

The CDF-type metal efflux protein EmfA is essential for *Brucella abortus* 2308 resistance to manganese toxicity and is a critical virulence determinant in experimentally-infected mice.

By

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April, 2019

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Divalent cationic metals are important trace nutrients for all bacteria because metals serve as cofactors for proteins involved in numerous cellular functions. Despite the fact that metals play an essential role in bacterial physiology, metals become harmful to bacteria if accumulated in excess. To prevent metal-induced toxicity, bacteria employ a variety of factors, such as metal-responsive transcriptional regulators and metal efflux proteins, that function to maintain proper intracellular metal homeostasis. The *Brucella* strains are Gram-negative, pathogenic bacteria that cause the severe febrile zoonotic disease, brucellosis, and these bacteria require the metal manganese for growth and during host infection. While the manganese acquisition protein, MntH, is essential for the capacity of *Brucella spp.* to maintain sufficient manganese nutrition, the mechanisms by which these bacteria prevent manganese toxicity have not been determined. The studies of this thesis were performed to identify how *Brucella spp.* cope with manganese toxicity and whether these factors are essential for *Brucella* virulence.



The CDF-type metal efflux protein EmfA is essential for *Brucella abortus* 2308 resistance to manganese toxicity and is a critical virulence determinant in experimentally-infected mice.

A Thesis

Presented to the Faculty of the Department of Biology  
East Carolina University

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Master of Science in Molecular Biology and Biotechnology

By:

Matthew J. Johnsrude

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## Dedication

This work is dedicated to my grandmother, Wanda Johnsrude. Her constant support, encouragement, and belief in my abilities is something I will always draw inspiration from.

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## Chapter 1: Introduction

The *Brucella spp.* are Gram-negative, pathogenic bacteria that cause the zoonotic disease brucellosis. Currently, ten distinct *Brucella* species have been characterized and are named based on their native hosts, which consists of both wild and domestic mammals. Of these species, *B. abortus*, *B. melitensis*, and *B. suis* are predominantly involved in human brucellosis, mainly through contact with infected animals, animal products, or laboratory exposure. Human exposure to the *Brucella spp.* results in a debilitating, febrile illness that is only treatable through extensive antibiotic therapy. There is no safe, effective vaccine available for human brucellosis at the time of this document and the disease remains endemic in several regions around the world despite the fact that effective eradication and preventative measures are available. Until further characterization of the physiology and pathogenesis of the brucellae yields viable drug targets against these highly-infectious microorganisms, these strategies remain the best option for preventing outbreaks of human and animal brucellosis.

### History of the Genus *Brucella*

Brucellosis, the febrile and debilitating disease caused by the *Brucella spp.*, is an important world-wide zoonosis that is thought to have originated before written historical accounts. Efforts to characterize the paleoepidemiology of brucellosis have uncovered a 2.3-2.5 million-year-old skeleton exhibiting patterns of the disease; however, discerning one infectious disease from another using skeletal evidence is imprecise and has placed limitations on these approaches<sup>1</sup>. Further archaeological findings suggest brucellosis first reached endemic levels in Europe during the Roman era and sustained

its prevalence throughout the Middle Ages<sup>1</sup>. The first official documentation of brucellosis was written by J. A. Marston, an assistant surgeon with the British Royal Army, who described an unknown illness in his 1861 Army Medical Department report<sup>2</sup>. At the time, Marston was stationed at a naval port on the Mediterranean archipelago nation of Malta that functioned as a nearby field hospital for the ensuing Crimean War<sup>3</sup>.

Roughly a decade later, the British army physician, Captain David Bruce, confirmed Marston's work by effectively differentiating this uncharacterized disease, or 'Malta Fever', from other enteric fevers. With the aid of Mary Elizabeth Steele, Bruce's wife and previous technician of the renowned microbiologist Robert Koch, Bruce examined the spleen of an infected soldier in which he observed large quantities of a single coccus<sup>3</sup>. Bruce then experimentally infected a primate with the bacterium, which confirmed that this coccus was the causative agent of Malta Fever<sup>4</sup>. In 1893, Bruce officially designated the newly-discovered microorganism as *Micrococcus melitensis*<sup>3</sup>.

By this time, hundreds of servicemen were displaying symptoms of Malta Fever each year. After testing many possible leads, Themistocles Zammit, a physician and native of Malta, hypothesized that infected goats were contributing to the transmission of Malta Fever and successfully isolated the bacterium from these animals' blood and milk<sup>5</sup>. Dr. Zammit's recognition of animal vectors and their role in the transmission of brucellosis was a major milestone in the characterization of Malta Fever.

Meanwhile, the Danish veterinarian, Bernhard Bang, was investigating a seemingly unrelated phenomenon associated with his country's livestock. Of unknown cause, entire populations of cattle spontaneously aborted premature calves in the late stages of pregnancy. In 1897, Bang's investigation culminated with the identification of

an abnormal substance between a pregnant female cow's uterus and fetal envelope<sup>6</sup>. Microscopic analysis of the unusual matter enabled Bang to observe small bacilli located both intracellularly and extracellularly throughout the substance<sup>6</sup>. Bang isolated the bacillus, which he deemed *Bacillus abortus*, and experimentally determined its Gram-negative and nonmotile nature<sup>6</sup>.

In the late 1910s, the work of Bruce and Bang collided. Alice C. Evans, a U.S. microbiologist whose work focused on bacterial contamination of milk, proved that *Micrococcus melitensis* of Malta Fever and *Bacillus abortus* of Bang's Disease are indistinguishable<sup>7</sup>. Crediting Captain Bruce as the first to discover the bacterium, Evans consolidated the microorganisms under the genus *Brucella*<sup>7</sup>.

The highly infectious nature of the *Brucella spp.* led to military interest in weaponizing these bacteria during the 20<sup>th</sup> century. The appeal of the *Brucella* strains for biological warfare stemmed from both the high efficacy at which these organisms infect humans via the aerosol route and the low mortality rate (<5%) of individuals diagnosed with brucellosis<sup>8</sup>. Several major national programs for offensive biological warfare were attracted by these 'ideal' parameters and began developing strategies to use *Brucella suis* as an agent to cause a prolonged, incapacitating disease requiring a significant portion of uninfected troops to provide medical care<sup>8</sup>. In 1952, *B. suis* was the first biological agent weaponized by the U.S. military<sup>9</sup>. Ultimately, President Nixon discontinued the American offensive biological warfare program in the early 1970s.

Certain *Brucella spp.* (e.g. *B. melitensis*, *B. suis*, and *B. abortus*) continue to pose a potential bioterrorism risk and remain non-Tier 1 Select Agents according to the Center for Disease Control and Prevention (CDC). In a hypothetical bioterrorism model,



an aerosol cloud of *B. melitensis* would cause 82,500 cases and \$477.7 million in economic damage per 100,000 individuals exposed<sup>10</sup>. Efforts to characterize the physiology of the *Brucella* strains are currently underway to mitigate both the economic and public health impact of this pathogenic species.

### The worldwide impact of brucellosis

Several natural hosts of the *Brucella spp.* are important food animals of agricultural industries throughout the world. Animal brucellosis, especially caused by *B. melitensis*, remains a significant global economic concern due to the capacity of these bacteria to cause abortion and infertility in their natural hosts<sup>11-13</sup>. At the time of this study, animal brucellosis is prevalent in countries within the Mediterranean, Latin America, Africa, Middle East, and Asia, where survey data of bovine brucellosis indicate that upwards of 39% of cattle are currently infected with *Brucella abortus* in these regions<sup>11,13</sup>. An estimated 25% reduction in milk production by dairy cow carriers of *B. abortus* and the inability to use meat from infected food animals both contribute to the projected \$600 million annual economic losses that result from animal brucellosis<sup>14</sup>.

Many countries, including the U.S. and most of Europe, have successfully reduced incidence rates for animal brucellosis through the implementation of eradication programs. Specifically, these measures include the identification, quarantine, and depopulation of brucellosis-affected herds, alerting the public of potentially infected animal products, consistent surveillance and vaccination of livestock, and proper safety guidelines and equipment for those in contact with natural hosts of the *Brucella* strains<sup>15</sup>. As shown in Figure 1.1, human brucellosis remains endemic in several regions

around the world and a majority of the approximately 500,000 annual human brucellosis cases are documented in countries where animal brucellosis is uncontrolled<sup>16</sup>.

Accordingly, human brucellosis persists as the world's most common zoonosis.

Direct contact with infected animals is the most common mode of transmission for human and animal brucellosis. Exposure to infected placental tissue or decaying animal carcass are also well-documented sources of infection within the agricultural setting<sup>16</sup>. Outside of animal husbandry, *Brucella* infection is an occupational hazard in clinical and laboratory settings that provide treatment for the disease and conduct biomedical research on these bacteria, respectively. Many countries implement strict safety guidelines within these workplaces and restrict research to Biosafety Level 3 containment to mitigate these risks of exposure<sup>17</sup>. The public is also at risk of infection through the consumption of raw dairy goods or undercooked animal tissue<sup>16</sup>. Given the low infectious dose (10-100 cells) of the *Brucella* strains, regularly testing of and pasteurizing animal products are essential for preventing major brucellosis outbreaks<sup>15</sup>.

Individuals diagnosed with brucellosis invariably experience severe febrile symptoms. Other constitutional symptoms and the enlargement of lymph nodes, spleen, and liver are typically observed in patients with this disease as well<sup>16</sup>. Osteoarticular, reproductive, and central nervous system complications, among others, are common in patients diagnosed with brucellosis, which exemplifies the fact that this disease can affect nearly every organ system of the body<sup>16</sup>. Treatment of brucellosis requires the use of multiple antibiotics for a minimum six-week period; however, the efficacy of this treatment option is inconsistent, and the absence of viable human vaccination for those at risk of infection contributes to the disease's prevalence worldwide<sup>17</sup>. Although vaccine

development is achievable, knowledge of the molecular pathogenesis of the *Brucella* strains is incomplete<sup>16</sup>. Until characterization of the molecular nature of the *Brucella* spp. yields potential candidates for drug targets, public awareness and preventative measures remain the best course of action for controlling brucellosis.

### Taxonomy of the genus *Brucella*

*Brucella* spp. are members of the  $\alpha$ -2 subgroup of the  $\alpha$  proteobacteria, a phylogenetic division of bacteria that is comprised of animal pathogens and plant pathogens and symbionts<sup>18</sup>. Bacteria that belong to the genus *Brucella* are Gram-negative, facultative intracellular parasites that rely on host organisms for their replication and transmission. Accordingly, *Brucella* spp. are not found in the open environment<sup>19</sup>. The ten recognized *Brucella* species are named by natural host preference, which includes *B. melitensis* (goats and sheep), *B. abortus* (cattle and bison), *B. suis* (swine), *B. ovis* (sheep), *B. canis* (dogs), *B. neotomae* (wood rats), *B. ceti* (cetaceans), *B. pinnipedialis* (pinnipeds), and *B. microti* (common vole)<sup>20</sup>. The *Brucella* strains are also categorized based on differences in their lipopolysaccharide composition (see below) which determines whether strains are virulent (smooth) or avirulent (rough) in humans<sup>21,22</sup>. Biomedical research on the *Brucella* strains focuses on *B. melitensis*, *B. suis*, and *B. abortus* given their importance in both food animal and human health<sup>23</sup>.

## Establishing host infection

Upon ingestion, the *Brucella* strains' initial point of contact with the host is the oral, gastrointestinal, or respiratory mucosa<sup>24</sup>. Limited information regarding this process is currently available, however, studies have shown that the *Brucella* strains employ adhesins that mediate adhesion to epithelial cell surfaces and that these bacteria are taken up by endocytosis during their transepithelial migration<sup>24,25</sup>. Successful infiltration across both the mucosal and submucosal tissue layers results in the uptake of *Brucella spp.* by a variety of professional phagocytes, including dendritic cells, M cells, and neutrophils<sup>24</sup>. At the onset of infection, the brucellae are generally localized to the mononuclear phagocyte system found in lymphatic tissues<sup>12</sup>.

Ingestion by macrophages is an essential process for the pathogenesis of the *Brucella* strains as these bacteria naturally replicate within and require macrophages for their systematic dispersal in the host. Of the organ systems that these bacteria colonize during host infection, the reproductive system is particularly important in animal brucellosis because *Brucella* strains target placental trophoblasts of pregnant female animals and cause the abortion or premature birth of their offspring. These reproductive complications are important for the transmission of *Brucella spp.* to other hosts as the expulsion of infected birthing tissues or fluids puts other local animals at risk of infection<sup>12</sup>. In addition, viable offspring born to infected females are diseased upon birth<sup>12</sup>.

The pathogenesis of *Brucella spp.* deviates from most pathogens because these bacteria do not employ the 'classical' virulence factors, such as exotoxins, capsules, or lysogenic phages<sup>23,26</sup>. Alternatively, many virulence determinants of the *Brucella* strains

function to minimize host detection and ‘hijack’ host cell machinery during infection<sup>12</sup>. A major contributor to *Brucella* virulence is the unique composition of their lipopolysaccharide (LPS). *Brucella* LPS has reduced endotoxin activity compared to standard enterobacterial LPS<sup>27</sup>. Furthermore, the elongated lipid A moiety of the *Brucella* LPS functions as a shield against various antimicrobial attacks and recognition of pathogen-associated molecular patterns<sup>28</sup>.

The capability of *Brucella spp.* to evade detection during host invasion directly impacts their chances of intracellular survival within macrophages. Specifically, studies have shown that the *Brucella* LPS does not activate the alternative pathway of the complement system, which allows these bacteria to avoid host detection via complement-mediated opsonization<sup>29</sup>. Although it is not possible for all *Brucella* cells to elude host recognition in this manner, non-opsonized brucellae facilitate their cellular uptake by interacting with host cell receptors that are not associated with complement-mediated phagocytosis<sup>30</sup>. During their intracellular life cycle, these non-opsonized *Brucella* cells encounter considerably less phagolysosomal fusion, an antimicrobial function of macrophages that accounts for a large portion of the estimated 70-85% of brucellae that are eliminated by these host cells<sup>31,32</sup>.

### *Brucella* intracellular survival

Regardless of the precise mechanism by which these bacteria undergo phagocytosis, all intracellular brucellae reside within specialized phagosomal compartments, aptly referred to as the *Brucella*-containing vacuole (BCV), while inside macrophages. As shown in Figure 1.2, the intracellular trafficking of the BCV is divided

into three phases; the endosomal BCV (eBCV), the replicative BCV (rBCV), and the autophagy-associated BCV (aBCV)<sup>33</sup>. Briefly and chronologically, each phase coincides with this species' capacity to modulate vacuolar trafficking, undergo replication, and facilitate their cellular release. *Brucella spp.* employ numerous virulence factors that function to 'hijack' host cell machinery, disrupt innate immune responses, resist host antimicrobial defenses, and prevent host cell self-induced apoptosis during their BCV life cycle<sup>33</sup>.

