Abstract

The iron response regulator Irr controls iron homeostasis in *Brucella*

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December 2014
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Members of the genus *Brucella* are small, Gram-negative intracellular bacterial pathogens that are capable of infecting a wide range of mammalian hosts including humans. *Brucella* primarily reside inside of host macrophages. As an intracellular pathogen, *Brucella* must overcome iron sequestration in the host cell by utilizing highly efficient iron transport systems. These systems must be tightly regulated, however, as excess intracellular iron is toxic to the bacterial cells. Most of the alpha-proteobacteria rely on a transcriptional regulator called the iron response regulator (Irr) to control the expression of their iron metabolism genes. The work presented in this dissertation provides evidence to support the proposition that the Irr protein is the main iron-responsive transcriptional regulator in *Brucella*. Irr serves as an activator of genes coding for products that are involved in iron acquisition and a repressor of genes for products that require high levels of iron for their function, or serve as iron export and storage proteins when cellular iron levels are low. Irr is a conditionally stable protein that is present when cellular iron levels are low, and is degraded when cellular iron levels are high. Irr activity is controlled by inactivation and degradation through its interaction with heme, which is synthesized when cellular iron levels rise. *Brucella* has another iron-responsive regulator that is also found in some members of the alpha-proteobacteria called the rhizobial iron regulator (RirA). RirA is active

when cellular iron levels are high, and its regulon partially overlaps with that of Irr in *Brucella*. The activity of Irr when cellular iron levels are low, and the activity of RirA when cellular iron levels are high, ensures that cellular iron levels are maintained at physiological levels, protecting against iron starvation, and against iron related toxicity in *Brucella*.

The iron response regulator Irr controls iron homoeostasis in *Brucella*

A Dissertation
Submitted to the Graduate Faculty of the Brody School of Medicine at
East Carolina University

In Partial Fulfillment of the Requirements for the
Degree of Doctor of Philosophy
in the Department of Microbiology and Immunology

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Acknowledgments

Words cannot express my gratitude towards the Graduate School and the Department of Microbiology and Immunology at East Carolina University for providing me with the opportunity to pursue my graduate degree. I sincerely appreciate all of the time, energy, and resources that so many individuals have devoted towards my professional development. My achievements would not have been possible if it were not for the selfless dedication to all of those who I have had the pleasure of working with as a graduate student. In particular, I would like to thank Dr. Gemperline and Dr. McConnell from the graduate school for their help in my development as a student leader. I would like to thank my supervisor, Dr. Roop, for all that he has contributed to my maturation as a scientist. Throughout my academic career, Dr. Roop's mentoring has challenged and advanced my intellectual abilities, facilitating the development of my scientific and reasoning skills. I would like thank every member of the Roop laboratory, past and present, for their contributions to the work presented in this dissertation. The open and collaborative style through which the laboratory discusses and solves scientific problems has been an overwhelming positive and productive experience. I would be remiss if I did not thank my friends and family for their continued and unwavering support. It is difficult to imagine achieving a similar level of success without all that my parents, Emily, Lisa, James, Barrett, and Shannon have done for me.

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Chapter 1: Literature review

Brucella and brucellosis

The genus *Brucella* consists of small, Gram-negative intracellular bacterial pathogens that are capable of infecting a wide range of mammalian hosts including cattle, sheep and goats, pigs, dogs, and rodents. *Brucella* strains have also recently been recognized as important marine mammal pathogens. The brucellae replicate inside phagocytic cells of the reticuloendothelial system, particularly macrophages, and placental trophoblasts in pregnant animals (Roop et al., 2009). *Brucella* infections in their natural female hosts result in either abortion or birth of weakened offspring, and in males result in infertility (Enright, 1990). Humans can become infected with certain *Brucella* strains, but are incidental hosts. Worldwide, brucellosis is the most common bacterial zoonotic disease, with more than half a million people being infected annually (Pappas, 2010). Brucellosis in humans is a long-term, cyclic flu-like illness commonly referred to as undulant fever, and treatment requires a combination of different antibiotics for a prolonged period of time (Ariza et al., 2007). Most human brucellosis is caused by *B. melitensis*, although *B. abortus* and *B. suis* cause significant human disease as well (Pappas et al., 2006b). Human *Brucella* infections result from contact with infected animals or animal products. In countries where surveillance, vaccination and pasteurization of animal products are common, human brucellosis is rare (Ragan, 2002). In areas of the world where brucellosis is still endemic, mass vaccination of domesticated animals has been proposed as a cost effective way to decrease the human health burden and economic losses associated with the disease (Roth et al., 2003).

***Brucella* taxonomy**

Until recently, six species were recognized within the genus *Brucella* - *Brucella abortus*, *Brucella melitensis*, *Brucella suis*, *Brucella ovis*, *Brucella canis*, and *Brucella neotomae*. The natural hosts for *B. abortus* are cattle, bison, and elk, for *B. melitensis* are sheep and goats, for *B. suis* is pigs, for *B. canis* is dogs, for *B. ovis* is sheep and for *B. neotomae* is wild rodents. The number of *Brucella* strains has grown to now include those found in other mammals. These include *Brucella pinnipedialis* and *Brucella ceti* which have been isolated from seals and dolphins, respectively (Foster et al., 2007), *Brucella microti* which has been isolated from voles (Scholz et al., 2008) and *Brucella inopinata* which was isolated from a human breast implant (Scholz et al., 2010). While *Brucella* strains each have their own unique host preference, many strains have the capacity to cause infection in many different animals species.

Brucella strains share a remarkable degree of genetic homology with one another (Gandara et al., 2001). Whole genome sequence analysis of *B. abortus*, *B. melitensis*, and *B. suis* field isolates revealed sequence identity for most open reading frames was 99% or higher (Halling et al., 2005). While they share significant DNA homology, *Brucella* strains are phenotypically distinct and exhibit specific host preference and pathogenicity. This epidemiology dictates that *Brucella* strains be divided into distinct nomenspecies (Moreno et al., 2002). With the development of cost effective whole genome sequencing, an ongoing topic of discussion and research is an attempt to describe why there is such a great diversity of host preference, but with such a limited genetic diversity among *Brucella* strains.

