiljkovic et al., 1994). The resulting fragments were then labeled with [γ -³²P] ATP (Perkin Elmer) using the T₄ polynucleotide kinase reaction (Promega, Madison, WI, USA) and incubated in the presence of Mur.

DNase I footprint analysis

The DNase I footprint assay was performed as described previously (Díaz-Mireles et al., 2005) with slight modifications. Briefly, the oligonucleotide primers *mntH*-Murbox Forward and Reverse (Table 1) were individually labeled with [γ -³²P] ATP (Perkin Elmer) using the T₄ polynucleotide kinase reaction (Promega, Madison, WI, USA) prior to their use in PCR reactions to generate 110 bp DNA fragments encompassing the putative Mur box and flanking regions of

the *mntH* promoter. The PCR products were subjected to agarose electrophoresis and purified by gel extraction (Fermentas, Glen Burnie, MD, USA). DNA probes corresponding to 15,000 cpm of the forward labeled and reverse labeled templates were incubated separately in EMSA binding buffer supplemented with 100 ng/ml BSA and 50 ng/ml salmon sperm DNA (non-specific competitor) in the presence of 100 μ M MnCl₂ and increasing concentrations of the recombinant *Brucella* Mur protein. The reaction was incubated at room temperature for 30 minutes prior to treatment with 0.05 units of DNase I freshly diluted in 10 \times DNase I buffer (400 mM Tris-HCl [pH 8.0], 100 mM MgSO₄ and 10 mM CaCl₂) for 1 minute. This reaction was stopped by the addition of 5 mM EDTA and heating at 65 $^{\circ}$ C for 10 minutes. Reaction mixtures were ethanol precipitated and re-suspended in 4 μ l of formamide loading buffer (98% formamide, 10 mM EDTA [pH 8.0], 1 mg/ml xylene cyanol FF, 1 mg/ml bromophenol blue). Digested DNA fragments were separated on a denaturing 6% (w/v) acrylamide and 7 M urea sequencing gel in glycerol tolerant buffer (see above). Gels were dried in a vacuum dryer and subjected to autoradiography. Protected sequence was compared against the same 110 bp nucleotide sequence used in the reaction generated by the SequiTherm EXCELTM II DNA Sequencing kit (Epicentre, Madison, WI, USA).

Results

Mur represses *mntH* expression in *B. abortus* 2308 in response to increasing levels of Mn in a nutrient limited growth medium.

Previous studies showed that the addition of Mn to brucella broth (a complex nutritionally rich culture medium) represses β -galactosidase production from an *mntH-lacZ* fusion in *B. abortus* 2308 (Anderson et al., 2009), but the extent of that repression is limited and a considerable level of basal *mntH* expression is observed even when the brucella broth is supplemented with 500 μ M MnCl_2 . To further examine the relationship between *mntH* expression and Mn levels in *B. abortus* 2308, these experiments were repeated using the low manganese medium described by Anderson et al. (2009), which is less nutritionally complete than brucella broth. As shown in Figure 1A, the addition of 50 μ M MnCl_2 to low manganese minimal medium cultures reduces the level of β -galactosidase production by a *B. abortus* 2308 derivative carrying the plasmid-borne *mntH-lacZ* fusion to less than half that observed in the unsupplemented medium, and the addition of 250 and 500 μ M MnCl_2 reduces β -galactosidase production by this strain to essentially background levels. In contrast, manganese-responsive repression of the *mntH-lacZ* fusion is not observed in the isogenic *B. abortus mur* mutant Fur2 (Figure 1B), but restored when a plasmid-borne copy of *mur* was introduced into Fur2 (Figure 1B). Thus, in two separate sets of experiments employing two culture media with different compositions (Anderson et al., 2009; this study), genetic studies demonstrate a link between Mur and Mn-responsive regulation of *mntH* expression in *B. abortus* 2308.

Mur directly binds to the *mntH* promoter in *B. abortus* 2308.

A putative Mur recognition site (MRS) (Díaz-Mireles, 2005), 5'-AATGCAAATAGTTTGCAAT-3', lies upstream of the *mntH* coding region in the *B. melitensis*

16M genome sequence (Rodionov et al., 2006) and this same genomic arrangement occurs in *B. abortus* 2308 (Figure 2A). To evaluate the functionality of this MRS, primer extension analysis was first used to determine the transcriptional start site for *mntH* in *B. abortus* 2308 and define the promoter for this gene. As shown in Figure 2A, the putative MRS overlaps the -35 region of the *mntH* promoter, which is consistent with the predicted function of Mur as a repressor of *mntH* expression. Interestingly, the T residue identified as the transcriptional start site for *mntH* represents the middle nucleotide in the second codon of the *mntH* coding region annotated in the *B. abortus* 2308 and *B. melitensis* 16M genome sequences (Figure 2A). Because we were unable to detect a transcriptional start site for the *mntH* gene upstream of the T residue depicted in Figure 2A, we propose that an ATG start codon 81 nucleotides downstream of the annotated start codon (Figure 2A) is the authentic start codon for the *Brucella mntH* gene. This would produce a *Brucella* MntH protein that matches the N-terminus of the *E. coli* and *Salmonella* MntH proteins better than those annotated in the *B. abortus* 2308 and *B. melitensis* 16M genome sequences, but a shorter protein than that predicted in *B. japonicum*, a close phylogenetic relative (Figure 2B).

As shown in Figure 3, the *Brucella* Mur binds in a specific manner to a 110 bp DNA fragment representing the *mntH* promoter region in an EMSA. It was also noted in this assay that the addition of the chelator EDTA abrogated the capacity of Mur to bind to the *mntH* promoter, which is consistent with the proposition that the DNA binding activity of Mur requires its interaction with a metal co-factor (in this case Mn). A DNaseI footprint analysis determined that the Mur binding site in the *mntH* promoter is comprised of the 28 nucleotide sequence 5'-TACTTAATGCAAATAGTTTGCAACTGCA-3' (Figure 4), which overlaps the predicted MRS in the *mntH* promoter (Figure 2A).