The capacity to modulate BCV intracellular trafficking is essential for *Brucella spp.* during host colonization. To accomplish this task, the brucellae secrete effectors that directly interact with the host machinery involved in vacuolar trafficking. Cyclic  $\beta$ -1,2-glucans, for instance, interact with vacuolar membrane lipid rafts to inhibit prolonged lysosomal exposure of the BCV<sup>34</sup>. In addition, these bacteria encode a VirB type IV secretion system (T4SS) that releases *Brucella* secreted proteins (Bsp) which directly interact with host cell machinery to modulate BCV trafficking<sup>35</sup>. These factors, among others, allow *Brucella spp.* to escape the endolysosomal trafficking pathway and alter the intracellular trajectory of the BCV towards the endoplasmic reticulum<sup>36</sup>. Here, the brucellae convert the endosome-like BCV (eBCV) into an organelle that is permissive for their replication (rBCV) and, subsequently, one that mediates their release from the macrophage (aBCV)<sup>37,38</sup>.

Bacterial effectors that inhibit antigen-responsive immune processes are also important for these bacteria during their intracellular life cycle. The outer membrane protein (Omp) 25, for instance, inhibits host TNF- $\alpha$  production which results in a lessened stimulation of antigen-presentation and macrophage activation<sup>39</sup>. In addition,

the lipoprotein Omp19 functions as an inhibitor of CD64, a phagocytosis-inducing host cell receptor protein that is upregulated by antigen recognition<sup>40</sup>.

Despite these various strategies to disrupt host immune functions, *Brucella* strains encounter many host-mediated environmental stresses while inside macrophages. Accordingly, *Brucella spp.* have adapted the means to directly sense and cope with these conditions. The BvrS/BvrR two-component system, for example, functions as a crucial 'switch' that activates genes in response to acidic conditions<sup>41</sup>. Transcriptome analysis demonstrates that BvrR regulates approximately 127 genes, many of which represent critical virulence determinants for these bacteria, such as outer membrane proteins, stress response proteins, transport systems, chaperones, transcriptional regulators, and, notably, the VirB T4SS<sup>42</sup>. Other antimicrobial functions of macrophages expose phagocytized bacteria to harmful oxidative radicals and deprive intracellular pathogens of important metal nutrients (i.e. iron). To resist these host-mediated environmental stresses, the *Brucella* strains rely on enzymes that detoxify exogenously-derived O<sub>2</sub><sup>-</sup> and metal acquisition systems that compete for bioavailable metals within the phagosome<sup>43,44</sup>.

### Metals at the host-pathogen interface

During infection, hosts employ 'metal-withdrawal' defenses that sequester elemental metals, such as Fe and Zn, at host-pathogen interfaces to deprive pathogenic bacteria of trace nutrients that constitute basic physiologic needs for all microorganisms<sup>45-47</sup>. Referred to as nutritional immunity, these metal deprivation strategies are an important component of a host's innate immune response because

pathogens that are starved for metals cannot replicate or cause disease. Metal-chelating agents, such as lactoferrin and calprotectin, are secreted by host immune cells to limit the availability of extracellular Fe, Mn, and Zn<sup>45,46</sup>. Intracellular pathogens are also subjected to metal limitation during their intracellular life cycle through the activity of the vacuolar membrane-embedded cation metal transporter, Nramp1 (natural resistance-associated macrophage protein 1), which removes free metals from the phagosomal compartment<sup>47</sup>.

To combat host-mediated metal limitation, bacteria have adapted numerous strategies to maintain sufficient metal nutrition during host infection. The causative agent of Lyme disease *Borrelia burgdorferi*, for example, does not require Fe for its physiologic or pathogenic functions; therefore, negating host Fe deprivation strategies<sup>48</sup>. In addition, certain bacteria encode several metal-dependent proteins with identical function but each cofactors with a different transition metal<sup>49</sup>. This ensures that essential cellular processes are functional should one metal's intracellular levels be insufficient relative to others. Although these strategies alleviate the effects of host-mediated metal deprivation, no pathogenic bacterium, including the *Brucella* strains, can sustain growth or host colonization without a basal level of metal nutrition.

### Bacterial manganese acquisition strategies

To ensure physiologic metal requirements are met, bacteria employ high-affinity metal acquisition systems to import metals from their immediate environment. These systems are especially important for pathogenic bacteria because they must compete



against the host for bioavailable metals. Accordingly, genes encoding metal import systems typically represent critical virulence determinants for these bacteria<sup>50-52</sup>.

In addition to iron and zinc, host 'metal-withdrawal' defenses limit the bioavailability of the metal manganese (Mn) during host infection<sup>47</sup>. Bacteria employ Mn-specific divalent cation transporters to compete against the host for free Mn. At the time of this study, two principle types of bacterial Mn importers have been characterized. The ABC-type (ATP-binding cassette) Mn import system consists of a periplasmic metal-binding protein, a permease functioning to transport the divalent metal ion across the inner membrane, and a cytosolic ATPase to provide energy for this transport<sup>53</sup>. Other bacteria employ the MntH importer, a membrane-embedded Nramp1 family protein that facilitates Mn uptake via proton-coupled transport<sup>54</sup>.

Divalent Mn importers are ubiquitous among bacteria. *Lactobacillus plantarus*, for instance, has genes encoding five Mn acquisition systems in its genome; however, this bacterium appears to be an outlier as most bacteria are estimated to possess just 1-2 Mn importers<sup>55</sup>. *Brucella spp.* employ a MntH homolog that functions as the sole high-affinity Mn importer in these bacteria. Anderson et al. showed that the loss of a functional MntH decreases the capacity of *B. abortus* to grow under Mn-deplete conditions and that an isogenic *B. abortus mntH* mutant displays severe attenuation in experimentally-infected mice<sup>56</sup>. The fact that a Mn importer is essential for the virulence and physiology of the *Brucella* strains indicates that Mn is an important trace nutrient for these bacteria.

## The biological role of manganese

Divalent cationic metals, such as iron (Fe), magnesium (Mg), zinc (Zn), and copper (Cu), are essential trace nutrients for bacteria. The predominant biological role of metals is to serve as cofactors for enzymes involved in a variety of important cellular processes including DNA replication, transcription, metabolism, and energy generation<sup>57</sup>. Individual metal species also possess additional functionality that is unique to each metal. Coordination of free Mg to biological membranes and nucleic acids increases the stability and structural integrity of these macromolecules, whereas the capacity for Fe to exist in two oxidation states (Fe<sup>2+</sup> and Fe<sup>3+</sup>) allows this metal to be extremely versatile in biochemical reactions<sup>58,59</sup>.

The metal Mn is equally important for the nutrition of most bacteria. Many bacteria, including *Borrelia burgdorferi*, *Bradyrhizobium japonicum*, and the *Brucella* strains, rely on Mn as a micronutrient during all stages of growth, but certain bacteria appear to only require this metal situationally<sup>60-62</sup>. *Escherichia coli*, for instance, selectively expresses Mn acquisition systems during periods of oxidative stress<sup>63</sup>. Under these conditions, cytosolic Fe can participate in Fenton chemistry to generate harmful reactive oxygen species (ROS). By cofactoring Fe-dependent proteins with Mn in lieu of Fe, bacteria can mitigate the effects of intracellular Fenton chemistry while retaining enzymatic function<sup>63</sup>. Free divalent Mn can also form complexes with small metabolic anions, such as lactate and phosphate, that can reduce O<sub>2</sub><sup>-</sup> radicals in a protein-independent manner<sup>64</sup>.

Aside from these circumstantial functions of Mn, this metal primarily serves as a cofactor for Mn-dependent proteins, several of which have been characterized in

*Brucella*. The superoxide dismutase, SodA, is an anti-oxidant enzyme that detoxifies endogenous ROS generated by the electron transport chain and is well-conserved among bacterial species<sup>65</sup>. The pyruvate kinase PykM also requires Mn as a cofactor, which suggests Mn is important for carbon metabolism in bacteria that employ these proteins<sup>61</sup>. Notably, SodA and PykM both play critical roles in the physiology and virulence of *Brucella* strains<sup>66,67</sup>.

The metal Mn may also be integral for the 'stringent response' of the *Brucella* strains as the bacterium's sole stringent response regulator, Rsh, is a homolog of the Mn-utilizing *Staphylococcus* Rel stringent response regulator<sup>68</sup>. Should *Brucella* Rsh cofactor with Mn, these bacteria would require sufficient intracellular Mn levels to cope with amino acid deprivation and the inhibition of tRNA amino acylation. Other possible Mn-dependent processes of the *Brucella* strains include the supply of phosphatidylcholine to the cellular membrane and global transcriptional regulation by Cyclic-di-GMP. Enzymes linked to both processes are characterized in the *Brucella* strains and homologs of these proteins require Mn cofactors in other bacteria<sup>69-72</sup>.

## Manganese toxicity and homeostasis

A caveat to the nutritional benefit of metals is that metals are damaging to bacterial cells when accumulated in excess. Metal-induced toxicity is largely the result of surplus metal atoms outcompeting other metal species for their native protein binding sites, and improper metalation can lead to the inactivation of the affected proteins and their associated cellular processes<sup>73,74</sup>. Furthermore, certain metal species are harmful to cells at lower intracellular concentrations than others. The Irving-Williams Series

(Mg/Ca < Mn < Fe < Co < Ni < Cu > Zn), which represents the relative stability of transition metals in metal-ligand complexes, indicates that certain metals naturally form stronger metal-ligand complexes and, therefore, are more likely to displace metals that are lower on the scale from proteins<sup>75</sup>.

Although Mn is lower on the Irving-Williams Series relative to other transition metals, excess intracellular Mn is harmful to bacteria. Information regarding proteins that are specifically targeted by surplus Mn ions is scarce, however, studies examining the effects of Mn toxicity in *Bradyrhizobium japonicum* demonstrate that enzymes involved in carbon metabolism and heme biosynthesis exhibit reduced activity under these conditions<sup>76</sup>. In addition, studies have shown that excess intracellular Mn causes severe disruption in the cell cycle of *Streptococcus pneumoniae* and reduces the capacity of *Staphylococcus aureus* to resist oxidative stress<sup>77,78</sup>.

Computational-based approaches estimate that roughly one third of the bacterial proteome is comprised of metal-utilizing proteins which emphasizes the extent to which metal toxicity can cause harm to bacteria<sup>79</sup>. To resist metal toxicity, bacteria employ metal-responsive regulators, metal efflux proteins, metallochaperones, and metal storage proteins (Figure 1.3)<sup>80</sup>. Metal efflux proteins function to remove excess metals from the cell, whereas metallochaperones and metal storage proteins ensure the proper metalation of proteins and prevent uncontrolled metal reactivity, respectively<sup>81-83</sup>. Metal-responsive transcriptional regulators are centrally involved in the selective expression of factors that confer resistance to metal toxicity<sup>84</sup>. For many bacterial pathogens, the capacity to prevent metal toxicity is regarded as equally important as metal acquisition strategies for their virulence<sup>77,78</sup>.

## Manganese-responsive transcriptional regulators

Metal homeostasis systems enable bacteria to maintain sufficient metal nutrition and prevent metal toxicity, and these processes are generally orchestrated by metal-responsive proteins that regulate the transcription of genes encoding metal transporters and other metal-utilizing proteins<sup>84,85</sup>. The DNA-binding affinity of many 'metal-sensing' transcriptional regulators is reversibly activated by direct metal-binding<sup>86,87</sup>. When complexed with metals, these regulators selectively activate or repress (or both) gene transcription which allows bacteria to strategically respond to intracellular metal levels<sup>84,88</sup>. Although certain metal-responsive transcriptional regulators are functional in the unbound state, those that respond to Mn are generally active when complexed with this metal<sup>89,90</sup>.

Most, if not all, bacterial Mn-responsive transcriptional regulators belong to either the DtxR or Fur protein families. Originally characterized as a regulator of toxin production, DtxR (diphtheria toxin repressor) of *Corynebacterium diphtheriae* is the primary Fe-responsive transcriptional regulator of this bacterium and functions to repress Fe uptake genes in response to excess intracellular concentrations of this metal<sup>91</sup>. Certain bacteria, including *B. subtilis*, *E. coli*, and the *Salmonella spp.*, employ MntR, a Mn-responsive DtxR-type regulator, which functions as a repressor of Mn acquisition genes when this protein is complexed with Mn<sup>85,92,93</sup>. The DNA binding site of MntR, known as the MntR box, overlaps -10 promoter elements upstream of genes encoding both MntH-type and ABC-type Mn importers to inhibit transcription of these genes<sup>92</sup>. Studies have also shown that this Mn-responsive protein activates *B. subtilis* Mn efflux gene transcription when bound to Mn, which indicates that MntR coordinates

the selective expression of factors that maintain sufficient Mn nutrition and those that prevent Mn toxicity<sup>84</sup>. To date, there is no indication that a MntR homolog is encoded by the *Brucella* genome and MntR-type regulators appear to be poorly represented among close phylogenetic relatives of these bacteria. Accordingly, *mntR* genes have only been identified in two  $\alpha$  proteobacteria (*Rhodobacter capsulatus* and *Mesorhizobium loti*)<sup>94</sup>.

The second class of Mn-responsive transcriptional regulators that is well-conserved in bacteria belong to the Fur protein family. Similar to the regulatory mechanism of DtxR-type proteins, the DNA binding activity of Fur, or ferric uptake regulator, is dependent on direct interactions with Fe. Once bound to Fe, this regulatory protein represses the transcription of several Fe-utilizing genes, most notably, genes encoding Fe-acquisition systems<sup>88</sup>. Other Fur-like regulators, such as Nur and Zur, are specifically activated by other transition metals, however, the diversity of this protein family is best represented by members that directly interact with heme or H<sub>2</sub>O<sub>2</sub><sup>95-98</sup>. Bacteria, including the *Brucella* spp., that do not employ a MntR homolog typically encode the Mn-responsive Fur-type regulator Mur, or manganese uptake regulator. *Brucella* Mur is the only Mn-responsive transcriptional regulator that has been identified in this bacterium and functions as a repressor of *mntH* gene transcription when complexed with Mn, which is consistent with the function of Mur homologs in other bacteria<sup>99</sup>. The regulatory network of *Brucella* Mur, however, is not fully characterized and it remains unclear whether *Brucella* Mur plays an important role in this bacterium's resistance to Mn toxicity.

## Manganese efflux proteins

Although metal-responsive transcriptional regulators coordinate the downregulation of metal acquisition systems in response to elevated intracellular metal concentrations, these regulatory proteins are unable to protect a bacterial cell from a sudden exposure to biocidal concentrations of metals. Furthermore, the repression of metal uptake gene transcription is unable to accommodate for sudden decreases in the optimal intracellular concentration of a metal when the current intracellular metal concentration has surpassed this optimal concentration. To prevent metal toxicity under these conditions, bacteria employ metal efflux proteins that facilitate the removal of excess metals from the cell. Over the past decade, studies examining bacterial resistance to metal toxicity have shown that metal efflux proteins play an equally important role as metal-responsive transcriptional regulators in this process. In addition, genes encoding metal efflux proteins are often critical virulence determinants for pathogenic bacteria<sup>77,100</sup>.