Animal brucellosis

Animal brucellosis is an economically important disease in many parts of the world. While brucellosis remains endemic in Africa, the Mediterranean, the Middle East, parts of Asia, Latin America, the use of control and surveillance programs have eradicated or virtually eradicated brucellosis in countries with strong public health and veterinary infrastructures (Gul & Khan, 2007; Fefai, 2002). There are three elements to effective control of animal brucellosis -- 1) surveillance to identify infected animal herds; 2) prevention of transmission to non-infected animal herds; and 3) eradication of the reservoirs to eliminate the sources of infections (Avila-Calderon et al., 2013). Bovine brucellosis eradication has been achieved in many countries using a test-and-slaughter policy and the United States is now considered-brucellosis free (Minas et al., 2004). However, brucellosis is still present in wild elk and bison herds in the Yellowstone National Park area, which poses a risk for transmission to domestic animal herds in this region (Rhyan et al., 2013).

Not all countries have the infrastructure to perform surveillance and test-and-slaughter programs and vaccination has been proposed as a cost effective and efficacious mechanism to reduce the brucellosis burden in animal populations (Avila-Calderon et al., 2013). Vaccination of animals prior to infection can reduce infection prevalence and the consequent risk of human disease (Rubach et al., 2013; Avila-Calderon et al., 2013). Vaccination of young animals over a period of 15 years in Greece resulted in a significant decline in the number of abortions in sheep and goats and a reduction in human infections. Shortly after stopping the vaccination program the frequency of brucellosis in animals and humans quickly increased, underscoring the need for continued animal vaccination to reduce brucellosis prevalence (Minas et al., 2004).

B. abortus S19 is a live, smooth, attenuated vaccine strain used for control of bovine brucellosis. There are significant drawbacks to this strain as a vaccine, however, as it can cause disease in humans, and does not permit discrimination of infected animals from vaccinated animals because of a strong immune response against the lipopolysaccharide O-polysaccharide component (O-side chain) of the *Brucella* outer membrane which is an antibody target for serological diagnostic tests (Avila-Calderon et al., 2013). *B. abortus* RB51 is a live rough vaccine strain used to prevent bovine brucellosis that lacks the O-side chain, permitting the serologic distinction between vaccinated and naturally infected cattle but is still capable of causing disease in humans (Avila-Calderon et al., 2013). The live vaccine strain *B. melitensis* Rev. 1 is currently used for the control of brucellosis in goats and sheep, but is still capable of causing disease in humans and induces a positive antibody response in serological tests in vaccinated animals (Avila-Calderon et al., 2013). An active area of research is in the development of more efficacious *Brucella* vaccines for domestic animals such as cattle, sheep, and goats that do not have the ability to cause disease in humans and will not confuse diagnostic test results (Moriyon et al, 2004).

Human brucellosis

The World Health Organization estimates that there are 500,000 new cases of human brucellosis annually, making it one of the most frequent zoonoses worldwide and a global public health problem (Pappas et al., 2006b). Transmission to humans occurs through the consumption of infected, unpasteurized animal products, through direct contact with infected animal parts and through the inhalation of infected particles (Pappas et al., 2005). Because of the classic zoonotic route of transmission to humans from animals, dairy industry professionals, shepherds,

laboratory personnel, and those who consume unpasteurized dairy products are the most at-risk groups for acquiring brucellosis. The association between infected animals and human infections is well-defined, as there is a significant correlation between the elimination of animal brucellosis and declining rates of human brucellosis (Minas et al., 2004).

Human brucellosis is a debilitating, febrile disease that is easily misdiagnosed and can persist for years if left untreated. A spiking, or relapsing fever is nearly always associated with this disease and it is not uncommon to see enlargement of the spleen and liver. Peripheral arthritis of the knees, hips, ankles and wrists, sacroiliitis (inflammation where the lower spine and pelvis connect), and spondylitis (inflammation of the vertebrae) are the most common complications of human brucellosis regardless of the *Brucella* strain causing the infection (Bosilkovski et al., 2004; Ariza et al., 1993; Solera et al., 1999). Less than ten percent of brucellosis cases present with central nervous system involvement such as meningitis, encephalitis and brain abscesses (Shakir et al., 1987). The principal cause of mortality in human brucellosis patients is endocarditis, which is rare (Reguera et al., 2003).

The prevalence of animal brucellosis in the United States is very low, along with the incidence of human infection. Most cases of brucellosis in the United States are acquired through international travel or the consumption of food imported from endemic areas (Pappas et al., 2006b). Laboratory diagnostics are performed on patients presenting with clinical symptoms and recent travel to areas of the world where brucellosis is endemic (Galinska & Zagorski, 2013). The definitive diagnosis of brucellosis is through culture. However, the speed and lack of efficacy of culture-based diagnosis are compounding factors that make it difficult. Serology is reliable and is always used as a backup to culture in the diagnosis of human brucellosis (Araj, 2010). The World Health Organization recommends the use of doxycycline for six weeks in

combination with either gentamicin for seven to ten days, streptomycin for two to three weeks or rifampin for six weeks. While these regimens are effective at resolving most infections, relapse is a characteristic feature of human brucellosis (Ariza et al., 1995). The main risk factors associated with relapse of brucellosis

Brucella as 'Select Agents'

Brucella strains have characteristics that make them attractive candidates for use as biological weapons. These include the availability of virulent strains isolated from infected animals in endemic areas, high infectivity via the aerosol route, storage stability, the lack of an efficacious human vaccine, the debilitating nature of the infection, the difficulty in treating the infection and easy dissemination into the environment (Doganay & Doganay, 2013). *Brucella* has been shown to survive for eighty seven days in milk and up to three weeks in goat cheese, which can serve as an example of intentional food contamination with *Brucella* (Falenski et al., 2011). Because of these characteristics, *B. melitensis*, *B. abortus* and *B. suis* are classified as 'non-Tier'1 Select Agents by the Centers for Disease Control and Prevention (Pappas et al., 2006a).

Brucella strains are intracellular pathogens

Brucella strains can efficiently colonize cells of the monocyte/macrophage lineage and their ability to cause chronic disease in animals and humans is directly related to the ability of the brucellae to persist for long periods of time within host cells and to resist the host immune response (Martirosyan et al., 2011). Our understanding of *Brucella* interactions with host cells has mostly relied on *in vitro* studies using macrophages and to a lesser extent, trophoblasts, both

of which are known to serve as host cells for *Brucella* during natural infections (Roop et al., 2009).