In an attempt to identify specific subsets of nucleotides in the MRS that are required for Mur binding, the capacity of synthetic double-stranded DNA fragments representing the *mntH* promoter to bind to Mur was evaluated in an EMSA. Only when a core set of 11 nucleotides was changed from CAATAGTTG to TCTGCTACGT did one of the synthetic *mntH* promoter fragments lose its capacity to bind to Mur (Figure 5).

Discussion

Mur proteins belong to the Fur superfamily of bacterial metalloregulators (Lee and Helmann, 2007). These proteins show a considerable amount of amino acid conservation with the prototype and namesake protein in this superfamily, the ferric uptake regulator Fur, but they appear to lack a zinc-binding site that is characteristic of most bacterial Fur proteins (Bellini and Hemmings, 2006) also unlike Fur. The activity of Mur as a transcriptional repressor is modulated *in vivo* by manganese instead of iron. Mur proteins have been described exclusively in the α -proteobacteria, where Mur regulates the expression of manganese acquisition genes in *Rhizobium leguminosarum* (Díaz-Mireles et al., 2004) and *Sinorhizobium meliloti* (Chao et al., 2004; Platero et al., 2007). Recent studies have also shown that proteins currently designated as Fur are functioning as manganese-responsive, instead of iron-responsive, regulators in *Bradyrhizobium japonicum* (Hohle and O'Brian, 2009; 2010) and *Agrobacterium tumefaciens* (Kitphati et al., 2007), and thus they appear to be functional Mur homologs as well.

The gene designated BAB1_1668 in the *B. abortus* 2308 genome sequence is predicted to encode a Fur homolog. This gene was originally cloned during an attempt to identify the iron-responsive regulator of the siderophore biosynthesis genes in *B. abortus* 2308 by genetic complementation (Phillips et al., 1996). A plasmid-borne copy of this gene restores iron-responsive repression of the *fiu-lacZ* fusion in the *E. coli fur* mutant H1780 (Hantke 1987), but when the corresponding gene was deleted from *B. abortus* 2308 by gene replacement, the resulting mutant (designated Fur2) exhibited wild-type regulation of its siderophore biosynthesis genes (Roop et al., 2004). Subsequent studies have shown that a recombinant version of this *Brucella* Fur homolog does not bind to the promoter regions of the iron-responsive *dhbC* (Bellaire et al., 2003) or *bhuA* (Paulley et al., 2007) genes in an EMSA (E. Menscher,

unpublished data). These findings further support the proposition that this protein is not serving as an iron-responsive transcriptional regulator in *B. abortus* 2308. Instead, data presented in this and a previous report (Anderson et al., 2009) indicate that the gene encoded by BAB1_1668 is serving as a Mur.

As demonstrated in this report, Mur binds to the *mntH* promoter region in *B. abortus* 2308 and directly regulates the expression of this gene in a manganese-responsive manner. The 30 nt sequence in the *mntH* promoter protected by Mur in the DNase I protection studies contains a motif (5'-AATGCAAATAGTTGCAA-3') that matches well with the consensus Mur-responsive sequence (MRS) or "Mur-box" defined by Rodionov et al. (2006) and MRSs identified upstream of the *mntH* gene in *B. japonicum* (Hohle and O'Brian, 2009), the *sitA* gene in *R. leguminosarum* (Díaz-Mireles et al., 2005) and the *mntA/sitA* genes of *S. meliloti* (Chao et al., 2004; Platero et al., 2007) (Figure 6). It is notable in this regard that the location of the region of the *mntH* promoter protected by Mur in *B. abortus* 2308 (-55 to -24 with respect the transcriptional start site) is very similar to the location of the region protected by Mur in the *B. japonicum mntH* promoter (-56 to -22 with respect to the transcriptional start site) (Hohle and O'Brian, 2009). Selective regional mutagenesis of the MRS in the *Brucella mntH* promoter sequence identified an 11 nt motif in this region that is critical for Mur binding. These findings are similar to those obtained with Fur in *E. coli* (Escolar et al., 1999) and *Caulobacter* (da Silva Neto et al., 2009) where groups of nucleotides rather than individual nucleotides have been shown to be responsible for the binding of Fur to "Fur boxes".

Manganese is an essential micronutrient for *Brucella* strains (Sanders et al., 1953; Evenson and Gerhardt, 1955), and the importance of MntH for the efficient acquisition of this divalent cation *in vitro* and *in vivo* has been documented experimentally (Anderson et al., 2009). Like

most other biologically beneficial metals, manganese can be toxic if accumulated beyond the levels needed by the bacterial cell, although the potential toxicity of this metal appears to be considerably less than that of other beneficial metals such as iron, zinc, nickel or copper (Papp-Wallace et al., 2006; Waldron and Robinson, 2010). The extent to which Mur protects *B. abortus* 2308 from manganese toxicity is presently under investigation.

In order to fully understand the importance of Mur in the physiology of *Brucella* strains, it will be important to determine the extent of the Mur regulon. *mntH* and *irr* are the only genes that have been shown to be directly regulated by Mur in *B. japonicum* (Hohle and O'Brian, 2010), and the bioinformatics based study performed by Rodionov et al. (2006) suggests that the same may be true in *Brucella*. The iron response regulator Irr (Small et al., 2009) controls the expression of iron metabolism genes in many α -proteobacteria including *Brucella* strains (Martínez et al., 2006). Mur regulates the expression of *irr* at the transcriptional level in *B. japonicum* (Hohle and O'Brian, 2010), while manganese stabilizes the Irr protein (Puri et al., 2009), providing coordination between cellular manganese levels and the expression of the iron metabolism genes in this bacterium. Evidence for indirect effects of manganese on the expression of iron metabolism genes has also been reported for *A. tumefaciens fur* (Kitphati et al. 2007) and *S. meliloti mur* mutants (Chao et al., 2004), suggesting that similar regulatory links may exist in these bacteria as well.