Three classes of Mn efflux proteins have been characterized in bacteria. *Neisseria* MntX, *Xanthomonas* YebN, and *Escherichia* MntP are Mn-specific exporters belonging to the LysE protein family of transporters and are important for these species' capacity to grow under conditions of increased exogenous Mn<sup>101-103</sup>. Structurally, each monomer of a LysE-type Mn exporter is characterized by two functional transmembrane domains that are comprised of three membrane-spanning helices. A second type of Mn exporter is MneA, which appears to be phylogenetically related to a class of eukaryotic calcium efflux pumps embedded in the Golgi apparatus; however, this Mn efflux protein

has only been identified in *Vibrio cholerae*<sup>104</sup>. Potential homologs of *V. cholerae* MneA and their frequency in other bacterial species are currently unknown.

Metal exporters belonging to the cation diffusion facilitator (CDF) protein family are ubiquitous among prokaryotic and eukaryotic cells. Members of this superfamily of metal efflux proteins are further categorized based on their metal-specificity and these various substrate-defined subclasses indicate that the efflux activity of CDF-type exporters can be specific for any and/or several of the biologically-relevant metals<sup>105</sup>. *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Deinococcus radiodurans* all employ the CDF-type Mn efflux protein, MntE, which plays an essential role in these species' tolerance of excess environmental Mn<sup>106,107</sup>. Recently, Cubillas et. al. identified another CDF-type Mn efflux protein<sup>108</sup>. Despite apparent homology to the *S. pneumoniae* MntE, the *Rhizobium etli* EmfA (efflux of Mn and Fe) was shown to be a CDF-type metal efflux protein with both Mn and Fe specificity, and this study indicates that EmfA and MntE belong to different subgroups within the CDF protein family. At the time of this study, only one other EmfA ortholog has been characterized, but the efflux activity of *Sinorhizobium meliloti* SmYiiP appears to be Mn-specific<sup>109</sup>.



## Statement of the Problem

Many bacteria require Mn-responsive transcriptional regulators and Mn efflux proteins to maintain proper Mn homeostasis during conditions of excess environmental Mn. While the *Brucella* strains do encode the Mn-responsive transcriptional regulator Mur, preliminary studies suggested that this 'Mn-sensing' protein may not play a role in this bacterium's resistance to Mn toxicity. To identify other factors that contribute to this process, a search of the publicly available *B. abortus* 2308 genome sequence was performed, which resulted in the identification of a possible gene predicted to encode a homolog of the *Rhizobium etli* EmfA. The studies that form the basis for this thesis were performed to address three hypotheses: a) that the *Brucella* EmfA ortholog protects these bacteria from Mn toxicity, b) that this protein is an important virulence determinant, and c) that the corresponding *Brucella* gene is regulated in a Mn-dependent manner.

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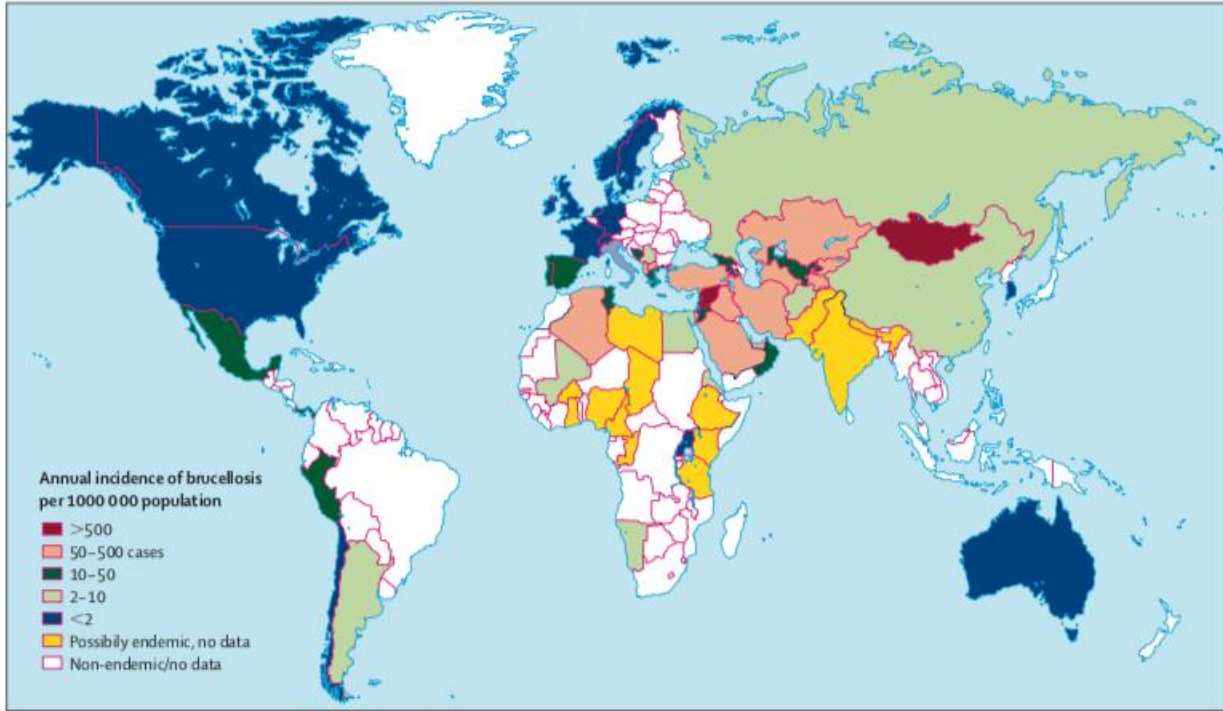
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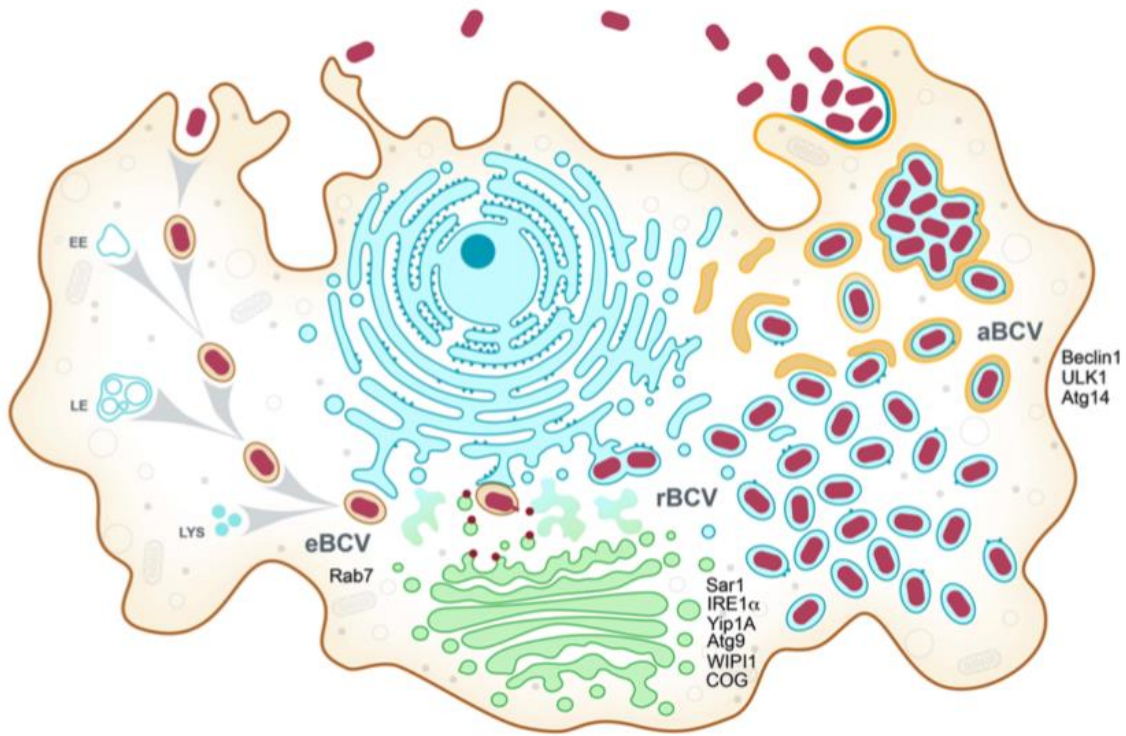
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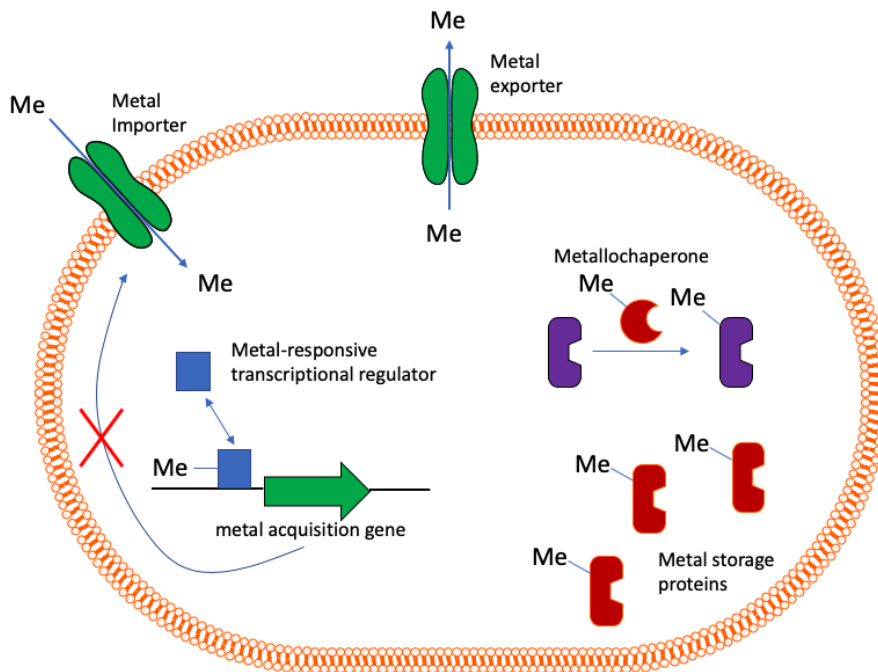
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**Figure 1.1:** The worldwide incidence of brucellosis as presented by Pappas et al. (2006)<sup>11</sup>. Human brucellosis is currently endemic in regions of the Mediterranean, Middle East, Asia, Africa, and South America. Typically, those regions with the highest annual incidence of human brucellosis are areas where animal brucellosis remains uncontrolled.



**Figure 1.2:** The intracellular life cycle of the *Brucella*-containing vacuole (BCV) as presented by Celli et al. (2019)<sup>33</sup>. The BCV life cycle is divided into three stages (endosomal BCV, replicative BCV, and autophagy-associated BCV) that coincide with the *Brucella* strains capacity to modulate vacuolar trafficking machinery, undergo intracellular replication, and facilitate their egress from the macrophage.



**Figure 1.3:** Illustration of bacterial proteins involved in metal homeostasis. Bacteria employ a variety of factors to maintain proper metal homeostasis. Metal transporters facilitate the import or efflux of metal nutrients and the selective expression of these metal transport genes are typically coordinated by a metal-responsive transcriptional regulator. Metallochaperones and metal storage proteins ensure that proteins are properly metallated and prevent free metals from outcompeting other metal species for their native protein binding sites, respectively.

## Chapter II: The cation diffusion facilitator family protein EmfA confers resistance to manganese toxicity in *Brucella abortus* 2308 and is an essential virulence determinant in mice.

The gene designated BAB\_RS23470 in the *Brucella abortus* 2308 genome is predicted to encode an ortholog of the cation diffusion facilitator family protein EmfA which has been linked to resistance to Mn toxicity in *Rhizobium etli*. A *B. abortus emfA* null mutant derived from strain 2308 displays increased sensitivity to elevated levels of Mn in the growth medium compared to the parent strain, but wild-type resistance to Fe, Mg, Zn, Cu, Co and Ni. Inductively-coupled plasma mass spectroscopy also indicates that the *B. abortus emfA* mutant retains significantly higher levels of cellular Mn after exposure to this metal than the parent strain, which is consistent with the proposed role of EmfA as a Mn exporter. The *B. abortus emfA* mutant exhibits significant attenuation compared to strain 2308 in both C57BL/6 and C57BL/6<sup>NRAMP+/+</sup> mice, and notably, this attenuation is more pronounced in mice with a functional Nramp1 protein. Comparative phenotypic analysis of mutants also suggests that EmfA plays a much more important role in maintaining Mn homeostasis and preventing the toxicity of this metal in *Brucella* than does the Mn-responsive transcriptional regulator Mur.

### Introduction

Metals are essential trace nutrients for all bacteria. By and large the most widespread use of metals is to support the structure and catalytic function of cellular proteins. Numerous important physiological processes including DNA synthesis,

metabolic pathways, and energy generation, rely on enzymes with metal co-factors to function properly<sup>1</sup>. To accommodate the portion of their proteome that requires metal ions, bacteria employ high affinity metal acquisition systems to import bioavailable metals from their immediate environment. Obtaining sufficient intracellular levels of certain metals such as iron (Fe), manganese (Mn) and zinc (Zn) is especially difficult for bacteria that are mammalian pathogens because these bacteria must overcome the so-called 'metal withdrawal defenses' of their hosts, which actively limit the availability of metal nutrients<sup>2-5</sup>. Accordingly, high affinity Fe, Mn and Zn acquisition systems typically represent essential virulence determinants for bacterial pathogens<sup>6-9</sup>.

Despite their role as critical micronutrients, metals become toxic if accumulated in excess of biological needs. Metal-induced toxicity is largely the result of surplus cytosolic metal atoms outcompeting other metal species for their native protein binding sites<sup>10</sup>. Improper protein metalation can lead to the loss of the affected proteins' function, effectively disrupting the many cellular functions carried out by metal-dependent proteins<sup>11,12</sup>. Computational studies estimate that roughly one third of bacterial proteins require metal co-factors, which further emphasizes the extent to which metal toxicity causes harm<sup>13</sup>. Other mechanisms of metal toxicity are specific to individual metal species. Excess accumulation of Fe, for example, results in the generation of harmful oxidative radicals by means of intracellular Fenton chemistry<sup>14</sup>.

Bacteria actively regulate intracellular metal homeostasis by means of high-affinity metal importers, metal-responsive transcriptional regulators, and metal efflux proteins. These systems operate in a concerted manner to both maintain sufficient metal nutrition and prevent excess accumulation of intracellular metals. Metal-

responsive transcriptional regulators, for instance, control the expression of metal acquisition systems to ensure these systems operate only under conditions of metal deprivation, and ensure that metal exporters are only functional under conditions of metal excess<sup>15</sup>. Furthermore, bacterial species encode metal-specific chaperones and storage proteins to facilitate proper protein metalation and protect against uncontrolled reactivity of intracellular metals, respectively<sup>16,17</sup>.