There are two types of infections caused by *Brucella* that result in different pathologies. During an acute infection, which occurs in pregnant animals, the brucellae replicate to a very high density within placental trophoblasts resulting in third trimester abortion of the fetus (Smith et al., 1962). The large numbers of brucellae in the aborted fetal material is important for transmission between natural hosts. During a chronic infection, the brucellae reside within macrophages, where they replicate slowly resulting in a prolonged infection that is important for the maintenance of disease reservoirs in wild or domestic animal herds (Roop et al., 2009).

Brucella intracellular trafficking

Macrophages are an important site for *Brucella* intracellular replication (Roop et al., 2009). Over 90% of the brucellae internalized by macrophages are quickly killed, but the remaining bacteria establish an intracellular niche that allows for replication (von Bargen et al., 2012). The events that occur beginning with *Brucella* entry into the macrophage and ending with *Brucella* gaining access to its replicative niche in a vacuole within the endoplasmic reticulum are known as *Brucella* trafficking (see figure 1.1).

Disruption of lipid rafts on the macrophage cell surface reduces the ability of *Brucella* to survive within macrophages early in the infection indicating that lipid raft mediated entry is required for these bacteria to begin an infection (Naroeni & Porte, 2002). Upon entry into host cells, the brucellae reside in *Brucella* containing-vacuoles (BCVs) that interact with components of the endocytic pathway (von Bargen et al., 2012). Early BCVs have high levels of cholesterol and flotillin-1, which are involved in lipid raft signaling associated with phagosome maturation

and interactions with the endocytic pathway (Arellano-Reynose et al., 2005). Immediately after internalization, the BCVs undergo very transient interactions with early endosomes (Celli et al., 2003). At this time, the *Brucella* two-component regulator BvrR/BvrS controls the expression of genes coding for surface components, outer membrane proteins and those involved in lipopolysaccharide modifications that are necessary for proper entry in to the phagosome and prevention of prolonged fusion of the BCV with the endocytic pathway (Sola-Landa et al., 1998; Lopez-Goni, 2002; Guzman-Verri et al., 2002). The cholesterol-rich lipid rafts present on the BCV membrane are also modified by cyclic β-1,2-glucan, which is secreted from *Brucella*, and is thought to prevent prolonged interactions with lysosomes (Arellano-Reynoso et al., 2005).

As *Brucella* trafficking continues, BCVs lose early endosomal markers and acquire the late endosomal/lysosomal membrane protein LAMP1 when vacuolar acidification occurs (Porte et al., 1999; Celli et al., 2003). The BCVs eventually acquire late endosomal markers which are necessary for further BCV trafficking demonstrating that to reach their replicative niche, the maturation of BCVs requires controlled and limited fusion events with late endosomes and lysosomes (Starr et al., 2008). BCV acidification induces the expression of *Brucella* genes that are important for intracellular trafficking. The *virB* operon that codes for a Type IV secretion system (T4SS) is required for the establishment of the *Brucella* replicative niche and is one of the sets of genes whose expression is induced in response to BCV acidification (Boschiroli et al., 2002). The Type IV secretion system encoded by the *Brucella virB* genes is homologous to Type IV DNA transfer systems found in *Agrobacterium tumefaciens* and *Legionella pneumophila* (Alvarez-Martinez & Christie, 2009). The *Brucella* Type IV secretion system secretes effector molecules to control BCV trafficking through the macrophage endocytic pathway preventing them from fully fusing with the lysosomes and promoting their sustained interactions with the

endoplasmic reticulum (Celli et al., 2003; Starr et al., 2008). The inability of *virB* mutants to establish an intracellular niche in the macrophage explains their attenuation in the mouse model of infection (Hong et al., 2000) and in goats (Zygmunt et al., 2006) underscoring the important role of proper intracellular trafficking for *Brucella* infections to be successful.

Following the transient fusion events with the endocytic pathway and acidification, the BCVs fuse with the endoplasmic reticulum and return to a neutral pH resulting in a safe niche for intracellular persistence (Celli et al., 2005). *Brucella* has also been isolated from endoplasmic reticulum-associated compartments in placental trophoblasts from *B. abortus* infected cattle and goat tissues (Anderson & Cheville, 1986) indicating that ability of BCVs to properly traffic to the endoplasmic reticulum is vital for the brucellae to reach an intracellular niche for replication in cell types that propagate both acute and persistent infections.

The need for iron

With the exceptions of *Lactobacillus plantarum* (Archibald, 1983) and *Borrelia burgdorferi* (Posey and Gherardini, 2000), nearly all living organisms require iron to survive. Iron participates in major biological processes such as photosynthesis, nitrogen fixation, methanogenesis, hydrogen production and consumption, respiration, metabolic pathways, oxygen transport, gene regulation and DNA biosynthesis (Andrews et al., 2003). One quarter to one third of all known proteins require metals for proper folding and function, with iron serving as a cofactor for many of these proteins (Waldron & Robinson, 2009). Iron can participate in redox reactions involving the transfer of electrons because of its ability to transition between the reduced ferrous form (Fe^{2+}), and the oxidized ferric (Fe^{3+}) form. This redox activity makes it a

versatile prosthetic component for incorporation into proteins that function as catalysts or electron carriers (Andrews et al., 2003).

Iron toxicity

While iron is an essential element in most biological systems, it can catalyze the formation of potentially toxic reactive oxygen species. The cyclic interaction of the reduced (ferrous, Fe^{2+}) and oxidized (ferric, Fe^{3+}) forms of iron with hydrogen peroxide produces reactive, damaging hydroxyl radicals (**OH**$^{\cdot}$) in a process known as Fenton chemistry. (1) Ferrous iron reacts with hydrogen peroxide producing ferric iron, a hydroxyl radical and a hydroxyl anion (Haber & Weiss, 1932). (2) Ferric iron reacts with hydrogen peroxide producing ferrous iron, a peroxide radical, and a proton (Fenton, 1894). The generation of hydroxyl radicals is particularly problematic for cells because it is more damaging to DNA than hydrogen peroxide or peroxide radicals (Imlay and Linn, 1988). Moreover, there are no known antioxidants that can detoxify hydroxyl radicals directly in cells (Imlay et al., 1988). Endogenous superoxide occurs at the surface of bacterial cytoplasmic membranes as a byproduct of respiration (Kurtz, 2006) and in the presence of free iron, the Fenton reaction occurs. For these reasons, cells tightly regulate intracellular iron concentrations in an effort to avoid reactive oxygen species mediated cell damage.