During their residence in host macrophages the brucellae must deal with both endogenous ROS generated by their respiratory metabolism as well as exogenous ROS generated by the NADPH activity of the host phagocyte (Roop et al., 2009). Efficient manganese transport mediated by MntH has been linked to the ROS resistance in *B. abortus* 2308 (Anderson et al., 2009), and Irr is required for optimal expression of the siderophore biosynthesis (Martínez et al.,

2006) and heme transport genes (E. S. Anderson, unpublished data) in this bacterium. Having the capacity to modulate *irr* expression and Irr activity such that iron acquisition genes are only maximally expressed in *Brucella* strains when these bacteria possess protective levels of manganese would thus be beneficial in their intracellular niche in the host because it would potentially reduce the risk for ROS toxicity due to iron-mediated Fenton chemistry. Thus, it will be important to determine if the same coordination between manganese and Irr activity that occurs in *B. japonicum* exists in *Brucella*, and if Mur play an active role in maintaining this coordination.

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Table 3.1 Primers used in this study

<u>Primer designation</u>	<u>Sequence (5' – 3')</u>
<i>mntH</i> -Forward	AACATACTCCCCCTACTCCCTTATTC
<i>mntH</i> -Reverse	GTATCAGATCGGCGTTCTATTTCTT
rMur Forward	CGCGGTCTCAGCGCATGAACAAGCCATACACC
rMur Reverse	GCGCGGTCTCATATCAGGATTTCAATTTTTTGC
<i>mntH</i> -Murbox Forward	TTCCGCCCCCGCCGTTGAC
<i>mntH</i> -Murbox Reverse	GGACATGATCCATGGGGTAC
Murbox control F	AATTACTTAATGCAAATAGTTTGCAACTGCATAAG
Murbox control R	GATCCTTTATGCAGTTGCAAAC TATTTGCATTAAGT
Murbox mutation #1 F	AATTACTTAATGTCTGTAGTTTGCAACTGCATAAG
Murbox mutation #1 R	GATCCTTTATGCAGTTGCAAAC TACAGACATTAAGT
Murbox mutation #2 F	AATTACTTAATGTCTGCTATTTGCAACTGCATAAAG
Murbox mutation #2 R	GATCCTTTATGCAGTTGCAAATAGCAGACATTAAGT
Murbox mutation #3 F	AATTACTTAATGCAAATAGCAGTCAACTGCATAAAG
Murbox mutation #3 R	GATCCTTTATGCAGTTGACTGCTATTTGCATTAAGT
Murbox mutation #4 F	AATTACTTAATGCAAAC TACAGTCAACTGCATAAAG
Murbox mutation #4 R	GATCCTTTATGCAGTTGACTGTAGTTTGCATTAAGT
Murbox mutation #5 F	AATTACTTAATGTCTGCTACAGTCAACTGCATAAAG
Murbox mutation #5 R	GATCCTTTATGCAGTTGACTGTAGCAGACATTAAGT
<i>mur</i> Forward	AGATGCTCAATTGCCGCACC
<i>mur</i> Reverse	CCCGTTGGACAGTTAATGCG
<i>mntH</i> – PE	TTGAAGGCTGGCGTCGCGAGCGTGGAG
<i>mntH-lacZ</i> Forward	GAGCGGGCCATCCTTCTGAA
<i>mntH-lacZ</i> Reverse	GTTGCCGGGATCCATATAACC
<i>recA</i> Forward	TCGCAATGAATTTTGAGAC
<i>recA</i> Reverse	TGGAACAAAATTGCGAAAT

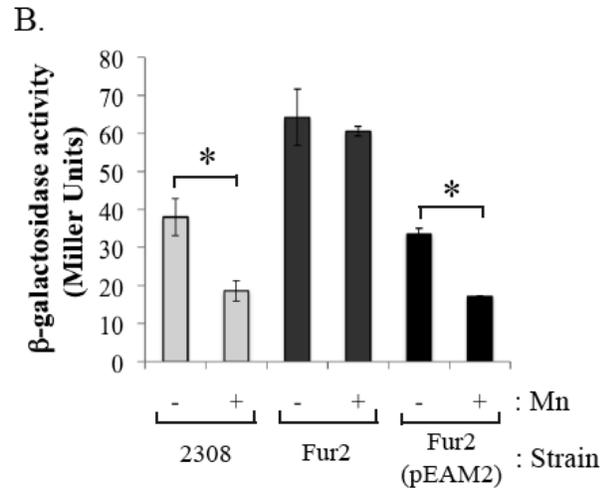
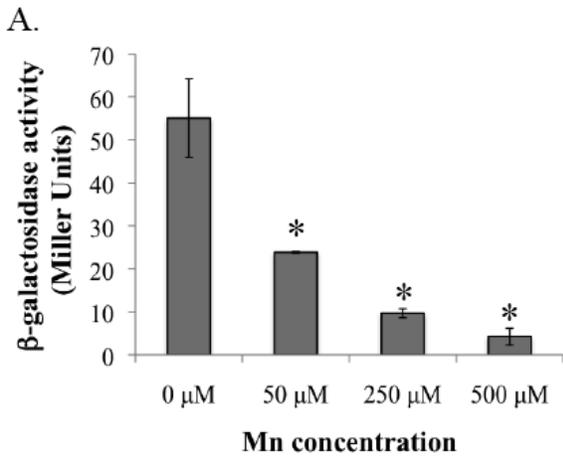
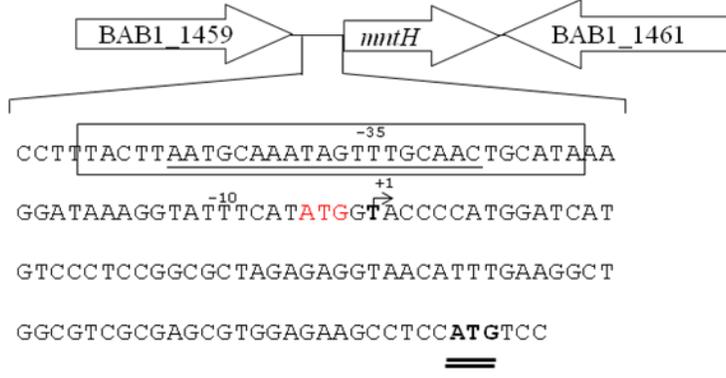