The *Brucella spp.* are Gram-negative bacteria that cause abortion and infertility in food animals<sup>18</sup>. Humans are also at risk of infection as brucellosis is the world's most prevalent zoonotic disease and this disease is endemic in areas of the world where brucellosis in food animals is not controlled by efficient eradication programs<sup>19</sup>. The metal manganese (Mn) is an essential micronutrient for *Brucella* strains<sup>20</sup>, presumably because several cellular proteins that are required for the basic physiology and virulence of these bacteria are Mn-dependent<sup>21,22</sup>. To resist Mn deprivation, the *Brucella* strains rely on a single high affinity Mn importer, MntH, but the mechanisms that these bacteria use to resist Mn toxicity are unknown<sup>23</sup>. Expression of the *mntH* gene is repressed by a prototypical Fur-like transcriptional repressor, Mur, in response to increasing intracellular Mn concentrations<sup>24</sup>. The role that Mur plays in preventing Mn toxicity in *Brucella*, however, has not been experimentally determined. The present study sought to understand how *Brucella abortus* 2308 copes with increasing intracellular Mn and determine the importance of preventing Mn toxicity during host infection.



## Results

A *B. abortus mur* mutant does not exhibit increased sensitivity to excess environmental Mn.

The *B. abortus mur* mutant EAM001 was grown on media supplemented with increasing levels of Mn to examine the role of Mur in this bacterium's resistance to Mn toxicity. As shown in Figure 2.1, the *B. abortus mur* mutant displays the same level of resistance to excess Mn in the growth medium as the parental 2308 strain. To further examine Mur's contribution to Mn homeostasis in *Brucella*, inductively coupled plasma mass spectrometry (ICP-MS) was employed to measure cellular Mn levels of *B. abortus* 2308 and the *mur* mutant during growth in a rich medium and after exposure to exogenous Mn in this medium. As shown in Figure 2.2, the parent strain and *mur* mutant exhibit similar cellular levels of Mn under both conditions. These data suggest that Mur plays a limited role in protecting *B. abortus* 2308 from Mn toxicity, or that additional factors compensate for the loss of a functional Mn-responsive transcriptional repressor in this strain.

An ortholog of the *Rhizobium* EmfA protein confers resistance to Mn toxicity in *Brucella*.

The gene designated BAB\_RS23470 in the *B. abortus* 2308 genome is predicted to encode a 302 aa protein that shares 64% identity and 76% similarity at the amino acid sequence level with the EmfA protein of *Rhizobium etli*<sup>25</sup>. EmfA is a clade IV member of the cation diffusion facilitator (CDF) protein family of metal exporters, and phenotypic analysis of a *R. etli emfA* mutant indicates that this protein plays an

important role in protecting this bacterium from Mn toxicity. To determine if the *Brucella* EmfA ortholog performs a similar function, *B. abortus* 2308 and the isogenic *emfA* mutant JEP061 were grown on Schaedler agar plates supplemented with increasing levels of Mn (Figure 2.3). The *B. abortus emfA* mutant exhibits growth restriction at considerably lower levels of exogenous Mn in this assay than the parental 2308 strain, and genetic complementation of the *emfA* mutant with a plasmid-borne copy of the *emfA* gene restores the resistance of this mutant to Mn to the same level as that of 2308. Notably, a *B. abortus emfA mur* double mutant displays the same level of sensitivity to Mn as the *emfA* mutant in this assay. These experimental findings indicate that EmfA plays a primary role in protecting *Brucella* strains from Mn toxicity, while the role of Mur in this process is currently unclear.

The designation 'Emf' stands for efflux of Mn and Fe, and EmfA homologs have been proposed to be able to transport both Mn and Fe out of bacterial cells<sup>25</sup>. To examine the possibility that EmfA might be able to protect *Brucella* strains from toxic levels of metals other than Mn, *B. abortus* 2308 and the *emfA* mutant were grown on agar plates supplemented with increasing levels of Fe, Mg, Zn, Ni, Co and Cu. As shown in Figure 4, both strains displayed equivalent levels of resistance to these metals. This suggests that EmfA plays a specific role in protecting *Brucella* from Mn toxicity.

The phenotype of the *B. abortus emfA* mutant (e.g. increased sensitivity to Mn) is consistent with the proposed function of the corresponding gene product as a CDF-type Mn exporter. To evaluate this proposed function more directly, we used ICP-MS to measure cellular Mn levels in *B. abortus* 2308, the *emfA* mutant and complemented

mutant during routine cultivation in brucella broth (a rich medium), and following a brief exposure to exogenous Mn in this growth medium (Figure 2.5). *B. abortus* 2308 and the *emfA* mutant display equivalent levels of cellular Mn after growth to mid-log phase in brucella broth, but interestingly, the levels of Mn detected in the complemented *emfA* mutant MJJ012 are significantly lower under these conditions than those found in either the parent strain or mutant. This is presumably the result of the increased number of copies of *emfA* in the complemented mutant. Specifically, pMR10 was used for this complementation, and this plasmid is maintained at 2-4 copies per genome in *Brucella* strains<sup>26</sup>. In contrast, the *B. abortus emfA* mutant exhibits significantly higher levels of cellular Mn following exposure to this metal in brucella broth than does either the parent strain or complemented mutant. Both of these experimental findings support the proposed function of EmfA as a Mn exporter in *Brucella*.

EmfA is required for the wild-type virulence of *B. abortus* 2308 in mice.

Several bacterial CDF-type Mn exporters have been linked to virulence<sup>27,28</sup>. To assess the importance of EmfA for the virulence of *B. abortus* 2308, the spleen colonization profiles of this strain, the *emfA* mutant and complemented mutant were examined in C57BL/6 and C57BL/6<sup>NRAMP1+/+</sup> mice. The latter mice were included in this study because the divalent cation transporter Nramp1 has been shown to be important for restricting Mn availability to bacterial pathogens during their residence in host macrophages, and long-term intracellular persistence in macrophage is a key determinant of *Brucella* virulence<sup>29,30</sup>. Remarkably, the *B. abortus emfA* mutant only

displays statistically significant attenuation in mice that possess a functional Nramp1 (Figure 2.6).

## Discussion

Many bacteria require both Mn-responsive transcriptional regulators and Mn efflux proteins to maintain proper Mn homeostasis and prevent Mn toxicity<sup>31-33</sup>. With no evidence suggesting that a functional Mur is required for the resistance of *B. abortus* 2308 Mn toxicity, the possibility that this bacterium employs additional factors to prevent the over accumulation of this metal was examined. The studies reported here demonstrate that the previously uncharacterized *B. abortus* 2308 BAB\_RS23470 gene encodes a functional homolog of the *Rhizobium* Mn exporter EmfA, and that this protein is essential for wild-type resistance of excess environmental Mn.

To our knowledge, only two other EmfA orthologs (*R. etli* EmfA and *S. meliloti* SmYiiP) have been characterized at the time of this study and, in all cases, the loss of a functional EmfA (or SmYiiP) caused these bacteria to display increased sensitivity to Mn toxicity<sup>25,34</sup>. Furthermore, the data presented here are consistent with previous studies suggesting that these proteins are Mn exporters. As mentioned previously, *R. etli* EmfA is reportedly an efflux protein with Mn and Fe specificity, although, it remains unclear whether *B. abortus* EmfA functions as an exporter of both metals. Of note, *in vitro* analysis of *S. meliloti* SmYiiP metal transport demonstrated that this exporter's Mn transport rate increased in the presence of a H<sup>+</sup> diffusion gradient, whereas the transport rates for other metals, including Fe, remained unchanged<sup>34</sup>. While no experimental evidence linking *B. abortus* EmfA to Fe efflux was obtained during this study, further evaluation is required to determine this protein's substrate specificity.

The data presented here indicate that *B. abortus* EmfA (along with the other  $\alpha$  proteobacterial EmfA orthologs) functions similarly to other CDF-type Mn exporters<sup>27,28</sup>. Although computational-based approaches clearly suggest that *B. abortus* EmfA is a CDF family protein, several structural features that confer Mn-specificity to *S. pneumoniae* MntE, a well-characterized CDF-type Mn exporter, are noticeably absent from the *B. abortus* EmfA amino acid sequence. This further supports the conclusion put forth by Cubillas et al.<sup>25</sup> that EmfA and MntE belong to different substrate-defined clades within the CDF protein family<sup>35</sup>.

Mammalian hosts employ 'metal-withdrawal' defenses that are essential for these organisms to combat pathogenic bacteria during infection<sup>5,29,36</sup>. Accordingly, factors that sequester free Mn play an important role in the host immune response because they deprive these bacteria of bioavailable Mn from both extracellular and intracellular environments<sup>8,29</sup>. For the *Brucella* strains, ingestion by host macrophages is an essential component to their basic biology and pathogenesis as these bacteria naturally replicate within and require macrophages to sustain host infection<sup>30</sup>. The severe attenuation of a *B. abortus* *mntH* mutant in mice and cultivated macrophages suggests that this bacterium requires sufficient Mn nutrition throughout the course of an infection<sup>23</sup>. The evidence presented in this study indicates that Mn efflux is equally important to *B. abortus* virulence as Mn acquisition, which is consistent with the fact that Mn efflux proteins are critical virulence determinants for other pathogenic bacteria.

The pronounced attenuation demonstrated by the *B. abortus* *emfA* mutant in mice that encode a functional copy of Nramp1 relative to those that do not is of considerable interest because this finding appears to be in conflict with the proposed

role that Nramp1 plays as a host defense against other intracellular pathogens. Specifically, studies have shown that Nramp1-mediated efflux of Mn and other transition metals from the phagosomal compartments of macrophages limits the availability of essential metal nutrients at the host-pathogen interface<sup>29</sup>. Significant attenuation of a *Salmonella* Typhimurium *mntH sitABCD* double mutant (which lacks both high-affinity Mn acquisition systems), for example, is exclusively seen in mice that encode a functional Nramp1<sup>37</sup>. This suggests that this bacterium requires high affinity Mn importers to specifically compete against the activity of Nramp1. While previous studies examining the role of Nramp1 during *Brucella* infections have shown that this divalent cation transporter plays a limited role in protecting experimentally-infected mice, the experimental evidence presented here suggests that *Brucella* spp. require a Mn exporter in an environment where Nramp1 presumably limits availability of this metal.

One possible explanation for this paradoxical finding is that Nramp1 may further limit the availability of Fe relative to that of Mn within the phagosome. Should these conditions arise, the reduced availability of Fe would likely cause the bacterium's intracellular Fe concentration to decrease more so than its intracellular Mn levels. The intracellular concentrations of these two metals are co-dependent in other bacteria<sup>28,38</sup>; therefore, it is logical to consider that the Mn efflux activity of *B. abortus* EmfA would enable these bacteria to reduce intracellular Mn levels in order to maintain a proper balance in this bacterium's Mn:Fe ratio. This proposal, in part, is supported by the fact that a *Neisseria meningitidis* *mntX* mutant becomes more sensitive to exogenous Mn at lower levels of environmental Fe<sup>39</sup>. Further studies are required to investigate the

specific role of *Brucella* EmfA during host colonization and whether this bacterium's intracellular Fe and Mn levels are co-dependent.

The transcription of genes encoding Mn efflux proteins is regulated by Mn-responsive transcriptional regulators in many bacteria<sup>28,40</sup>. The Mn-responsive transcriptional network of these bacteria typically relies on a single 'Mn-sensing' transcriptional regulator that inversely regulates genes involved in Mn uptake and efflux<sup>33,40</sup>. This enables these bacteria to strategically activate or repress metal-utilizing genes in response to Mn availability. Preliminary studies suggest that the *B. abortus emfA* gene is not regulated by this bacterium's sole Mn-responsive transcriptional regulator, Mur, nor is *B. abortus emfA* gene transcription responsive to increases in exogenous Mn. At the time of this study, no mode of regulation has been identified for *B. abortus emfA* which suggests that this gene is either expressed constitutively or regulated in a non-canonical fashion.

The lack of a Mn-sensitive phenotype in the *B. abortus mur* mutant is inconsistent with previous studies examining Mn-responsive transcriptional regulators employed by other bacteria<sup>31,41,42</sup>. Based on the results obtained from this study, it appears that the *Brucella* Mur does not play a major role in resisting Mn toxicity. While Mur does function to repress Mn acquisition in response to sufficient levels of the metal in *B. abortus* 2308, the data presented here suggests that a functional Mur is not essential to the basic physiology of this bacterium<sup>24</sup>. With no indication that *B. abortus* 2308 requires a Mn-responsive transcriptional regulator under conditions of excess exogenous Mn, it is possible that *Brucella* Mur functions primarily as a mechanism of energy conservation

## Materials and Methods

### Bacterial strains and culture conditions

Unless otherwise noted, *Brucella abortus* 2308 and derivatives of this strain were cultivated on Schaedler agar supplemented with 5% defibrinated bovine blood (SBA) at 37°C with 5% CO<sub>2</sub>, or in brucella broth at 37°C with shaking at 250 rpm. *Brucella* stock cultures were maintained in brucella broth supplemented with 25% glycerol and stored at -80°C.

For all growth conditions on a solid medium with supplemented manganese, Schaedler agar (without defibrinated bovine blood) was prepared with additions of an MnCl<sub>2</sub> stock solution prior to solidification. Anhydrous MnCl<sub>2</sub> was solubilized into H<sub>2</sub>O and subsequently filter sterilized prior to its addition to Schaedler agar. All growth conditions on a solid medium with supplemented iron, magnesium, zinc, nickel, cobalt, or copper were prepared in a similar manner. Schaedler agar was stored at room temperature until use to minimize condensation.

For all growth conditions requiring a liquid culture supplemented with manganese, 50 mL of brucella broth was added to a 500 mL flask and supplemented with MnCl<sub>2</sub> as described above. Bacterial cultures were incubated at 37°C with shaking at 250 rpm.

### Construction of the *B. abortus mur*, *emfA*, and *mur emfA* mutants

The *B. abortus emfA* null mutant was constructed from *B. abortus* 2308 using the non-polar unmarked gene excision strategy described by Caswell et al<sup>43</sup>. Approximately 1000 bp upstream of the *emfA* gene (BAB\_RS23470) to the second codon was



incorporated into the pNTPS138 plasmid along with a fragment containing the last two codons of this gene's coding region to approximately 1000 bp downstream of *emfA* gene. Fusion of the two incorporated fragments at the first two and last two codons of the *emfA* coding region was performed to generate an in-frame deletion of the gene. The plasmid was electroporated into *B. abortus* 2308 and deletion of the *emfA* coding region was confirmed by screening using PCR. The resultant mutant strain with the mutated *emfA* locus was designated JEP61. All mutants employed by this study were constructed in an identical manner and are listed in Table 2.1. The nucleotide primers used for construction of the pNTPS-based plasmid is shown in Table 2.2.

#### Genetic complementation of the *B. abortus emfA* mutant

A copy of the parental *emfA* allele was reintroduced into the *emfA* mutant background in order to confirm the link between the *emfA* mutation and phenotype exhibited by the *B. abortus emfA* mutant strain. The *B. abortus* 2308 *emfA* gene was cloned into the pMR10 plasmid using the procedures described by Caswell et al.<sup>43</sup> to generate the pMJ12 plasmid which was subsequently electroporated into the *B. abortus emfA* mutant. The nucleotide primers used to construct the pMR10-based plasmid, pMJ12, are shown in Table 2.2. The *B. abortus* JEP61 derivative with the plasmid-borne copy of *emfA* was designated MJJ012 (Table 2.1).