(1) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \mathbf{OH}^{\cdot} + OH^-$

(2) $Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + OOH^{\cdot} + H^+$

Iron restriction in the host

Nearly all organisms require iron to carry out physiological functions, but the potential for toxicity resulting from excessive accumulation of iron dictates the careful control of cellular iron levels. Sequestration of iron by mammals serves not only to prevent iron toxicity, but also to limit iron availability to pathogens in a biological process known as nutritional immunity. Nutritional immunity in mammals involves sequestration of iron in the extracellular environment and the sequestration and compartmentalization of iron in the intracellular environment (Hood & Skaar, 2010). In mammals, most iron is complexed with heme, which is a component of the oxygen transport protein hemoglobin. The serum proteins haptoglobin and hemopexin bind to free hemoglobin and heme that is not contained within red blood cells (Hood & Skaar, 2010). Extracellular iron that is not incorporated into heme is bound by serum transferrin or at mucosal surfaces by lactoferrin and intracellular iron is stored in a complex with ferritin (Hood & Skaar, 2010). The activity of these proteins ensures that both extracellular and intracellular niches have limited iron availability for pathogens to access.

There are also specific mechanisms utilized by mammals to further exacerbate the limitation of iron availability to intracellular pathogens. When macrophages are infected by intracellular pathogens they enhance the production and activity of the macrophage iron export protein ferroportin to limit the amount of cellular iron available to the invading bacteria (Nairz et al., 2007; Paradkar et al., 2008). Upon the induction of innate immunity stimulated by internalization of a pathogen, IFN-γ activation of host macrophages reduces the production of transferrin receptors on the surface of activated macrophages limiting the amount of iron coming into the macrophage (Byrd & Horwitz, 1989). IFN-γ also stimulates the activity of Nramp1 (natural resistance-associated macrophage protein 1), which is a protein that is highly conserved

among mammals that pumps iron out of the phagosomal compartment severely depleting the amount of iron available for an invading intracellular pathogen (Cellier et al., 1995; Cellier et al., 2007; Hackam, et al., 1998). The combination of iron sequestration mechanisms employed by host macrophages limits iron availability to intracellular pathogens in an attempt to limit replication and persistence.

Brucella iron transport systems

Brucella pathogenesis depends on the ability of the brucellae to survive and replicate within host phagocytic cells. Because of the dependence on this intracellular lifestyle for survival, *Brucella* must obtain nutrients required for growth from the host. IFN-γ is critical for limiting *Brucella* replication in the host (Sathiyaseelan et al., 2000). Upon activation of host macrophages by IFN-γ, iron restriction in the phagosomal compartment becomes exacerbated further restricting the availability of iron to invading pathogens. To overcome iron restriction, effective intracellular pathogens utilize highly efficient iron acquisition systems to transport this nutrient into the bacterial cell. Three iron transport systems have been characterized in *Brucella*, a heme transport system, a ferrous iron transport system and a siderophore-mediated iron transport system, and there is also a predicted iron citrate transport system that has yet to be characterized (see Figure 1.2).

Heme transport

Heme is synthesized in many human cell types and can also be obtained from the diet (Hamza and Dailey, 2012). Heme is a critical cofactor for cellular functions such as oxygen

transport, enzymatic reactions and cellular respiration. Heme molecules contain roughly 70% of the iron in the human body (Bridges and Seligman, 1995). Because such a high proportion of iron is held within heme molecules in mammals, pathogenic bacteria have evolved mechanisms to transport host heme into their cells to utilize as an iron source.

Heme transport systems have been well-studied in a number of Gram-negative bacteria such as *Yersinia enterocolitica* (Stojiljkovic & Hantke, 1992), *Yersinia pestis* (Hornung et al., 1996), *Shigella dysenteriae* (Eakanunkul et al., 2005; Burkhard & Wilks, 2007), and *Pseudomonas aeruginosa* (Lansky et al., 2006; Ho et al., 2007; Tong & Guo, 2007). The overall structures of the proteins that transport heme across the outer membrane are similar among bacteria, with a membrane-spanning beta-barrel and extracellular loops that bind to heme (Wilks & Burkhard, 2007). Heme transport is a process that requires energy, and the TonB/ExbB/ExbD system is responsible for transducing the proton motive force of the cytoplasmic membrane to the outer membrane transporters providing the energy needed to move heme molecules across the outer membrane (Krewulak & Vogel, 2011). Once transport of the heme molecule through the outer membrane occurs, ABC transport systems then continue the movement of heme through the periplasm, across the inner membrane and into the cytoplasm (Runyen-Janecky, 2013). These ABC transport systems consist of a high affinity periplasmic ligand-binding protein that is responsible for moving heme between the outer and inner membranes, two subunits of a cytoplasmic membrane permease, and a membrane ATPase to supply energy for transport (Runyen-Janecky, 2013). Once heme is inside of the bacterial cell, enzymes called heme oxygenases cleave the heme to release the iron to be used for cellular processes (Runyen-Janecky, 2013).

A major role of the mammalian macrophage is to recycle senescent erythrocytes (Bratosin et al., 1998). This process results in a considerable flux of heme through these phagocytes (Crichton et al., 2002). Some of this heme is transported to the endoplasmic reticulum (Taketani et al., 2005). The chronic form of *Brucella* infections observed in mammals is characterized by prolonged intracellular persistence in host macrophages (Roop et al., 2009). BCVs traffic through the macrophage endocytic pathway and eventually fuse with the endoplasmic reticulum (Celli et al., 2005), making the possibility of heme becoming a relevant iron source for these bacteria an attractive proposition (see Figure 1.1). *Brucella* strains have the capacity to acquire heme from the host and use it as an iron source (Almiron et al., 2001; Paulley et al., 2007). In *Brucella*, the BhuATUV heme transport system has been characterized and the absence of BhuA causes attenuation in the mouse model of chronic infection (Paulley et al., 2007; Ojeda dissertation, 2012).