Figure 3.1 β -galactosidase production from a plasmid-borne *mntH-lacZ* transcriptional fusion. *B. abortus* 2308 (A and B), Fur2 [2308 *mur*] (B) and Fur2 (pEAM2) (B) following 48 h cultivation in low manganese minimal medium (-) and this medium supplemented with 50 μ M MnCl₂ (+) in panel B. The data are representative of multiple (≥ 3) experiments from which equivalent results and statistical trends were obtained. Significance - * = $P < 0.05$ for comparisons of A) 2308 vs. 2308 + MnCl₂ and B) 2308 vs Fur2 under the same cultured conditions.



<i>Brucella abortus</i> MntH	(1) -MVPHGSCPSGAREVTFEQRRERGEASMSDVHRTIRVNRSGSKFRRALSFPGYLAV
<i>Bradyrhizobium japonicum</i> MntH	(1) ----MDARSPDLTTDAAGWRTDVPTTKSLAEVNASVALPTAGVWRRLLAEVPGYLVS
<i>Escherichia coli</i> MntH	(1) -----MTNYRVESSSGRAARKMRLALMGPAFIAAI
<i>Salmonella typhimurium</i> MntH	(1) -----MTDNRVENSSGRAARKLRALMGPAFIAAI

Figure 3.2 Mapping the 5'-end of *mntH* (A) Genetic organization of *mntH* and surrounding genes in *B. abortus* 2308. The transcriptional start site for *mntH* identified by primer extension is indicated in bold-face print with an arrow and a “+1” designation above it. The predicted Mur-binding site (or MRS) is underlined and the Mur binding region identified by DNase I footprint analysis in this study is enclosed in a box. The predicted *mntH* start codon annotated in the *B. abortus* 2308 genome sequence in GenBank is denoted in italics with a single underline and the ATG that is proposed to be the authentic *mntH* start codon based on the experimental findings presented in this report is depicted in bold face print with a double underline. (B). A comparative alignment of the N-termini of the predicted products of the *B. abortus mntH* gene based on the current GenBank annotation and the revised MntH sequence (starting with “M” indicated in bold-face and underlined) with the MntH proteins of *E. coli*, *Salmonella* and *Bradyrhizobium japonicum*. The revised annotation based on the work presented in this report is depicted in bold face and underlined.

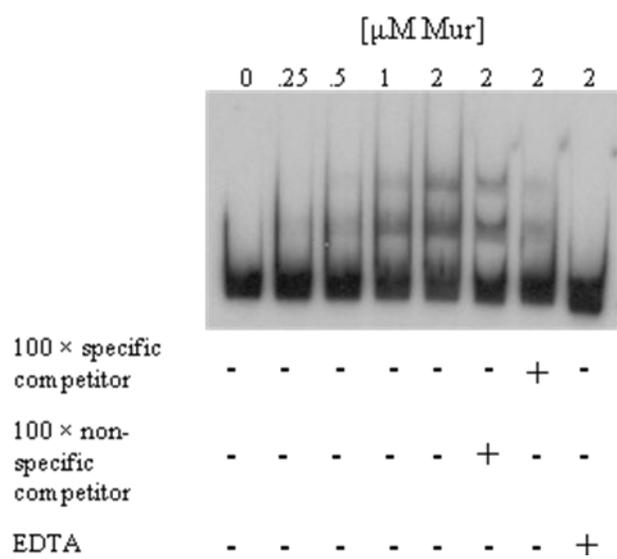
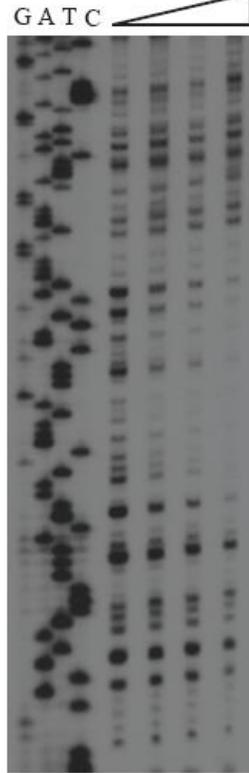


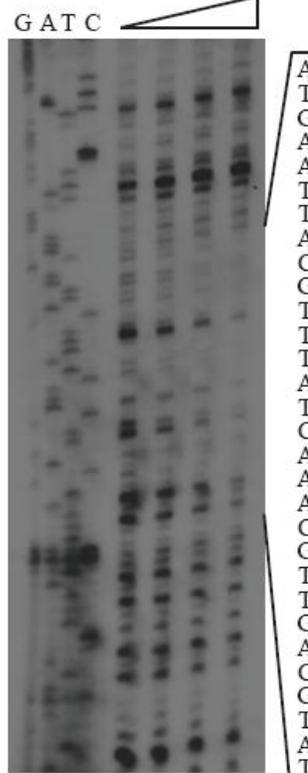
Figure 3.3 Mur binds to the *mntH* promoter. Recombinant Mur binds the promoter region of *mntH* in *B. abortus* 2308 in an EMSA. The concentration of recombinant Mur added to the reactions is shown above the lanes, and the +/- symbols below the lanes indicate whether the reactions contain an unlabeled DNA fragment representing the *mntH* promoter region (specific inhibitor); an unlabeled DNA fragment representing the upstream region of *recA* (non-specific inhibitor), and/or the chelator EDTA.