#### Growth of *B. abortus* strains in metal-supplemented media

*Brucella abortus* 2308, JEP61(2308  $\Delta emfA$ ), MJJ012 (JEP61 [pMJ12], EAM001(2308  $\Delta mur$ ), and JEP62 (2308  $\Delta emfA \Delta mur$ ) were cultivated on SBA for 48 hours. Cells were harvested into phosphate-buffered saline (PBS) and the cell

suspensions adjusted to an OD<sub>600</sub> of 0.15 (10<sup>9</sup> CFU/mL). These cell suspensions were diluted to a final cell density of 10<sup>4</sup> CFU/mL and 25 µL of the bacterial suspensions were placed on Schaedler agar plates containing 0, 10, 50, 100, 500, or 1000 µM MnCl<sub>2</sub> (final concentration). After an incubation period of 72 h at 37°C with 5% CO<sub>2</sub>, the relative growth of each strain was examined.

The same experimental procedures were used to evaluate the capacity of *B. abortus* 2308 and the *emfA* mutant JEP61 to grow on Schaedler agar plates supplemented with 10, 100, 500, or 1000 µM FeCl<sub>3</sub>, ZnSO<sub>4</sub>, NiCl<sub>2</sub>, MgCl<sub>2</sub>, CoCl<sub>2</sub>, or CuCl<sub>2</sub> (final concentration). These experimental procedures were also used to evaluate the capacity of *B. abortus* 2308 and the *mur* mutant EAM001 to grow on Schaedler agar plates supplemented with 0, 1, 5, 10, 15, or 20 mM MnCl<sub>2</sub> (final concentration).

Inductively coupled plasma mass spectrometry for measurement of total cell-associated manganese

Inductively coupled plasma mass spectrometry (ICP-MS) was used to determine the total amount of cell-associated Mn of the *B. abortus* strains. Cells from liquid cultures of *B. abortus* 2308, EAM001, JEP61, and MJJ012 were harvested into fresh brucella broth and adjusted to an optical density of 0.15 (10<sup>9</sup> CFU/mL). The adjusted suspensions were used to inoculate 50 mL of brucella broth in 500 mL flasks and were grown to mid-log phase at 37°C with shaking at 250 rpm. A baseline for total cell-associated Mn was taken by harvesting 5 mL of each culture by centrifugation, washing the resultant pellet with 10 mL sterilized H<sub>2</sub>O, and resuspending it in 1 mL NO. The NO-hydrolyzed cell preparations were stored at -80°C.

Cultures of each strain were then supplemented with 50  $\mu\text{M}$   $\text{MnCl}_2$ . ICP-MS sample preparation as described above was performed after 2 h of incubation (37°C with shaking at 250 rpm) with increased Mn. Prior to removal from the BSL-3 containment, all samples were boiled for 30 minutes and kill tests were conducted to confirm that no live organism was removed from the facility. A Bradford assay was performed on all cultures from both timepoints to determine total cellular protein in order to compare the amount of cell-associated Mn per ng total bacterial protein.

#### Experimental infection of mice

The virulence of the *B. abortus* strains in C57BL/6 and C57BL/6<sup>NRAMP+/+</sup> mice was evaluated using the methods described by Gee et al<sup>26</sup>. Briefly, mice were infected with  $5 \times 10^4$  brucellae via the intraperitoneal route, and at two- and five-weeks post infection the number of brucellae present in the spleens of these mice was determined. The animal use protocol (K167a) under which these experiments were performed was reviewed and approved by the East Carolina University Animal Care and Use Committee.

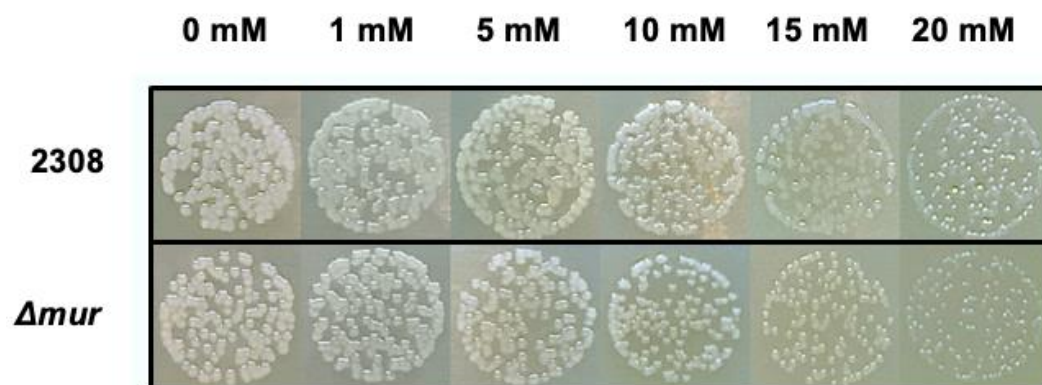
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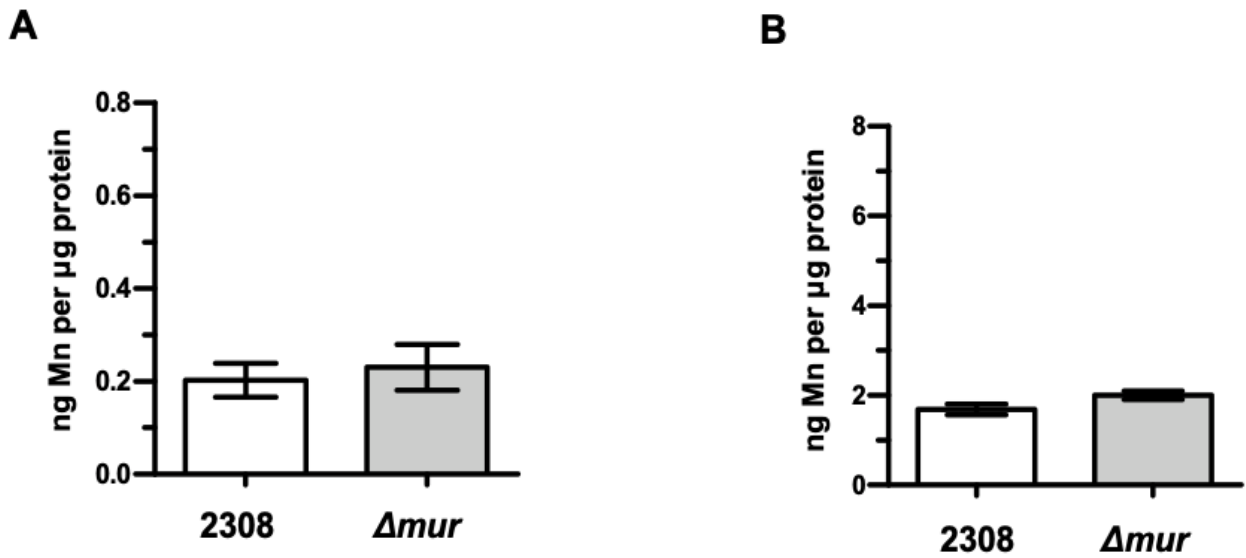
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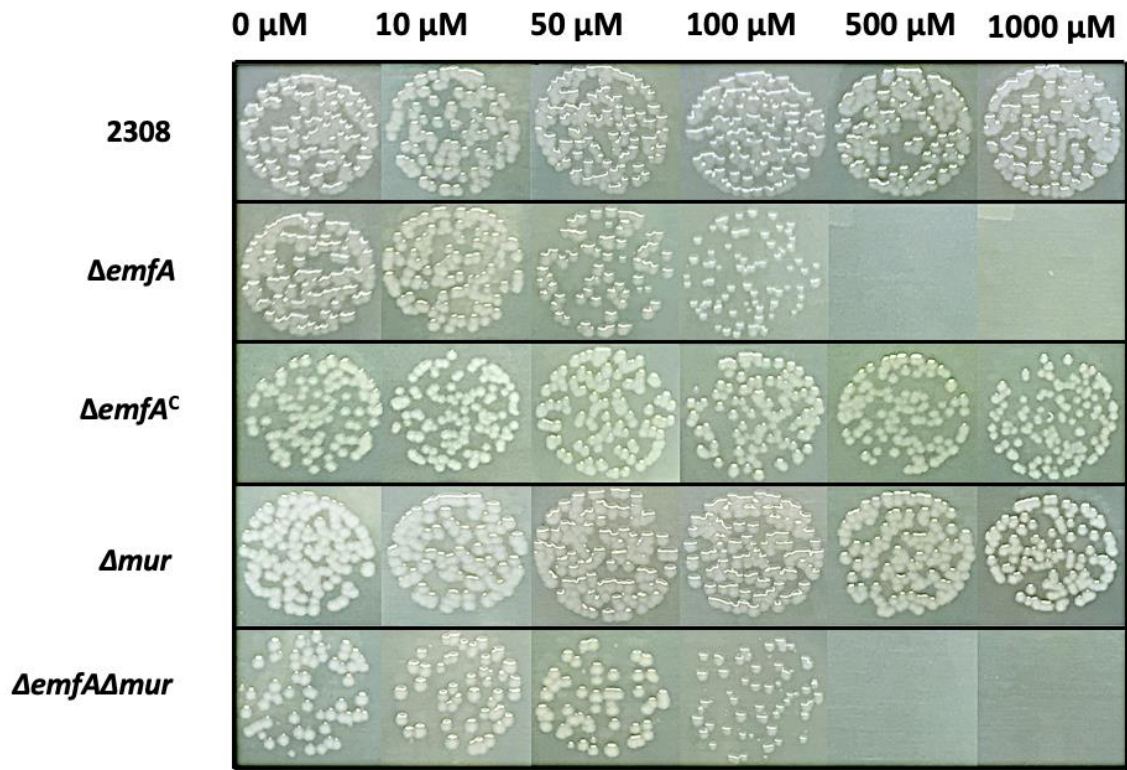


**Figure 2.1:** *B. abortus* 2308 does not require Mur for growth in the presence of excess exogenous Mn. Twenty-five  $\mu\text{L}$  suspensions of *B. abortus* 2308 and the isogenic *mur* mutant EAM001 were placed on Schaedler agar plates supplemented with  $\text{MnCl}_2$  and growth was observed following 72 h of incubation at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$ .

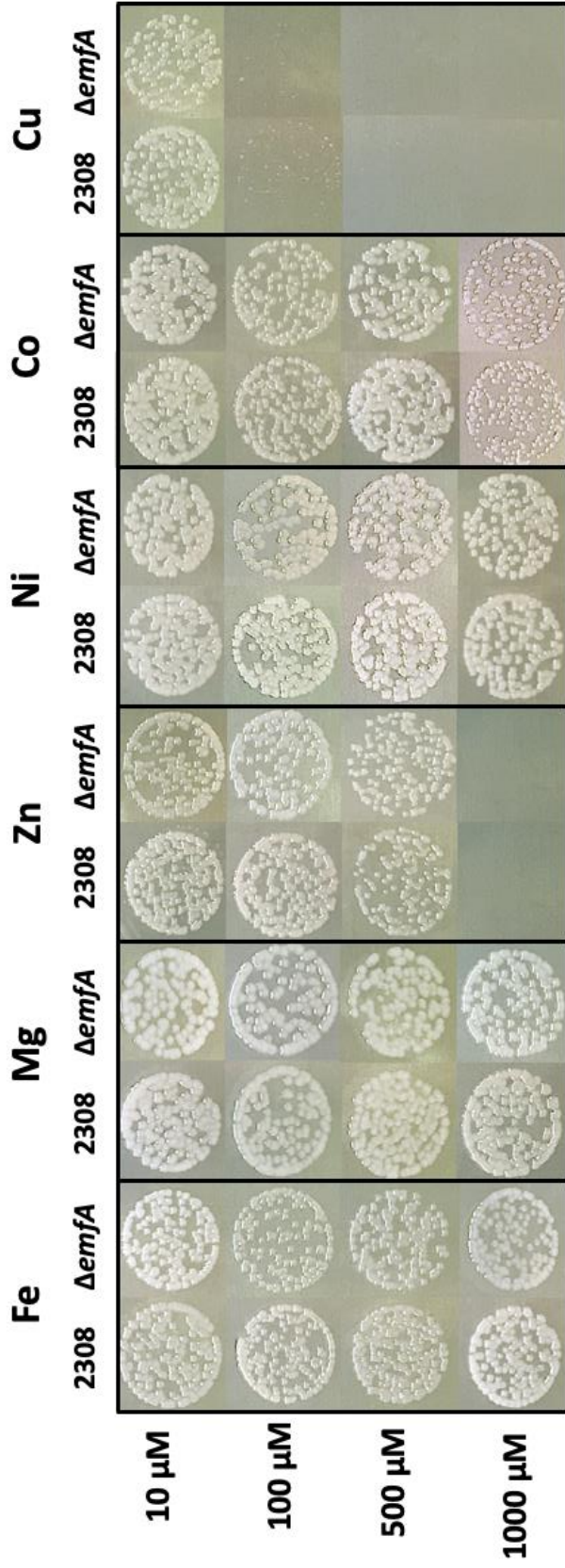




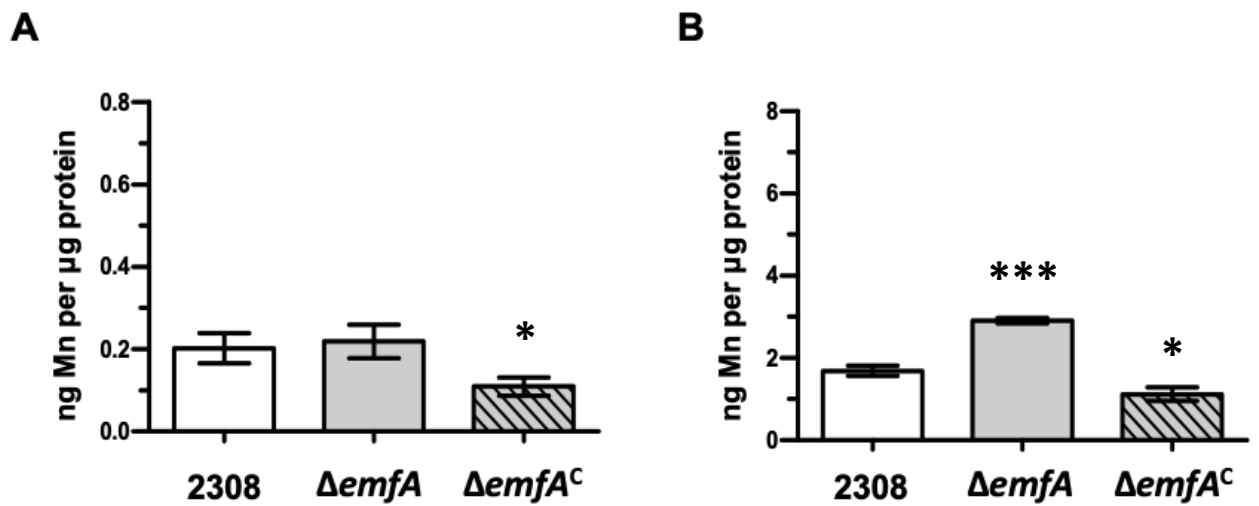
**Figure 2.2:** The *B. abortus mur* mutant contains similar cellular Mn levels as the wild-type. The cellular Mn levels of *B. abortus* 2308 and the *mur* mutant EAM001 were measured following growth to mid-log phase in Brucella broth (A) and subsequent 2 h exposure to 50 μM MnCl<sub>2</sub> in this medium (B). Cellular Mn content was determined by ICP-MS and cellular protein levels were determined using the Bradford assay.