Ferrous iron transport

Once the brucellae are internalized by macrophages, they reside in acidic compartments called endolysosomal *Brucella*-containing vacuoles (Starr et al., 2012). Because these compartments are acidic in nature, the equilibrium of ferrous vs. ferric iron will be more in favor of ferrous iron, making ferrous iron a potentially biologically relevant iron source for the intracellular brucellae (see Figure 1.1) (Roop et al., 2011). The ferrous iron transporter FtrABCD has recently been characterized in *B. abortus* and is essential for virulence in the mouse model of infection (Elhassanny et al., 2013). The proposed model for FtrABCD ferrous iron transport starts with ferrous iron diffusing across the outer membrane through a porin where it is bound by FtrA in the periplasm. Ferrous iron is then oxidized to ferric iron before being

transferred to FtrC for translocation across the cytoplasmic membrane. FtrD restores the redox balance of the transporter by accepting and passing on electrons released from the oxidation of iron to an intracellular oxidant (Elhassanny et al., 2013). Like most genes coding for iron transport systems, expression of the *Brucella ftrABCD* operon is elevated in response to low cellular iron levels (Elhassanny et al., 2013). These genes also have the capacity to respond to low pH in the extracellular environment and the homologous system in *Bordetella* exhibits a similar expression pattern (Elhassanny et al., 2012; Brickman & Armstrong, 2012). Linking *ftrABCD* expression to low extracellular pH levels makes sense because a reduced pH environment favors the presence of soluble ferrous iron over ferric iron (Crichton, 2009). Because of the acidification of the early *Brucella*-containing vacuoles (Starr et al., 2012), ferrous iron serves as an important iron source for these bacteria during an acute infection (Elhassanny et al., 2013).

Siderophore-mediated iron transport

Many bacteria synthesize and secrete low molecular weight (150-2000 Da) (Schalk et al., 2012) ferric iron-specific chelating agents called siderophores during growth under iron limiting conditions (Ratledge & Dover, 2000) to gain access to iron present in their extracellular environment (Chu et al., 2010). Iron-bound siderophore complexes are large and cannot pass through porins in Gram-negative bacteria. Instead, they are imported by specific transport systems that are made up of an outer membrane protein that transports the iron-bound siderophore complex across the outer membrane in a TonB-dependent manner, a periplasmic binding protein (PBP) to shuttle the complex to the inner membrane and an ABC transporter that moves the complex across the cytoplasmic membrane (see Figure 1.2) (Noinaj et al., 2010).

Two siderophores have been identified in *Brucella* -- the catechol, 2,3-dihydroxybenzoic acid (2,3-DHBA), and brucebactin, which is a more complex, 2-3-DHBA-based siderophore (Lopez-Goni et al., 1992; Gonzalez-Carrero et al., 2002). The genes coding for the enzymes responsible for the synthesis of the *Brucella* siderophores have been identified and lie within a single genetic locus comprised of three operons. The *dhbCB* and *A* genes code for enzymes that synthesize 2,3-DHBA (Bellaire et al., 1999) and the *dhbE* and *B*, *vibH* and *entD* gene products convert 2,3-DHBA to brucebactin (Gonzalez-Carrero et al., 2002; Bellaire et al., 2003a). The *Brucella* genome also encodes homologs of the TonB-dependent outer membrane proteins Fiu (Hantke et al., 1990) and Cir (Griggs et al., 1987) which transport siderophores in *E. coli*, and a homolog of the PBP ABC transporter complex FatBCDE that is responsible for transporting siderophore in *Vibrio anguillarum* (Koster et al., 1991). *B. abortus fatB* and *B. melitensis fatC* mutants cannot use brucebactin or 2,3-DHBA to acquire iron, indicating that the FatBCDE system is important for the transport of iron loaded siderophore into the cell (Gonzalez-Carrero et al., 2002; Danese et al., 2004).

Studies have indicated that the siderophore system is not required for *B. abortus* (Bellaire et al., 1999; Gonzalez- Carrero et al., 2002; Parent et al., 2002) or *B. melitensis* (Danese et al., 2004) virulence in experimentally infected mice (a model of chronic infection) (Roop et al., 2009). However, a *B. abortus dhbC* mutant that is unable to produce siderophore exhibits significant attenuation in pregnant cattle (a model of acute infection) (Bellaire et al., 2003; Roop et al., 2009). Brucellae replicate in placental trophoblasts during an acute stage infection (Roop et al., 2009). The four carbon sugar alcohol erythritol is a favored carbon source for *Brucella*, and the ruminant reproductive tract is rich in erythritol during the latter stages of pregnancy (Smith et al., 1962). The metabolism of erythritol results in the rapid proliferation of brucellae in

the ruminant placental trophoblasts which leads to a breakdown in the integrity of the placenta and eventual abortion (Smith et al., 1962). While metabolizing erythritol, *B. abortus* 2308 requires more iron than it does when using other carbon and energy sources (Bellaire et al., 2003b). It is thought that the reason behind the observation that a *dhbC* mutant is attenuated in pregnant animals is because there is an increased demand for iron during growth on erythritol in the placental trophoblast that the brucellae cannot overcome in the absence of the siderophore transport system.

Iron-responsive regulators

While efficient iron transport systems are required for intracellular pathogens to establish and maintain infections in hosts because of iron sequestration, unregulated iron transport can result in excessive cellular iron levels that are detrimental to the bacterial cells due to the production of damaging hydroxyl radicals. For this reason, the expression of genes involved in iron acquisition are tightly controlled and these genes are only expressed when bacterial cellular iron levels are low.

Fur

There is a group of metalloregulators that make up the Fur superfamily. Proteins belonging to the Fur superfamily are responsive to iron (Fur), zinc (Zur), manganese (Mur), nickel (Nur), peroxide stress (PerR) and heme (Irr). Many bacteria utilize the transcriptional regulator Fur (ferric uptake regulator) to control iron homeostasis (Troxell & Hassan, 2013). Fur was identified in *Escherichia coli* -- a *fur* mutant exhibits constitutive expression of genes coding

for iron acquisition systems (Hantke, 1981). A model for the most common mechanism of transcriptional control by Fur proteins is direct binding to specific sequences in the promoters of targets known as Fur boxes (Escolar et al., 1999) with iron binding directly to Fur serving as a co-repressor (Bagg & Neilands, 1987; Hantke, 2001). In the absence of iron, Fur is inactive which allows for the de-repression of target genes (see Figure 1.3).