A.



Forward strand

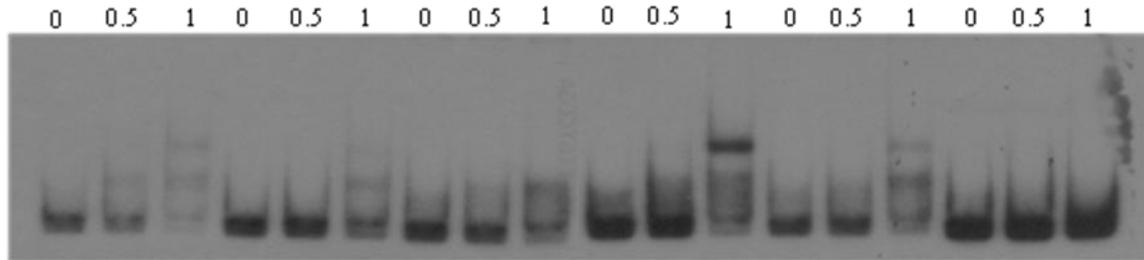
B.



Reverse strand

Figure 3.4 DNase I footprinting. Recombinant *Brucella* Mur binds a 30 base pair sequence upstream of the *B. abortus mntH* promoter . The triangle above the lanes indicates that the corresponding reactions contain increasing concentrations (0,1, 5 and 10 μ g) of recombinant *Brucella* Mur.

[μ M Mur]



Native

1.

2.

3.

4.

5.

Native. A C T T A A T G C A A A T A G T T T G C A A C T G C A T A

1. A C T T A A T G T C T G T A G T T T G C A A C T G C A T A

2. A C T T A A T G T C T G C T A T T T G C A A C T G C A T A

3. A C T T A A T G C A A A T A G C A G T C A A C T G C A T A

4. A C T T A A T G C A A A C T A C A G T C A A C T G C A T A

5. A C T T A A T G T C T G C T A C A G T C A A C T G C A T A

Figure 3.5 Mur binds a consensus sequence. EMSA analysis was used to map subsets of nucleotides present in the MRS of the *B. abortus* 2308 *mntH* promoter that are required for its interaction with Mur in an EMSA. The concentration of Mur added to the reactions is indicated above the lanes; the specific synthetic oligonucleotides (labeled “native”, 1, 2, 3, 4 and 5) used in the EMSA reactions are indicated below the lanes; and the nature of the nucleotide substitution in each synthetic oligonucleotide used in these reactions is shown at the bottom of the figure.

```

Bradyrhizobium MRS CCGATGCGGCCAGATGCAGTTGCAAATGAGTTGCAATAAGCTTCGACTTCGG-----
Brucella mntH MRS -----CCTTTACTTAATGCAAAT-ATTTGCAA-CTGCATAAAGGATAAA-----
Rhizobium sitA MRS1 -----ATCATTGTT-GCAATTCATTCTTAA-TTGCACAATAGTCGAAAACA
Rhizobium sitA MRS2 -----TCAAGCATTGTTGCAAATCATTCTGA--ATGCAACTTTGTTG-----
Sinorhizobium sitA MRS -----CTAGTT-GCAAATGCTTCTCA--TTGCA---TTGAC-----
Consensus          C A  ATT TTTGCAAAT ATTCTCAA  TGCA  T GAC

```

Figure 3.6 MRS alignment. Nucleotide alignment comparing identified Mur binding sites identified upstream *mntH* and *sitABC* in the α -proteobacteria (Díaz-Mireles et al., 2004; Kitphati et al., 2007; Platero et al., 2007).

CHAPTER IV: Summary and future directions

The results described in Chapter 2 of this thesis support the proposition that *Brucella* strains encode a single high affinity Mn^{2+} transporter, MntH. MntH is necessary for growth of *B. abortus* 2308 in the laboratory and for the establishment of infection in the animal model (Anderson et al., 2009). After *Brucella* are engulfed by macrophages, brucellae experience nutrient restriction (Roop et al., 2009), acidification (Boschioli et al., 2002) and oxidative challenge (Roop et al., 2009). Mn^{2+} uptake via MntH at this stage is necessary for survival (Anderson et al., 2009) most likely because *Brucella* depend upon Rsh, the bifunctional (p)ppGpp synthetase/hydrolase that requires manganese for activity. Rsh in *Brucella* activates the stringent response during amino acid starvation and nutrient restriction and is responsible for induction of the *virB* operon, a necessary component for proper trafficking to the ER. The inability to transport Mn^{2+} may then comprise the capacity of *Brucella* to adapt to harsh environmental conditions via Rsh which would have pleiotropic effects.