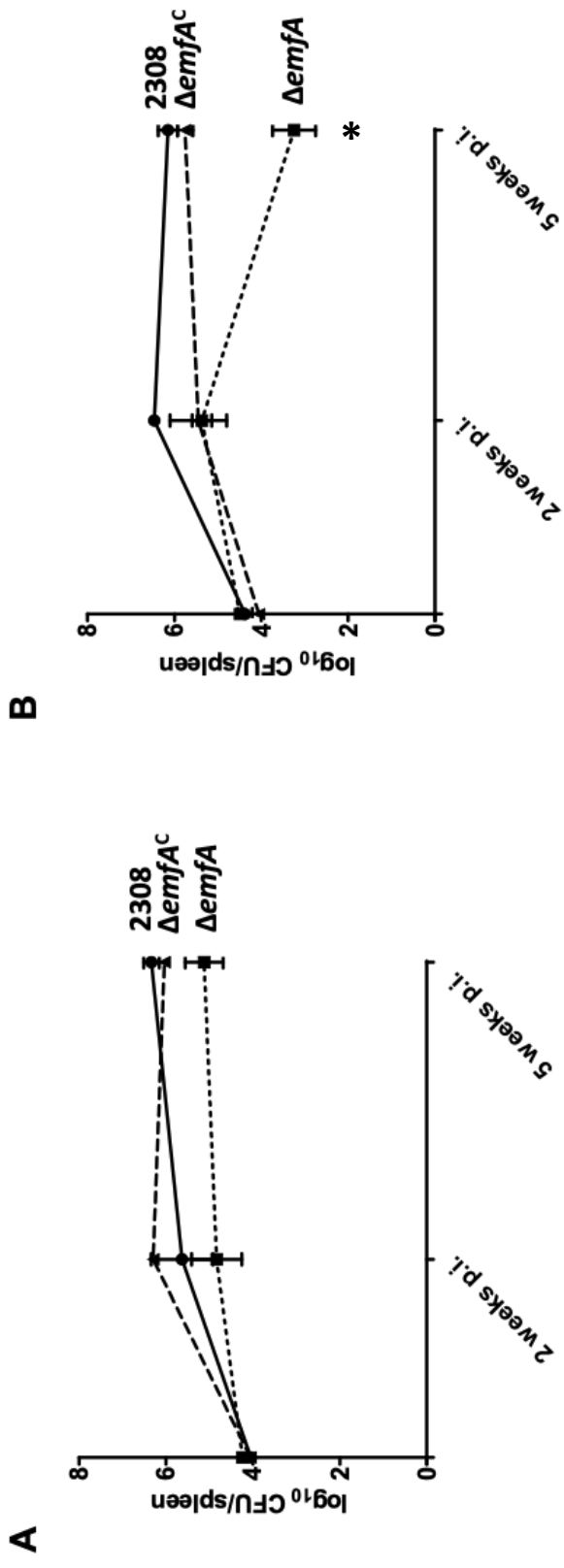
**Figure 2.3:** EmfA protects *B. abortus* 2308 from Mn toxicity. Twenty-five  $\mu\text{L}$  suspensions of *B. abortus* 2308 and derivative strains were placed on Schaedler agar plates supplemented with  $\text{MnCl}_2$  and growth was observed following 72 h of incubation at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$ .



**Figure 2.4:** EmfA does not protect *B. abortus* 2308 from Fe, Mg, Zn, Ni, Co, or Cu toxicity. Twenty-five  $\mu$ L suspensions of *B. abortus* 2308 and the isogenic *emfA* mutant JEP61 were placed on Schaedler agar plates supplemented with FeCl<sub>3</sub>, MgCl<sub>2</sub>, ZnSO<sub>4</sub>, NiCl<sub>2</sub>, CoCl<sub>2</sub>, or CuCl<sub>2</sub> and growth was observed following 72 h of incubation at 37°C with 5% CO<sub>2</sub>.



**Figure 2.5:** The *B. abortus emfA* mutant has increased cellular Mn content following Mn challenge compared to *B. abortus* 2308. Cellular Mn levels were measured in *B. abortus* 2308, the *emfA* mutant JEP61, and the complemented *emfA* mutant MJJ012 ( $\Delta emfA^C$ ) following growth the mid-log phase in brucella broth (A) and following a subsequent 2 h exposure to 50  $\mu\text{M}$   $\text{MnCl}_2$  in this medium (B). ICP-MS was used to measure cellular Mn levels and the Bradford assay used to determine cellular protein levels. Significance - \* $p < 0.05$  and \*\*\* $p < 0.005$  for comparisons of 2308 vs the *emfA* mutant or complemented *emfA* mutant using the Student's t-test.



**Figure 2.6:** The *B. abortus emfA* mutant displays attenuation in mice. Spleen colonization profiles of *Brucella abortus* 2308, the *emfA* mutant, and the complemented *emfA* mutant ( $\Delta emfA^C$ ) in C57BL/6 (A) and C57BL/6<sup>NRAMP<sup>+/+</sup></sup> (B) mice. Mice were infected with  $5 \times 10^4$  brucellae via the intraperitoneal route and evaluated at two weeks and five weeks post infection. Significance - \* $p < 0.05$  for comparisons vs 2308 using the Student's t-test.

**Table 2.1** Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference or source
<i>Brucella abortus</i>		
2308	Virulent challenge strain	43
EAM001	2308 $\Delta mur$	This study
JEP61	2308 $\Delta emfA$	This study
MJJ012	JEP61 with a plasmid-borne <i>emfA</i>	This study
JEP62	2308 $\Delta mur \Delta emfA$	This study
Plasmids		
pNPTS138	<i>sacB</i> -based gene replacement vector; Kanamycin resistance	44
pGEM-T Easy	Cloning vector; Ampicillin resistance	Promega
pMR10	Cloning vector; broad-host range; low copy number (2 to 4 copies per cell); kanamycin resistance	26
pMJ12	pMR10-based plasmid with functional copy of the <i>B. abortus</i> 2308 <i>emfA</i> gene; kanamycin resistance	This study

**Table 2.2** Oligonucleotide primers used in this study

<b>Primer</b>	<b>Sequence (5'--&gt;3')</b>
emfA up F BamH	GATGGA TCCGAAGGCAGGTTGACGATCAGCTTGTTG
emfA down R PstI	GATCTGCAGCCTTCGTCCATACGCTTGCCCAATTATTT
emfA up R	TTCGCCTGAGCCAAAATTACCATC
emfA down F	GTCCATAATGAACCTTTGGTTATCTTG
emfA comp EcoRI-R	GATGAATTTCTGGCTCAGGCCGAACGGAACC
emfA promoter PstI F	GATCTGCAGTAAC TTGAAGCCAGCCGCAGCCAAAACCTT
mur up F PstI	GATCTGCAGCATGGCCATCGGCAATCAGGGACTGAA
mur up R	GTTTCATGGATCGATTATGCACTGCATCGCCATCAGTTGC
mur down F	TCCTGACGGAAACCAATCCAGGAAGTTGGCGTTTTACGG
mur down R EcoRI	GATGAATTTCTCGAACTGCTGATCAATGACG

## Chapter III: Further examination of the Mn-responsive transcriptional network in *Brucella*.

*Brucella spp.* encode a functional Mur homolog that represses the gene encoding the Mn importer MntH when this transcriptional regulator binds Mn. In other bacteria, this regulatory process is critical for preventing Mn toxicity as bacterial mutants that lose the capacity to repress Mn acquisition gene transcription often exhibit growth restriction in the presence of excess exogenous Mn. This 'characteristic' phenotype of Mn-responsive regulatory mutants, however, is not displayed by a *B. abortus mur* mutant. Instead, the metal efflux protein EmfA appears to be the primary contributor to the resistance of these bacteria to Mn toxicity. The following studies were conducted to (A) examine the physiologic role of Mur in *Brucella* and (B) assess whether *emfA* expression is responsive to the stimuli that influence the selective expression of Mn efflux proteins in other bacteria.

### Introduction

Numerous bacteria employ Mn-dependent transcriptional regulators to strategically activate or repress metal transport genes in response to Mn bioavailability. Currently, two main types of Mn-responsive transcriptional regulators have been identified: MntR, a DtxR family protein; and Mur, a Fur family protein<sup>1-5</sup>. Each class of Mn-responsive regulators is broadly conserved and represented across bacteria, and both directly bind to, or 'sense', free Mn under conditions of sufficient (or excess) cellular concentrations of this metal<sup>6-11</sup>. While most, if not all, MntR- and Mur-type



regulators repress high affinity Mn import genes in response to adequate (or excess) cellular Mn levels, studies have also shown that certain Mn-responsive regulators activate Mn efflux genes under these conditions<sup>6,12</sup>. This suggests that Mn-responsive transcriptional regulators (that inversely regulate Mn import and efflux genes) are centrally involved in coordinating proper Mn homeostasis. Accordingly, many 'Mn-sensing' transcriptional regulators are essential for bacterial resistance to Mn toxicity<sup>3-5</sup>.

Mn is an essential micronutrient for the basic physiology and pathogenesis of the *Brucella* strains<sup>13,14</sup>. To acquire sufficient Mn and, presumably, to compete with host 'metal-withdrawal' defenses throughout the course of an infection, the *Brucella spp.* rely on the sole, high-affinity Mn importer MntH<sup>14</sup>. Menscher et al. demonstrated that these bacteria encode a Mur homolog that downregulates *mntH* expression in response to Mn-replete conditions<sup>11</sup>. Although this regulatory function is 'characteristic' of Mn-responsive transcriptional regulators, *B. abortus* Mur appears to play a minimal role in this bacterium's tolerance of excess environmental Mn (see Ch. II). This, in part, is demonstrated by the absence of an additive phenotype (compared to isogenic *emfA* mutant) displayed by a *B. abortus emfA mur* double mutant strain under conditions of increased environmental Mn and suggests the Mn exporter EmfA is the primary factor that provides this bacterium's resistance to Mn toxicity. Furthermore, the attenuation of isogenic *B. abortus emfA* and *mntH* mutants in mice indicates that maintaining proper Mn homeostasis is critical for these bacteria during host infection<sup>14</sup>. However, knowledge of the Mur regulon and regulation of *emfA* expression in *B. abortus* 2308 remains incomplete, which places limitations on our understanding of the physiologic importance of maintaining proper Mn homeostasis and the precise role that these

factors play in *Brucella spp.* virulence. This study sought to further characterize the Mn-responsive transcriptional network of *Brucella* and to identify environmental and transcriptional factors that influence the selective expression of the *emfA* gene.

## Results and Discussion

*emfA* expression is unresponsive to Mn in *B. abortus* 2308

As mentioned previously, certain bacteria encode a single Mn-responsive transcription factor that regulates the transcription of genes encoding both Mn uptake and Mn efflux proteins<sup>6,12</sup>. For these bacteria, Mn efflux proteins exhibit minimal expression under conditions of low Mn availability, whereas Mn efflux gene transcription is activated following exposure to increased exogenous Mn<sup>6,12</sup>. Due to the fact that the *B. abortus* metal efflux protein EmfA is essential for this bacterium's resistance of excess environmental Mn, it was expected that *emfA* transcription would increase in response to elevated levels of environmental Mn. To examine whether *emfA* is responsive to Mn, quantitative reverse transcriptase PCR (qRT-PCR) was employed to determine relative *emfA* expression in *B. abortus* 2308 before and after a 2-hour exposure to increased exogenous Mn. Figure 3.1 shows that this exposure does not increase *emfA* expression which suggests that this gene is unresponsive to the presence of excess environmental Mn.

While further studies are necessary to confirm that the *B. abortus emfA* gene is unresponsive to Mn, these data do indicate that *emfA* does not respond to a level of exogenous Mn that induces Mn efflux genes in other bacteria<sup>15</sup>. Of note, the *B. abortus mntH* gene was incorporated into this study as a control due to the fact that *mntH*

expression is downregulated in response to increased environmental Mn. These data also indicate that *emfA* expression is unresponsive to conditions that cause the repression of this bacterium's sole Mn acquisition gene which further suggests that EmfA production may not be dependent on intracellular Mn levels.

Mur does not function as a direct regulator of the *emfA* gene.

With no indication that *B. abortus emfA* expression is responsive to Mn under the tested conditions, the possibility that *emfA* is regulated by Mur was examined to assess whether this bacterium's only known Mn-responsive transcriptional regulator functions to regulate this Mn exporter. It was expected that Mur functions as a direct regulator of the *emfA* gene due to the fact that 'Mn-sensing' transcriptional regulators activate Mn efflux genes in other bacteria<sup>6,12</sup>. To assess this possible function of *B. abortus* Mur, qRT-PCR was conducted to measure *B. abortus emfA* gene transcription in a *mur* mutant background. As shown in Figure 3.1, the loss of Mur has no apparent effect on *emfA* expression in the presence or absence of Mn which suggests that Mur is uninvolved in *emfA* expression.

Computational analysis indicates that the *B. abortus emfA* promoter region does not contain a Mur recognition sequence (also known as the Mur box) which is consistent with gene expression studies demonstrating that *B. abortus emfA* gene transcription is unresponsive to the loss of a functional Mur (Figure 3.1)<sup>16</sup>. To dismiss the possibility that *B. abortus* Mur functions as a direct regulator of the *emfA* gene, electrophoretic mobility shift assay (EMSA) analysis was conducted to determine whether this protein displays specific binding affinity for the *B. abortus emfA* promoter region. This EMSA

confirmed that, under the conditions tested, Mur does not bind directly to the *emfA* promoter (Figure 3.2).

Menscher et al. demonstrated that *B. abortus* Mur binds upstream of the *mntH* coding region exclusively in the presence of Mn which is consistent with the regulatory mechanism of other members of the Fur protein family<sup>10,11,17</sup>. When complexed with a metal cofactor, Fur-like regulators undergo a conformational change that exposes the DNA binding motifs of these proteins<sup>18</sup>. As stated previously (see Ch. I), certain Fur family proteins can also bind chromosomal DNA in their unbound state<sup>19,20</sup>. In consideration of previous findings suggesting that *Brucella* Mur may function differently than other Mn-responsive regulators (see Ch. II), the possibility that Mur directly binds to the *emfA* promoter in the absence of Mn was also examined. EMSA analysis conducted in the presence of a metal chelator demonstrated that Mur does not bind to the *emfA* gene promoter under these conditions (data not shown). Collectively, these data indicate that *B. abortus* Mur does not bind upstream of the *emfA* coding region in its known, Mn-cofactored functional conformation nor does this protein bind the *emfA* promoter in the absence of Mn. Furthermore, these data may also indicate that *B. abortus* Mur is not required for this bacterium's resistance to Mn toxicity due to the fact that this protein does not induce *emfA* expression.