RyhB

Fur is required for the optimal expression of some of the genes coding for tricarboxylic acid cycle (TCA) enzymes such as succinate dehydrogenase (*sdhCDAB*) and the *sodB* gene, which codes for an iron dependent superoxide dismutase (Hantke, 1987; Dubrac and Touati, 2000). It was not until the discovery of a highly conserved small untranslated RNA (sRNA) named RyhB that a mechanism was described to explain the activation of genes by Fur (see Figure 1.3) (Masse & Gottesman, 2002). Portions of the sequence within RyhB are complementary to regions within each of its target genes. RyhB pairs with the *sdhCDAB* and *sodB* transcripts preventing translation of these mRNAs leading to the degradation of the transcripts. Fur directly represses *ryhB* transcription when cellular iron levels are high, allowing for the efficient translation of *sdhCDAB* and *sodB* (Masse & Gottesman, 2000). Most of the eighteen transcripts that code for fifty six proteins repressed by RyhB in *E. coli* code for iron dependent proteins (Masse et al., 2005). By repressing genes coding for non-essential proteins during iron starvation, RyhB activity prevents the production of iron dependent proteins beyond the cells ability to equip them with iron, allocating the limited available cellular iron for essential proteins such as those involved in nucleic acid and amino acid production whose genes are not regulated by RyhB (Masse et al., 2007).

DtxR

Gram-positive bacteria rely on the transcriptional repressor DtxR (diphtheria toxin regulatory protein) to regulate an array of genes whose products are involved in siderophore biosynthesis and iron acquisition (Boyd et al., 1990, 1990; Tao et al., 1994). Fur and DtxR share only 25% homology with each other but function in a similar fashion, requiring iron as a co-factor and binding directly to promoter regions of the target genes being repressed (Tao et al., 1992).

Mur

Fur homologs are present in most species of the alpha-proteobacteria, including *Brucella*, however, Fur is not the primary iron-responsive transcriptional regulator in these bacteria. In the alpha-proteobacteria, the Fur homolog has been renamed Mur (manganese-responsive regulator) because it is a manganese-responsive regulator and acts as a repressor of genes coding for manganese transport (*mntH*) (Diaz-Mireles, et al., 2004; Hohle & O'Brian, 2012; Menscher et al., 2012; Platero et al., 2007). The closest Fur homolog from the alpha-proteobacteria member *Bradyrhizobium japonicum* was originally identified by its ability to complement an *E. coli fur* mutant (Hamza et al., 1999). The only genes known to be directly regulated by the *B. japonicum* Fur homolog are the genes coding for the iron response regulator (Irr) and the high affinity manganese transporter (MntH) (Hohle & O'Brian, 2009; Hole & O'Brian, 2010).

RirA

The rhizobial iron regulator (RirA) was first identified and characterized as an iron-responsive transcriptional repressor in *Rhizobium leguminosarum* (Todd et al., 2002). A *R.*

leguminosarum rirA mutant exhibits constitutive expression of a number of operons whose products are involved in the synthesis or uptake of siderophores, or in the acquisition of other iron sources, that are normally repressed when cellular iron levels are high (Todd et al., 2002). There are RirA homologs in many of the alpha-proteobacteria, including *Sinorhizobium*, *Rhizobium*, *Mesorhizobium*, *Agrobacterium*, *Bartonella*, and *Brucella*, but not in *Bradyrhizobium* (Todd et al., 2002 & Rodionov et al., 2006).

There is no significant sequence homology between RirA and Fur, but RirA does share homology with the Rrf2 family of proteins (Todd et al., 2002; Johnston et al., 2007). IscR (iron sulfur cluster regulator) is a member of the Rrf2 family, and this protein represses the expression of genes involved in the assembly of iron sulfur clusters in proteins. IscR is itself an iron sulfur cluster protein and only binds to its target DNA sequences when charged with iron (Giel et al., 2006). RirA is thought to act in a similar manner to IscR in which, as cellular iron levels rise, RirA becomes co-activated by iron and binds to conserved DNA sequences called RirA-boxes within the promoter regions of target genes, resulting in transcriptional repression (Todd et al., 2005; Rodinov et al., 2006).

Irr

Irr (iron-responsive regulator) is a Fur homolog that was originally identified and has been most well studied in *Bradyrhizobium japonicum* where it was defined as a regulator of *hemB*, a gene coding for a heme biosynthesis enzyme. Transcription of *hemB* is repressed during growth under iron deplete conditions, and this iron-responsive control is lost in an *irr* mutant (Hamza et al., 1998). While it was originally characterized as a regulator of heme biosynthesis, a link between Irr and iron metabolism in general was later uncovered, indicating that Irr is an

important iron-responsive transcriptional regulator (Hamza et al., 2000; Wexler et al., 2003; Johnston et al., 2007). Whereas Fur and RirA are active regulators during growth in iron replete conditions, Irr is active as a transcriptional regulator when cells are grown under iron deplete conditions, where the protein binds to conserved sequences call ICE motifs (iron control elements) near the promoters of genes that are under its control (see Figure 1.4) (Rudolph et al., 2006). Irr has the ability to act as a positive or negative regulator in response to iron starvation. When the ICE motifs are in close proximity to the promoters of target genes, Irr is a repressor, physically blocking or impeding the DNA-dependent RNA polymerase from transcribing the gene. In contrast, when the ICE motifs are located upstream of the target promoter region, Irr is an activator (Rudolph et al., 2006; Johnston et al., 2007). The mechanism of Irr activator activity has not yet been determined, but it is believed to either recruit the DNA-dependent RNA polymerase to the promoter or alter the secondary structure of the DNA allowing the DNA-dependent RNA polymerase access to the promoter.

Bradyrhizobium japonicum Irr

The most well understood model describing how the activity of Irr is controlled in the alpha-proteobacteria is in *Bradyrhizobium japonicum*, which is a symbiont of soybean roots (see Figure 1.5). Whereas Fur directly senses intracellular iron levels through iron binding and subsequent activation of the protein, Irr responds to iron indirectly, through the biosynthesis of heme, whose intracellular concentration is correlated with internal iron availability. While heme can interact directly with Irr, the interaction is much more efficient when delivered by the enzyme ferrochelatase (Qi & O'Brian, 2002) (see Figure 1.5). The enzymatic activity of

ferrochelatase is to insert iron into protoporphyrin IX, making a heme molecule. When cellular iron levels are high, ferrochelatase binds to Irr and inhibits its activity (Qi & O'Brian, 2002). In these conditions, ferrochelatase synthesizes heme which then also binds to Irr, resulting in its degradation and derepression of the heme biosynthesis pathway. When cellular iron levels are low, ferrochelatase only binds to protoporphyrin IX, relieving the inhibition of Irr by ferrochelatase and Irr represses the heme biosynthesis pathway (Qi & O'Brian, 2002). Ferrochelatase binds directly to Irr, so when the heme molecule is made it is in close proximity to interact with, inactivate and degrade the Irr protein (Qi & O'Brian, 2002). This mechanism allows heme to act as a signaling molecule that cellular iron levels are rising without the need for cellular heme to accumulate in cells. Because Irr directly represses heme biosynthesis, heme biosynthesis and iron levels are linked through Irr activity.