Mur is the Mn^{2+} -specific regulator of *mntH* and this regulation by Mur is to assure the expression of *mntH* is minimized when physiologic Mn^{2+} requirements are met in *B. abortus* 2308. *mntH* expression early in the development of the BCV is not only beneficial but essential for this organism, however, too much Mn^{2+} can be toxic. Excessive Mn^{2+} accumulation has been shown to directly inhibit growth in bacteria (Silver et al., 1976), but no such inhibitory phenotype has yet to be observed in *Brucella* strains.

mntH expression is greatly increased in the presence of oxidative stress in the enterics (Kehres et al., 2000) and the same has been observed in *B. abortus* 2308 (Evan Menscher, unpublished data). The influx of Mn^{2+} during oxidative stress conditions provides *Brucella* with sufficient Mn^{2+} to possibly substitute for Fe^{2+} in proteins and decreases the potential for such

proteins to become damaged via Fenton reactions. During oxidative challenge it would therefore be detrimental to minimize *mntH* expression.

Previously, the *Brucella* Mur was predicted to regulate *mntH* and *irr* in *Brucella melitensis* 16 M (Rodionov et al., 2006). Irr is the primary iron-responsive regulator in the α -proteobacteria (Martinez et al., 2006) and in contrast to what is observed in *B. japonicum* where Mur binds the Irr promoter (Hohle and O'Brian, 2009) Mur in *B. abortus* 2308 does not bind to the *irr* promoter (Figure 1). However, upon closer review of the predictions by Rodionov et al. (2006), we realized that the gene this laboratory had cited as *irr1* (BMEI 1563) actually shares 99% gene identity to another *fur*-like gene, *perR* (BAB1_0393) in *B. abortus* 2308.

Brucella encodes PerR which is a H₂O₂ sensing paralog of the Fur superfamily. After discerning the nucleotides that comprise the Mur box in *Brucella*, it was discovered that a Mur-like motif which shares 94% nucleotide identity to the Mur box upstream of *mntH* was present upstream of *perR* (Figure 2). PerR (peroxide regulon repressor) was first identified in *B. subtilis* by the Helmann laboratory and is a repressor that utilizes a metal cofactor (Mn²⁺ and Fe²⁺) that undergoes a catalyzed oxidation reaction in the presence of H₂O₂ that allows for de-repression of peroxide stress genes (Mongkolsuk and Helmann, 2002). Interestingly, the Helmann laboratory has shown that Mn²⁺-PerR is inefficient at responding to H₂O₂ (Fuangthong et al., 2002). This finding, that PerR complexed with Mn²⁺ is an inefficient peroxide sensor is not surprising because the Mn²⁺ is electronically stable and will not undergo redox reactions with oxygen species in similar fashion to Fe²⁺. In the wild-type *Brucella* strain 2308, Mur represses MntH expression in a Mn²⁺-replete environment and data from our laboratory shows that Mur binds the *perR* promoter (Figure 3). It is now possible to suggest that Mur represses *perR* and this would limit the amount of peroxide repressor present in the cell (Figure 4a).

In this scenario, the peroxide regulon can be maximally expressed. However, in the *mur* mutant, dysregulation of *mntH* would allow for excessive accumulation of Mn^{2+} in the cell as well as increased PerR expression. This increase in intracellular Mn^{2+} would then serve PerR as a cofactor which would enable DNA binding and for this protein to serve as a repressor (Figure 4b); however, Mn^{2+} -PerR is inefficient in sensing peroxide stress and would continue to repress the peroxide regulon even in the presence of oxidative stress. This regulatory cascade may then explain the increased sensitivity to oxidative stress observed in the *mur* mutant previously in *B. abortus* 2308 (B. H. Bellaire dissertation, 2001). Therefore, the proposed regulatory cascade involving Mur regulating PerR supports an important biological role for Mur in metal ion homeostasis and peroxide defense. Mur mediated repression of *perR* would limit *Brucella* from producing excess PerR in Mn^{2+} replete conditions. Limiting the expression of *perR* in this environment would allow PerR to complex with Fe^{2+} which undergoes metal catalyzed oxidation (MCO) required for the derepression of PerR-regulated genes.

It is now evident that Mur regulation of *mntH* in *Brucella* is important. Mn^{2+} is required for survival of *Brucella* in the macrophage and for protection against oxidative stress, however, beyond its requirement in these instances, Mn^{2+} has the potential to interfere with important regulatory schemes involved in iron homeostasis and peroxide defense. Therefore, the data presented in this thesis support the proposition that deletion of *mur* or dysregulation of either MntH and/or PerR by mutation of the Mur boxes present upstream of these genes would result in the attenuation of *B. abortus*. The reduced virulence in these scenarios would be the result of excessive Mn^{2+} uptake via MntH which could act as a competitive inhibitor for other required transition elements thereby disrupting normal biological function; also the influx of Mn^{2+} can complex with PerR and render this metalloregulator an inefficient peroxide sensor. PerR- Mn^{2+} is

1000× less sensitive than PerR-Fe²⁺ in sensing peroxide stress (Lee and Helmann, 2006). Mn²⁺ could also complex with Irr as observed in *B. japonicum* (Puri et al., 2010) and for these very reasons proper regulation of MntH by Mur is necessary for normal growth of *Brucella* and for virulence.

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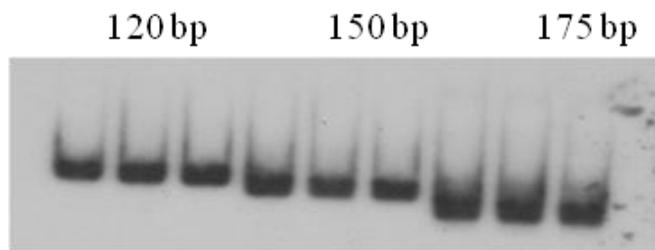
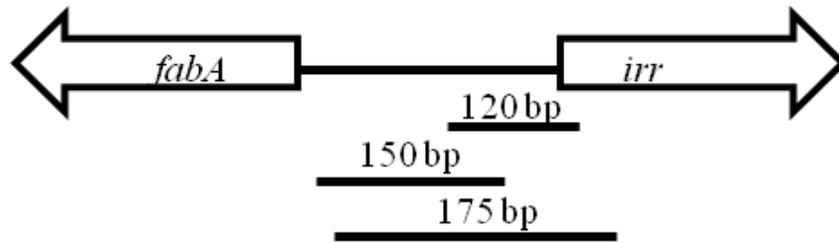
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0 0.5 2 0 0.5 2 0 0.5 2

μM $\text{Mur}_{B. abortus}$ + 100 μM MnCl_2

Figure 4.1 EMSA analysis with the *irr* promoter from *B. abortus* 2308 with Mur. Different size fragments encompassing the *irr* promoter were generated by PCR and radiolabeled with [γ - 32 P] ATP and incubated with increasing concentrations of the *B. abortus* Mur.