*emfA* transcription is unresponsive to oxidative stress.

Upon ingestion by macrophages, the brucellae reside within a specialized phagosomal compartment, known as the *Brucella*-containing vacuole (BCV), which is the natural replicative niche of these bacteria and is centrally involved in their capacity

to cause chronic infection<sup>21,22</sup>. Here, the *Brucella spp.* are exposed to reactive oxidative species (ROS) from the oxidative burst of macrophages in addition to the ROS generated from their own aerobic metabolism<sup>23</sup>. During periods of oxidative stress, elevated levels of intracellular Mn can be beneficial to bacteria given this metal's natural antioxidant properties and its role as a cofactor for enzymes that detoxify harmful O<sub>2</sub><sup>-</sup> radicals<sup>24</sup>. Accordingly, bacterial mutants that lack a functional Mn efflux protein typically display increased resistance to oxidative stress<sup>25-27</sup>.

Given the natural exposure of *Brucella spp.* to oxidative environments during their intracellular life cycle, it was predicted that *emfA* expression might decrease in response to exogenous oxidative stress. To assess this possibility, qRT-PCR was used to measure *emfA* transcription in *B. abortus* 2308 after a 2-hour exposure to H<sub>2</sub>O<sub>2</sub>. Figure 3.3 shows that *emfA* transcription is not significantly different in this bacterium after a 2-hour exposure to 5 mM H<sub>2</sub>O<sub>2</sub>. These data suggest that *emfA* expression is unresponsive to exogenous oxidative stress.

The generation of toxic O<sub>2</sub><sup>-</sup> radicals by aerobic metabolism is an additional source of oxidative stress for *Brucella* strains<sup>23</sup>. If accumulated in excess, H<sub>2</sub>O<sub>2</sub> can participate with Fe in intracellular Fenton chemistry which generates highly toxic hydroxyl radicals (see Ch. I)<sup>28</sup>. Many bacteria, including the *Brucella spp.*, employ the Mn-cofactored superoxide dismutase SodA which catalyzes the detoxification of these harmful molecules<sup>29</sup>. Furthermore, certain bacteria, such as *E. coli*, adapt to these conditions by replacing cellular Fe requirements with the less-reactive Mn<sup>24</sup>. To examine the possibility that *emfA* expression decreases in response to elevated levels of endogenous ROS, *emfA* transcription was measured via qRT-PCR in a *B. abortus ahpC*

*katE* double mutant background, in which increased levels of endogenous ROS has previously been determined<sup>30</sup>. Figure 3.3 shows that the *emfA* gene is unresponsive to increased endogenous ROS. Based on these data, it appears that *B. abortus emfA* gene transcription is unresponsive to oxidative stress.

Mur binds the promoter of an uncharacterized *Brucella* gene.

Despite the fact that *B. abortus* Mur represses this bacterium's sole, high-affinity Mn acquisition system, several findings from this study indicate that this bacterium's Mn-responsive regulator is not required for wild-type resistance to Mn toxicity. This suggests that the Mn-responsive transcriptional network of *B. abortus* 2308 may be atypical. Based on the fact that the repression of *mntH* expression by Mur is unnecessary for resisting Mn toxicity and that Mur is likely inactive under conditions of low intracellular Mn, it is logical to consider the importance of this protein for the physiology and pathogenesis of the *Brucella* strains. The *B. abortus* Mn-responsive transcriptional network, however, has not been fully characterized and further analysis of the Mur regulon may demonstrate the importance of this protein.

A comprehensive bioinformatic analysis of *Brucella* genome sequences performed by Rodionov et al. identified two consensus Mur recognition sequences (MRS) located upstream of the *mntH* gene and an uncharacterized *Brucella* gene<sup>16</sup>. A consensus MRS (5' - AATGCAAATAGTTTGC AAT - 3') overlaps the -35 position upstream of the *mntH* gene transcriptional start site, which is consistent with the function of Mur as a transcriptional repressor. *B. abortus* Mur directly binds to the MRS upstream of *mntH* coding region in the presence of Mn, however, the putative MRS

element located upstream of the aforementioned uncharacterized *Brucella* gene has not been experimentally determined<sup>11</sup>.

The uncharacterized *Brucella* gene is designated BAB\_RS17810 within the *B. abortus* 2308 genomic sequence. Algorithmic prediction suggests the putative BAB\_RS17810 gene product is a Fur family transcriptional regulator and this gene is currently annotated as 'irr2' in the publicly available *B. abortus* 2308 genomic sequence. Of note, the *Brucella spp.* encode a homolog of the iron response regulator, or Irr, that regulates Fe import, efflux, and storage genes in response to intracellular Fe levels<sup>31</sup>. At the time of this study, it is unclear whether the putative BAB\_RS17810 gene is annotated as 'irr2' due to the fact that an *irr* gene has already been identified within the *B. abortus* 2308 genome or if this gene's annotation is dependent on *irr2* genes encoded by other bacteria.

To further characterize *B. abortus* Mur, EMSA analysis was conducted to examine this protein's predicted binding affinity for the putative 'irr2' promoter. Figure 3.4 shows that *B. abortus* Mur binds in a specific manner to a ~250bp DNA fragment representing the putative 'irr2' promoter region in the presence of Mn. As expected, *B. abortus* Mur loses its binding affinity for this DNA fragment when a metal chelator is introduced to the binding conditions (data not shown), which is consistent with previous studies examining this protein<sup>11</sup>.

*irr2* gene transcription increases in a *B. abortus mur* mutant background.

The results shown in Figure 3.4 demonstrate that *B. abortus* Mur binds upstream of the *irr2* transcriptional start site in the presence of Mn. The fact that these data are

consistent with the original characterization of *B. abortus* Mur suggested that this Mn-responsive transcriptional regulator likely functions as a repressor of the putative *irr2* gene. To assess this possible regulatory function of *B. abortus* Mur, qRT-PCR was employed to measure '*irr2*' transcription in an isogenic *mur* mutant. Figure 3.5 shows that relative '*irr2*' gene expression is significantly higher in a *B. abortus mur* mutant background relative to the wild-type.

These results indicate that *B. abortus* Mur functions as a repressor of *irr2*. The significance of this finding, however, is currently limited by the fact that this gene is uncharacterized. Pilot studies were conducted to assess the role of this putative Fur family regulator in *B. abortus* resistance to Mn toxicity; however, a *B. abortus irr2* mutant does not exhibit growth restriction on Schaedler agar supplemented with up to 1 mM MnCl<sub>2</sub> (data not shown). Furthermore, preliminary computational-based attempts to determine this protein's identity using sequence alignment with various members of the Fur protein family are inconclusive due to the highly-conserved nature of Fur family proteins.

*Irr2* appears to repress *emfA* expression in *B. abortus* 2308.

As a result of this study, both MRS elements within the *B. abortus* 2308 genome that were predicted by Rodionov et al. have been confirmed via EMSA analysis<sup>16</sup>. While further studies are required to conclude that *B. abortus* Mur does not function as a direct regulator of other genes encoded by this bacterium, the metal-dependent regulatory mechanism in which *B. abortus* Mur represses *irr2* and *mntH* gene transcription in response to sufficient Mn levels is consistent with the 'classical' Fur model of metal-



dependent regulation<sup>17</sup>. Should this be the only mechanism by which *B. abortus* Mur can regulate gene transcription, it would not be possible for this protein to directly regulate both Mn uptake and Mn efflux genes in manner that would allow this bacterium to inversely express these genes in response to both 'low' and 'high' Mn conditions.

The results of this study, however, presented a possible indirect regulatory link between *B. abortus* Mur and the *emfA* gene. As demonstrated by this study, *B. abortus* Mur selectively represses the expression of a predicted transcriptional regulator (Irr2) under Mn-replete conditions. Although the function of this putative transcriptional regulator is unknown, it is logical to consider the possibility that Irr2 functions to repress *emfA* gene transcription. Under this proposed transcriptional network (Figure 3.6), *B. abortus* Mur would repress *irr2* expression, which would prevent Irr2 from downregulating *emfA* expression. To examine this possible regulatory function of Irr2, qRT-PCR was used to measure relative *emfA* gene expression in an *irr2* mutant background. Figure 3.7 shows preliminary data in which *emfA* gene transcription increased 3-fold in an *irr2* mutant background relative to the wild-type. Although further studies are required to properly characterize the regulatory link between Irr2 and *emfA*, this finding offers preliminary evidence that suggests a potential indirect regulatory link between the Mn-responsive transcriptional regulator Mur and *emfA*.

Based on the data presented by this study, it appears that the putative Fur family protein, Irr2, functions as a transcriptional repressor of the *emfA* gene. Many Fur family regulators directly 'sense' specific intracellular conditions by interacting with transition metals, heme groups, or H<sub>2</sub>O<sub>2</sub><sup>17,20,32-34</sup>. Therefore, it is likely that *Brucella* Irr2 becomes activate (or inactivate) following a direct interaction with an unknown stimulus, which

suggests that *emfA* expression may be regulated by a Mur-Irr2 multi-responsive transcriptional network. Although the findings of this study suggest that *emfA* expression is unresponsive to Mn and is not regulated by Mur, it is possible that the experimental conditions tested in this study were unable to identify the specific circumstances in which *emfA* is differentially expressed.

It is also important to consider the role that this putative multi-responsive transcriptional network plays in the physiology and pathogenesis of *Brucella* strains. At the time of this study, it appears that *Brucella* Mur directly regulates just two genes (*mntH* and *irr2*) encoded by these bacteria, but the regulon of *Brucella* Irr2 is unknown. Further characterization of the Irr2 regulon could demonstrate that other *Brucella* genes are indirectly regulated by Mur. Should Irr2 regulate virulence-related gene expression, it is possible that the Mur-Irr2 multi-responsive transcriptional network is important for these bacteria during host infection. Other regulatory proteins employed by *Brucella*, such as the BvrS/BvrR two-component system, induce virulence-related gene expression in response to environmental stressors (see Ch. I)<sup>35</sup>. Further studies are required to examine the possibility that Mn availability serves as a crucial environmental indicator that affects the expression of virulence-related genes.

## Materials and Methods

### Bacterial strains and culture conditions

Unless otherwise noted, *Brucella abortus* 2308 and derivatives of this strain (listed in Table 3.1) were cultivated on Schaedler agar supplemented with 5% defibrinated bovine blood (SBA) at 37°C with 5% CO<sub>2</sub>, or in brucella broth at 37°C with

shaking at 250 rpm. *Brucella* stock cultures were maintained in brucella broth supplemented with 25% glycerol and stored at -80°C.

#### Construction of the *B. abortus irr2* mutant

The *B. abortus irr2* null mutant was constructed from *B. abortus* 2308 using the non-polar unmarked gene excision strategy described by Caswell et al<sup>39</sup>. Approximately 1000 bp upstream of the *irr2* gene (BAB\_RS17810) to the second codon was incorporated into the pNTPS138 plasmid along with a fragment containing the last two codons of this gene's coding region to approximately 1000 bp downstream of *irr2* gene. Fusion of the two incorporated fragments at the first two and last two codons of the *irr2* coding region was performed to generate an in-frame deletion of the gene. The plasmid was electroporated into *B. abortus* 2308 and deletion of the *irr2* coding region was confirmed by screening using PCR. The resultant mutant strain with the mutated *emfA* locus was designated JEP98. All mutants employed by this study were constructed in an identical manner and are listed in Table 3.1. The nucleotide primers used for construction of the pNTPS-based plasmid is shown in Table 3.2.

#### Quantitative Reverse Transcriptase-PCR (qRT-PCR) analysis of *emfA* gene transcription.

Bacteria grown overnight in brucella broth were harvested into fresh brucella broth and adjusted to an OD<sub>600</sub> of 0.15 (10<sup>9</sup> CFU/mL). The adjusted suspensions were used to inoculate 50 mL of brucella broth in 500 mL flasks and were grown to mid-log phase at 37°C with shaking at 250 rpm. A baseline for *emfA* gene transcription was

taken by collecting 2 mL of each culture. A 2 mL aliquot of a 1:1 acetone:ethanol solution was added to each sample collected prior to storage at -80°C. For experimental parameters comparing *emfA* gene transcription from *B. abortus* derivative strains to the wild-type, no further treatment was conducted.

For studies analyzing relative *emfA* expression in response to increased environmental Mn conditions, bacterial cultures were then supplemented with stock solutions of MnCl<sub>2</sub> to reach final concentration of 50 µM at an estimated 48 mL volume. Stock solutions were prepared by solubilizing anhydrous MnCl<sub>2</sub> into H<sub>2</sub>O and were subsequently filter-sterilized prior to use. Once treated, cultures were incubated for an additional 2 h at 37°C with shaking at 250 rpm. Samples were collected in the same manner as described above. Cultures used for studies analyzing *emfA* gene transcription in response to increased exogenous H<sub>2</sub>O<sub>2</sub> were treated in a similar fashion with the exception of each culture being supplemented with H<sub>2</sub>O<sub>2</sub> to reach a final concentration of 5 mM.

Bacterial culture samples preserved in 1:1 acetone:ethanol at -80°C were thawed and total RNA/DNA was extracted using the methods described by Caswell et al.<sup>39</sup> and stored in 100% ethanol at -80 °C until proper tests were conducted to satisfy the requirements of liquid sample removal from BSL-3 containment. Once removed from BSL-3 containment, RNA samples were treated with DNase I using the DNA-free kit (Invitrogen) according to the manufacturers' protocols. Following DNase treatment, the concentration of RNA in each sample was determined by measuring absorbance (OD<sub>260</sub> and OD<sub>280</sub>) and 2 µg of mRNA transcripts were converted to cDNA by reverse transcription using SuperScript II RT (Invitrogen) with random hexamers as primers.

Quantitative PCR (qPCR) reactions were conducted using Sybr Green master mix (2x, Applied Biosystems), cDNA, and gene-specific primers for *emfA* (primers listed in table 3.2) according to the manufacturers' protocol. PCRs were carried out with an initial denaturation step at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min using the Biorad CFX96 Touch Real-Time PCR Detection System. Fluorescence readings were used to quantify the qPCR products. Relative *emfA* gene transcription (derived using CFX Maestro software) was compared using cDNA generated from untreated wild-type cultures ( $\Delta C=1$ ). Gene-specific primers for the reference genes 16S and GAP were used to normalize biological samples. Relative *mntH* expression was also measured as an experimental control when appropriate.