The rapid degradation of the Irr protein, which partially accounts for the inactivation of the protein in iron replete conditions involves oxidative damage (Yang et al., 2006a). However the protease responsible for its degradation has not yet been identified. The amino acid residues that are important for heme binding and subsequent degradation have been identified in the *B. japonicum* Irr protein. There is a heme regulatory motif (HRM) that is unique to *B. japonicum* Irr that binds to oxidized heme (Qi et al., 1999; Yang et al., 2004). The HRM is required for normal, rapid iron dependent degradation of Irr, but degradation can occur slowly without the HRM in a heme dependent manner (Yang et al., 2004). A second, internal HXH heme binding motif that is highly conserved among the alpha-proteobacteria Irr proteins, binds to heme in the reduced form. Mutations in this motif result in a stable Irr protein regardless of cellular iron or heme levels (Yang et al., 2004). Because the ability to bind to both oxidized and reduced heme is necessary for efficient Irr degradation, it is believed that the redox activity of heme may be

important for Irr turnover. Heme can react with oxygen to form reactive oxygen species that damage proteins (Aft & Mueller, 1984) which leads to the proposition that heme may catalyze the oxidation of Irr via a redox event with one heme molecule to one Irr protein, which is then targeted for degradation by a protease.

B. japonicum Irr affinity for ICE motifs (iron control elements) varies depending on the target gene (Jaggavarapu & O'Brian, 2014) (see Figure 1.6). Genes that are positively regulated by Irr, such as genes coding for products that are involved in iron acquisition, exhibit low Irr binding affinity to their ICE motifs. For negatively regulated genes, Irr affinity is high for genes important in managing stress associated with iron dependent chemistry, such as those coding for iron export or iron storage proteins, where strong repression is appropriate under low or moderate iron levels. Irr affinity is low for negatively regulated housekeeping genes that utilize iron for their activity such as those involved in heme biosynthesis whose expression must be maintained for growth. As cellular iron levels begin to rise, so too does heme biosynthesis. Heme binding to Irr partially inactivates the protein preventing it from regulating genes with low affinity binding sites while maintaining the ability to repress at the high affinity binding sites. Finally when cellular iron levels are high, Irr is degraded and there is a complete loss of repression of negatively regulated genes.

Rhizobium leguminosarum Irr

Rhizobium leguminosarum is a member of the alpha-proteobacteria and is a symbiont of peas, beans and clovers. *R. leguminosarum* genes that exhibit iron-responsive regulation are modulated by RirA and Irr (see Figure 1.5) (Todd et al., 2002; Todd et al., 2005). *R.*

leguminosarum RirA plays a more prominent role than Irr and is a repressor of genes whose products are involved in the synthesis or uptake of siderophores, heme, or other iron sources when cellular iron levels are high (Todd et al., 2002; Todd et al., 2005). When cellular iron levels are low, the *R. leguminosarum* Irr protein is a repressor of genes coding for products that require iron for their function and has no effect on the expression of genes coding for iron uptake systems (Todd et al., 2006). Both RirA and Irr respond to cellular iron levels indirectly. By linking RirA activity with iron sulfur cluster biogenesis, and Irr with the biosynthesis of heme, *R. leguminosarum* can regulate, to a very fine level, the expression of iron metabolism genes in response to cellular iron levels without the excessive accumulation of iron.

In the *R. leguminosarum* model, Irr is a stable protein in the presence of iron or heme and its regulatory activity on iron-responsive genes is controlled through loss of DNA binding activity upon heme binding (Singleton et al., 2010). *R. leguminosarum* Irr lacks the HRM found in *B. japonicum*, but does have the conserved HXH motif (Singleton et al., 2010) along with a second heme binding site that is involved in the oligomerization and inactivation of Irr (White et al., 2011). The difference between *B. japonicum* and *R. leguminosarum* Irr activity suggests that there are at least two distinct paradigms explaining how the regulatory activity of Irr and iron-responsive gene regulation in general, is controlled in the alpha-proteobacteria (see Figure 1.5).

Agrobacterium tumefaciens Irr

Agrobacterium tumefaciens is a plant pathogen and is a member of the alpha-proteobacteria. Similar to *B. japonicum*, *A. tumefaciens* Irr has the ability to repress the transcription of genes coding for iron utilizing processes, such as heme biosynthesis and iron sulfur cluster biosynthesis while activating genes coding for iron uptake systems when cellular

iron levels are low (Hibbing & Fuqua, 2011). *A. tumefaciens* also has a functional RirA protein and the *rirA* gene is directly repressed by Irr (Hibbing & Fuqua, 2011). Irr and RirA exhibit opposing and interlinked control of iron-responsive genes, with their regulons partially overlapping (Hibbing & Fuqua, 2011). Irr activity ensures that expression of genes coding for iron uptake systems occurs when cellular iron levels are low so the cell can maintain sufficient iron levels for physiological functions, while RirA is active when cellular iron levels are high ensuring that iron levels do not rise to dangerous levels. Having the ability of Irr to function when cellular iron levels are low and RirA functioning when cellular iron levels are high allows *A. tumefaciens* to have tight control over genes in response to cellular iron levels.

A. tumefaciens Irr lacks the HRM found in the *B. japonicum* Irr, but does appear to be degraded in response to rising cellular iron levels (Sukchawalit & Mongolsuk, 2014). The HXH heme binding motif is conserved in *A. tumefaciens* and mutation of these residues results in a form of Irr that has significantly diminished repressor activity, however its role in promoting Irr degradation has yet to be determined (Bhubhanil et al., 2012).