<i>B. abortus</i> MRS upstream <i>mntH</i>	(1)	-----TTAATGCAAAT-ATTGCAACTGCA-----
<i>B. abortus</i> MRS upstream <i>perR</i>	(1)	-----ATTGCAAATGGTTGCAATTGCA-----
Consensus	(1)	TAATTGCAAAT ATTGCAATTGCA

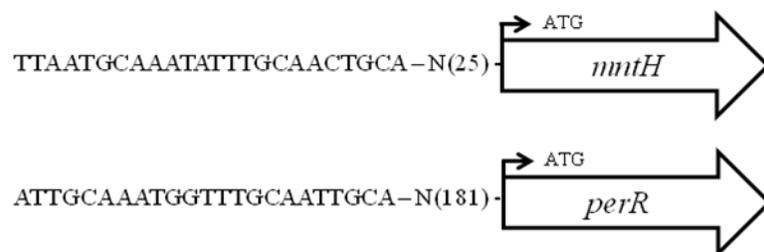
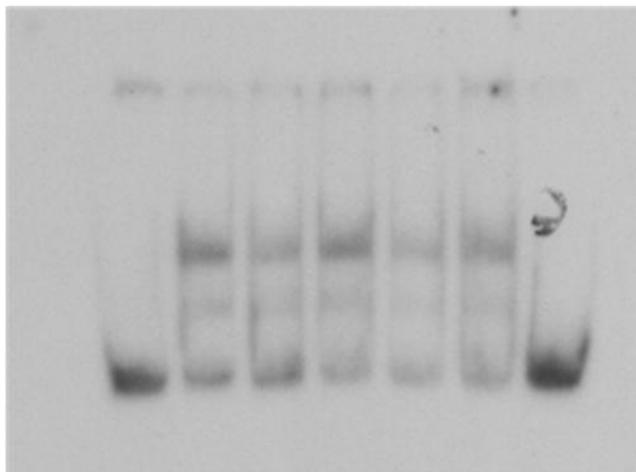


Figure 4.2 Mur boxes identified in *Brucella* Alignment of the Mur boxes found upstream *mntH* and *perR* in *B. abortus* 2308.

Protein
(μM)

0 0.3 0.6 1.2 1.2 1.2 1.2



50 \times specific
competitor

- - - - + - -

50 \times non-
specific
competitor

- - - - - + -

EDTA

- - - - - - +

Figure 4.3 Mur binds the *perR* promoter. EMSA analysis with the *Brucella* Mur and the upstream region of *perR* where a Mur-like motif is present. DNA fragments were generated by PCR and radiolabeled with [γ - 32 P] ATP and incubated with increasing concentrations [0, 0.3, 0.6, and 1.2 μ g] of the *B. abortus* Mur.