#### Electrophoretic Mobility Shift Assay (EMSA)

The methods used by Menscher et al.<sup>11</sup> (originally adapted from the methods described by Platero et al.<sup>36</sup>) were employed for the EMSAs conducted in this study. A ~250bp DNA fragment representing the *irr2* promoter region was amplified by PCR from *B. abortus* 2308 genomic DNA using the primers designated 'perR upF' and 'perR downR' (see Table 3.2). The amplified DNA fragment was end labeled with [ $\gamma$ -<sup>32</sup>P]ATP (Perkin Elmer, Waltham, MA) using T<sub>4</sub> polynucleotide kinase obtained from Promega (Madison, WI). An unlabeled version of this DNA fragment was used as a specific competitor in this reaction mixture, and a DNA fragment representing the *B. abortus* 2308 *btaE* (BAB\_RS16255) promoter was used as a nonspecific competitor.

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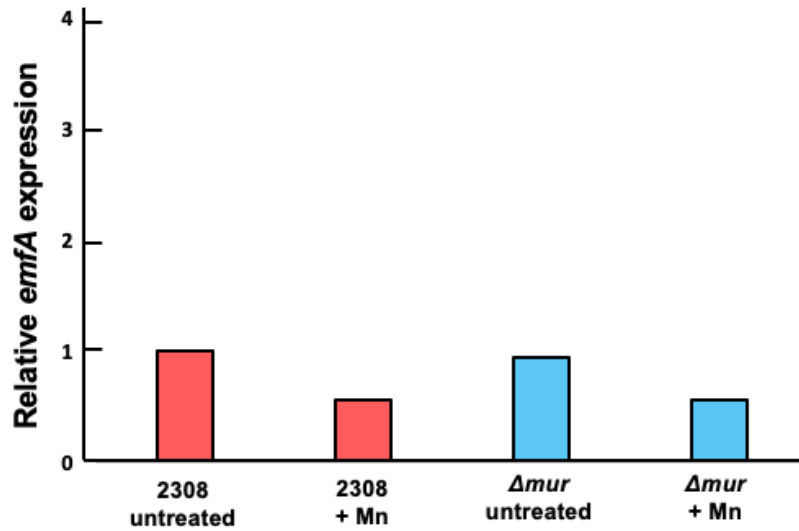
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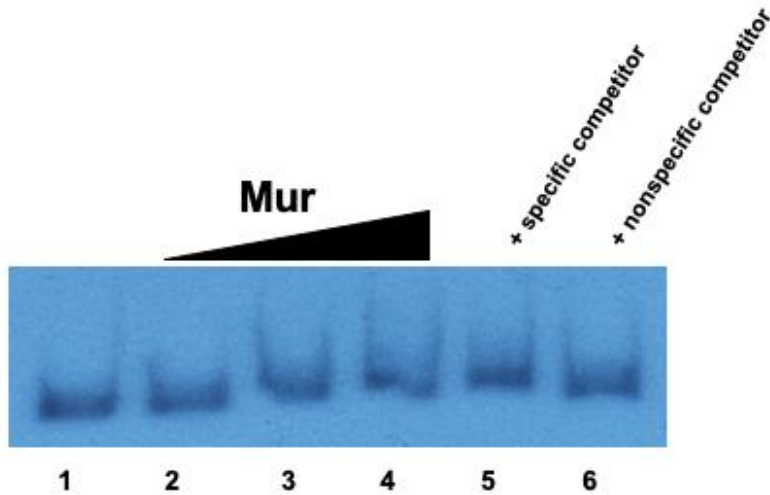
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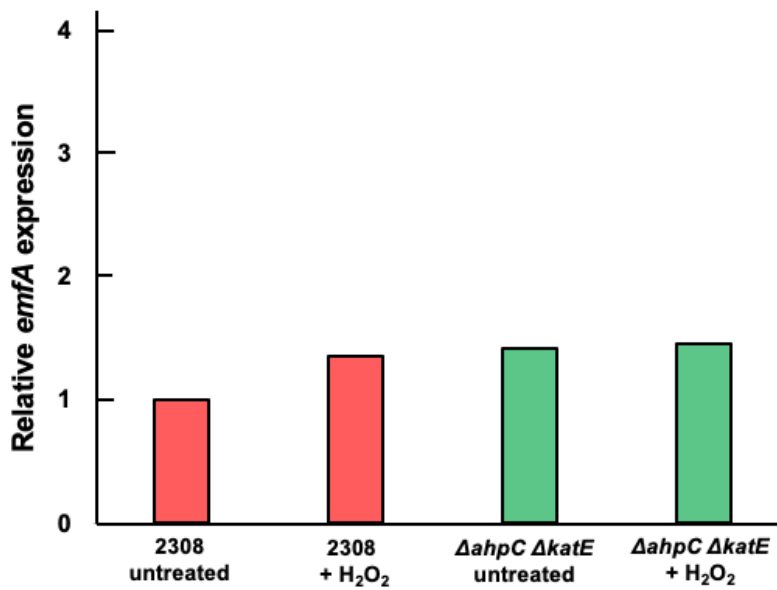
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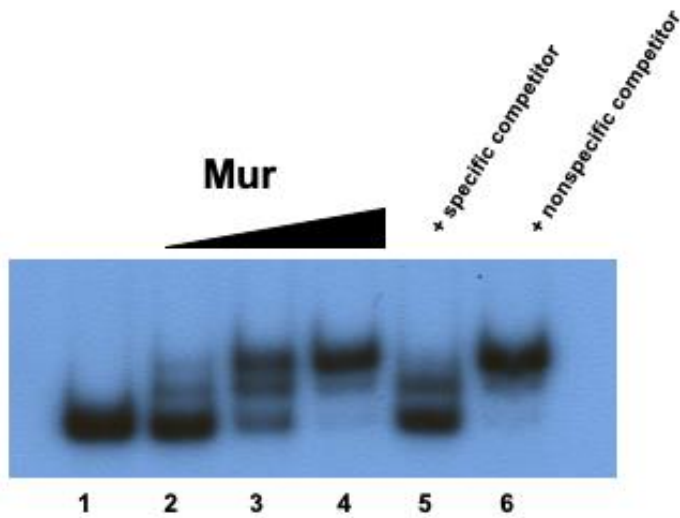
**Figure 3.1:** *B. abortus emfA* expression is unresponsive to Mn or the loss of a functional Mur. Relative expression of *emfA* in *B. abortus* 2308 and an isogenic *mur* mutant before (untreated) and after (+ Mn) a 2 h exposure to 50  $\mu$ M MnCl<sub>2</sub>. These experimental parameters were repeated three times.



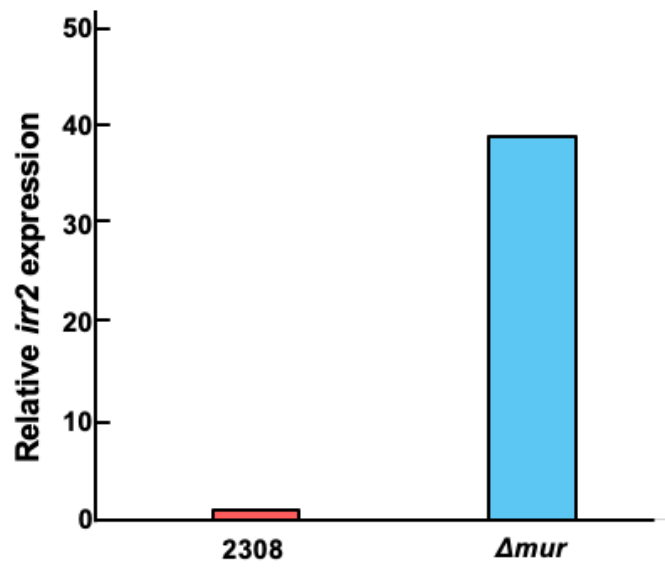
**Figure 3.2:** Mur does not bind to the *emfA* promoter in an electrophoretic mobility shift assay (EMSA). Lane 1,  $^{32}\text{P}$ -labeled *emfA* promoter-specific DNA fragment; Lanes 2 through 4,  $^{32}\text{P}$ -labeled *emfA* promoter-specific DNA fragment plus increasing concentrations (1x, 2x, 4x, respectively) of the Mur protein, Lane 5,  $^{32}\text{P}$ -labeled *emfA* promoter-specific DNA fragment plus the Mur protein plus ~200x unlabeled *emfA* promoter-specific DNA fragment (specific competitor), Lane 6,  $^{32}\text{P}$ -labeled *emfA* promoter-specific DNA fragment plus the Mur protein plus ~200x of unlabeled *B. abortus btaE* promoter-specific DNA (nonspecific competitor).



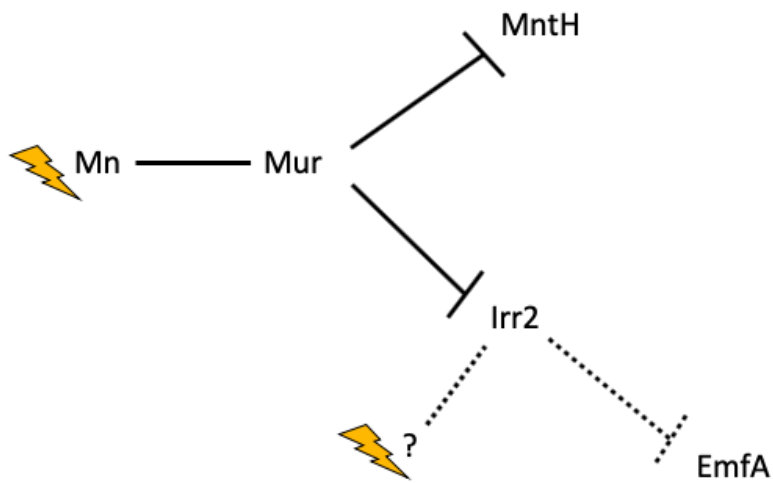
**Figure 3.3:** *B. abortus emfA* expression is unresponsive to exogenous or endogenous oxidative stress. Relative expression of *emfA* in *B. abortus* 2308 and an isogenic *ahpC katE* double mutant before (untreated) and after (+ H<sub>2</sub>O<sub>2</sub>) a 2 h exposure to 5 mM H<sub>2</sub>O<sub>2</sub>. These experimental parameters were repeated three times.



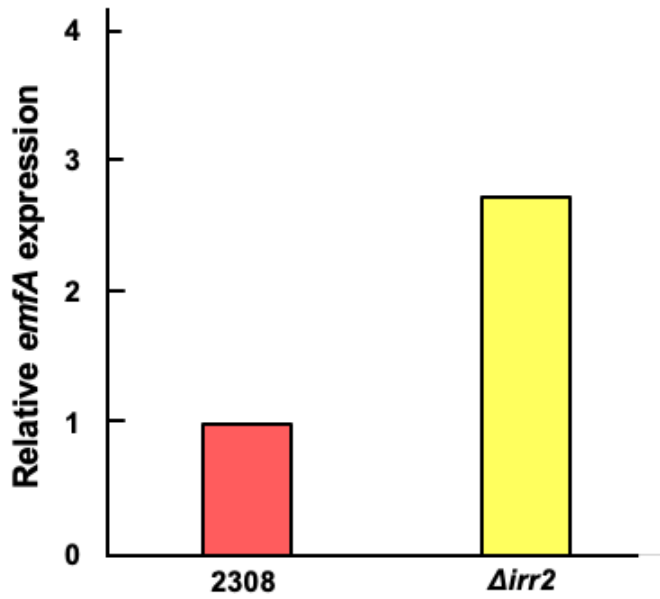
**Figure 3.4:** Mur binds directly to the *irr2* promoter in an electrophoretic mobility shift assay (EMSA). Lane 1,  $^{32}\text{P}$ -labeled *irr2* promoter-specific DNA fragment; Lanes 2 through 4,  $^{32}\text{P}$ -labeled *irr2* promoter-specific DNA fragment plus increasing concentrations (1x, 2x, 4x, respectively) of the Mur protein, Lane 5,  $^{32}\text{P}$ -labeled *irr2* promoter-specific DNA fragment plus the Mur protein plus ~200x unlabeled *irr2* promoter-specific DNA fragment (specific competitor), Lane 6,  $^{32}\text{P}$ -labeled *irr2* promoter-specific DNA fragment plus the Mur protein plus ~200x of unlabeled *B. abortus btaE* promoter-specific DNA (nonspecific competitor).



**Figure 3.5:** *B. abortus irr2* expression increases in an isogenic *mur* mutant. Relative expression of *irr2* in *B. abortus* 2308 and an isogenic *mur* mutant. These experimental parameters were repeated three times.



**Figure 3.6:** Proposed Mn-responsive transcriptional network of *B. abortus* 2308. This model depicts a possible indirect regulatory link between this bacterium's Mn-responsive transcriptional regulator and the Mn efflux protein EmfA. The proposed regulatory link is based on the identification of the putative Fur family transcriptional regulator 'Irr2' and its regulation by Mur at the level of transcription



**Figure 3.7:** *B. abortus emfA* expression appears to be upregulated in an isogenic *irr2* mutant. Preliminary assessment of relative *emfA* expression in *B. abortus* 2308 and an isogenic *irr2* mutant. These experimental parameters were repeated twice.



**Table 3.1** Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference or source
Strains		
<i>Brucella abortus</i>		
2308	Virulent challenge strain	36
EAM001	2308 $\Delta mur$	This study
JEP12	2308 $\Delta ahpC \Delta katE$	This study, based on 29
JEP98	2308 $\Delta irr2$	This study
Plasmids		
pNPTS138	<i>sacB</i> -based gene replacement vector; Kanamycin resistance	37
pGEM-T Easy	Cloning vector; Ampicillin resistance	Promega

**Table 3.2** Oligonucleotide primers used in this study

<b>Primer</b>	<b>Sequence (5'--&gt;3')</b>
emfA qPCR F	AGGTGAAACTCGATGAAGGTC
emfA qPCR R	GCAATGTTATCTCGGCCAATG
emfA EMSA F	TAACTTGAAGCCAGCCGCGAGCCAAACCTT
emfA EMSA R	GCGTCCATAATGAACCTTTGG
perR upF-BamHI	GATGGATCCATACCACCATTTGGGTCACCCG
perR downR-Hind	TGAAAGCTTCGCCCTGTTTC
perR downF	CAGCATGTGTCAGCCTCGTTTG
perR confR	CAGAGTAGCGAAACCCCTC
perR qPCRF	ACCGTGAATGCTGATCCTG
perR qPCRR	CAAAATCGGCATGTGACACC

## Appendix A: IACUC Approval Letter



Animal Care and  
Use Committee  
212 Ed Warren Life  
Sciences Building  
East Carolina University  
Greenville, NC 27834-4354

252-744-2436 office  
252-744-2355 fax

September 12, 2017

R. Martin Roop, Ph.D.  
Department of Micro/Immuno  
Biotech Building  
East Carolina University

Dear Dr. Roop:

Your Animal Use Protocol entitled, "Intracellular Persistence of *Brucella* Strains in Host Macrophages" (AUP #K167a) was reviewed by this institution's Animal Care and Use Committee on September 11, 2017. The following action was taken by the Committee:

"Approved as submitted"

**\*Please contact Aaron Hinkle at 744-2997 prior to hazard use\***

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP and are familiar with its contents.**

Sincerely yours,

Susan McRae, Ph.D.  
Chair, Animal Care and Use Committee

SM/jd

Enclosure