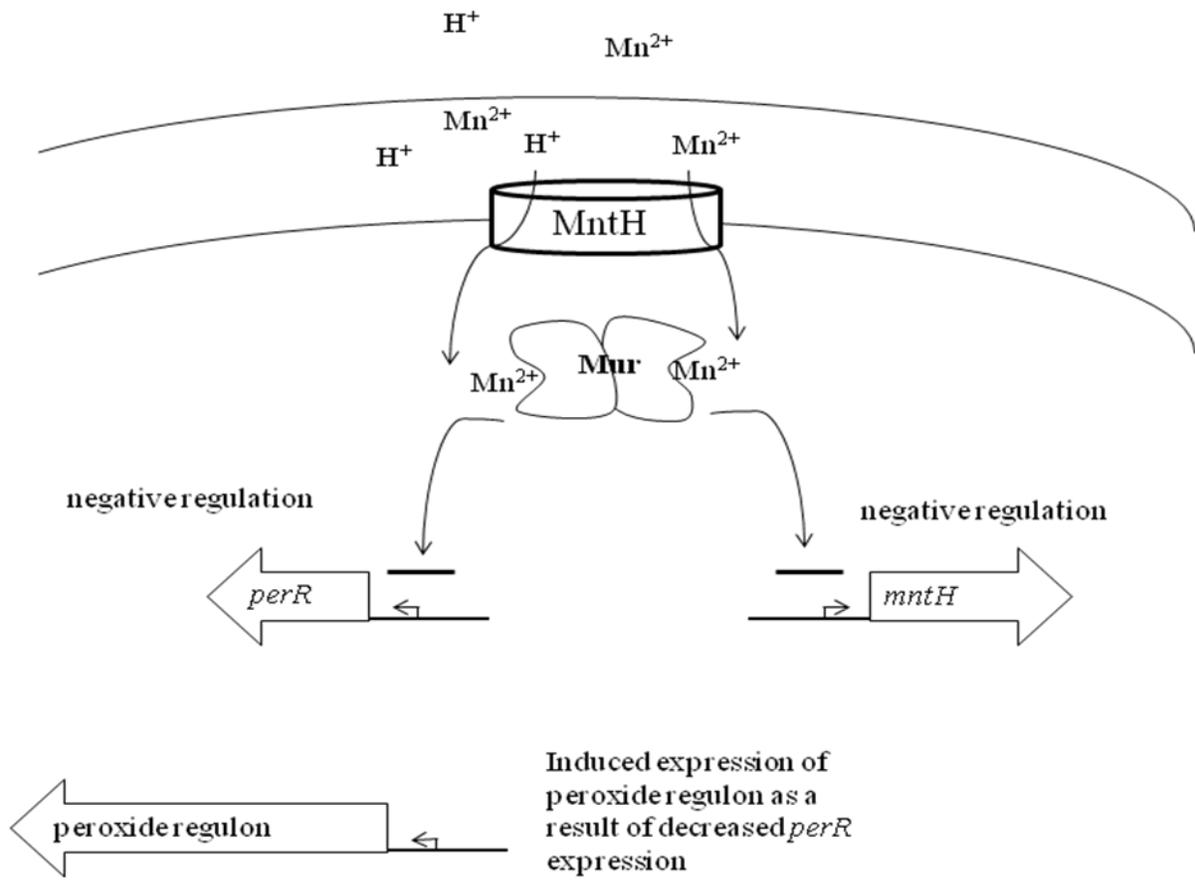


Figure 4.4a Mur regulation in *B. abortus* 2308. Model depicting the significance of Mur regulation in *Brucella*. Mur represses *mntH* and *perR* in Mn^{2+} - replete conditions and limits the accumulation of this metal and PerR in the cell.

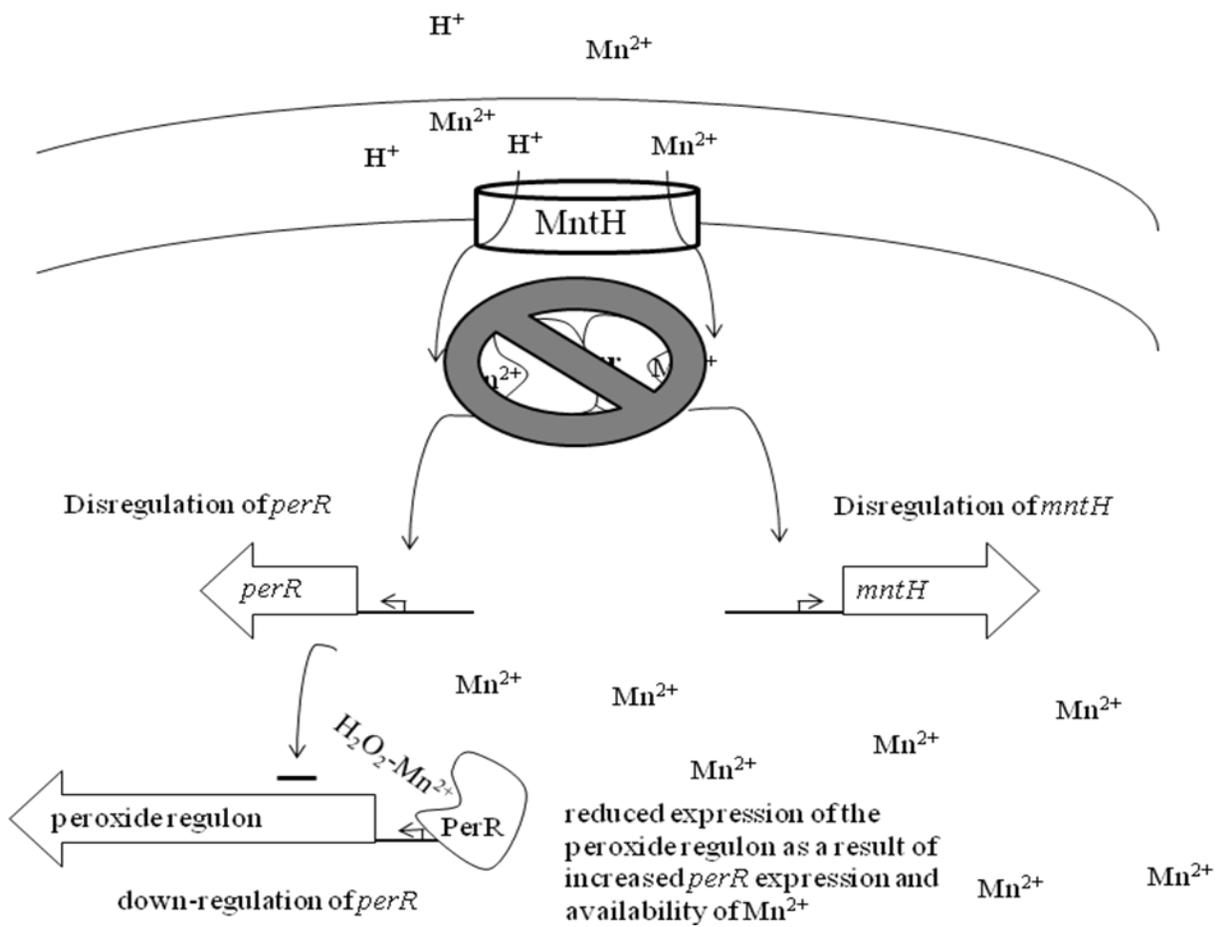


Figure 4.4b Mur regulation is necessary for normal peroxide defense in *B. abortus* 2308.

Model depicting the significance of Mur regulation in *Brucella*. In *Brucella* the absence of Mur causes the dysregulation of *mntH* and *perR*. Due to the inability to repress *mntH* expression, an excess of Mn^{2+} will be present in the cytoplasmic space where Mn^{2+} cofactors PerR. PerR complexed with Mn^{2+} represses genes of the peroxide regulon and is incapable of efficiently sensing peroxide stress and continues to repress the peroxide regulon in the presence of peroxide stress.