***Brucella* Irr**

The *Brucella irr* gene was originally identified by searching the *Brucella* genome for the open reading frame with the highest similarity to the *B. japonicum irr* gene (Martinez et al., 2005). The *Brucella irr* gene product was shown have the ability to repress heme biosynthesis and activate the expression of the genes coding for siderophore biosynthesis enzymes when the cells were grown under low iron conditions (Martinez et al., 2005; Martinez et al., 2006). These findings led to the proposition that Irr is a global iron-responsive regulator in *Brucella*. The

Brucella genome also encodes a RirA protein, and because the *Brucella* Irr protein directly regulates the expression of the genes coding for siderophore biosynthesis, it is possible that the activity of these two transcriptional regulators oppose one another and that their regulons may overlap as observed in *A. tumefaciens* (Hibbing & Fuqua, 2011).

While *Brucella irr* expression is not modulated by cellular iron levels, Irr protein is only detected when cellular iron levels are low (Martinez et al., 2005). The *Brucella* Irr protein also has the ability to bind to heme, suggesting that the activity of Irr may be controlled by a similar mechanism to that observed in *B. japonicum* (Martinez et al., 2005). *Brucella* Irr, like that of *R. leguminosarum* and *A. tumefaciens*, lacks the HRM that is associated with binding to the ferric form of heme and the turnover of Irr in *B. japonicum*. Because *Brucella* lacks the HRM, but does have the conserved HXH ferrous heme binding motif, elucidating how the activity of *Brucella* Irr is controlled will be an important contribution toward understanding iron-responsive gene regulation in the alpha-proteobacteria. Although through different mechanisms, the conserved HXH heme binding motif is important for controlling the activity of Irr from many different bacterial species in the alpha-proteobacteria, which may reflect divergent evolution of Irr from a common ancestor.

Iron source "availability"-responsive regulators

Pathogens encounter different iron sources in a host at different times during an infection. Genes coding for iron acquisition systems are often responsive to cellular iron levels and the presence of the cognate iron substrate. In the case of Fur regulated genes, de-repression of genes coding for iron acquisition systems occurs when cellular iron levels are low and a basal level of

transcription occurs. Optimal expression of these genes is dependent on a second signal, usually the substrate to be transported, and this regulation is mediated by a transcriptional activator. Priority regulation of iron acquisition systems according to iron source availability may allow faster adaptation to a changing host environment and thus a greater degree of pathogenic success.

DhbR

Most bacteria rely on Fur or Fur-like transcriptional repressors to regulate siderophore production and uptake (Crosa, 1997). It is not uncommon for bacteria to regulate siderophore biosynthesis at a second level where siderophore that has been transported back into the cell after chelating iron acts as a coactivator for induction of the siderophore biosynthesis genes and for the genes required for siderophore uptake (Brickman et al., 2001). A member of the AraC-like transcriptional activators called AlcR regulates genes whose products are involved in siderophore biosynthesis in *Bordetella pertussis* and *Bordetella bronchiseptica* using iron bound siderophore as a coactivator (Beaumont et al., 1998). Regulating the expression of the genes coding for siderophore biosynthesis and transport at two levels makes biological sense as a way to prevent the futile expression of genes coding for siderophore biosynthesis and transport proteins until the appropriate iron substrate is available in the environment. As part of the global iron starvation response, when cellular iron levels are low, Fur repression is abrogated, allowing for the de-repression, but not full activation, of genes coding for iron acquisition systems. Iron loaded siderophore is transported back into the cell and acts as a co-inducer for AlcR type transcriptional activators resulting in maximal expression of the genes coding for siderophore biosynthesis and transport.

The *dhbR* gene in *B. abortus* 2308 encodes a protein that exhibits significant homology with the *Bordetella* AlcR protein (Anderson et al., 2008). During growth under iron-deprived conditions, a *B. abortus dhbR* mutant exhibits 40% less siderophore production than the wild type strain. A genetic link between the decreased siderophore production and the absence of *dhbR* was made through the observation that the β-galactosidase activity produced by a *dhbC-lacZ* fusion in a *dhbR* or *dhbC* mutant is about half as much as produced in the wild type strain during growth in iron-deprived conditions (Anderson et al., 2008). Furthermore, recombinant DhbR binds directly to the promoter region of *dhbC*, showing that DhbR regulation is direct. Together these data indicate that DhbR is required for maximal expression of the genes coding for siderophore biosynthesis, and that siderophore acts as a co-inducer with DhbR during iron limitation (Anderson et al., 2008).

FecIR

In *Escherichia coli*, the ferric citrate uptake (Fec) system allows *E. coli* to grow on ferric citrate as the sole iron source. The *fec* genes that codes for Fec transport components are positively regulated by the extracytoplasmic function (ECF) σ factor FecI only under iron starvation conditions in the presence of the cognate substrate ferric citrate (Braun, 1997). ECF σ factors recruit DNA-dependent RNA-polymerase to specific genes and activate their expression in response to extracellular environmental signals (Brooks & Buchanon, 2008). The *fecI* and *fecR* genes are transcribed as a single operon and the expression of *fecIR* is repressed by Fur when intracellular iron levels are high (Braun, 1997). FecA is an outer membrane receptor that, upon recognition of extracellular ferric citrate, signals through the cytoplasmic transmembrane

protein FecR (Enz et al., 2000; Kim et al., 1997). FecR directly interacts with and activates FecI which then induces the expression of the *fec* genes (Enz et al., 2000; Mahren et al., 2002). Thus, when intracellular iron levels are high, Fur represses the expression of *fecIR*, so that there is no FecI to direct the DNA-dependent RNA polymerase to the promoter region of the genes coding for the Fec transport system. When intracellular iron levels drop, Fur is inactivated allowing for the synthesis of FecIR. When extracellular ferric citrate is present a signal is relayed from FecA, to FecR and finally to FecI, which in turn activates the transcription of the *fec* gene operon and ferric citrate can be transported into the cell. This mechanism ensures that the expression of the *fec* gene operon only occurs during low iron conditions in the presence of extracellular ferric citrate.

ChrSA

In *Corynebacterium diphtheriae*, the gene coding for the heme oxygenase HemO is repressed by iron through DtxR and activated by extracellular heme through ChrSA (Schmitt, 1997). DtxR binds directly to the *hemO* promoter to repress expression when cellular iron levels are high (Schmitt, 1997). Heme responsive activation of *hemO* is dependent on the two-component signal transduction system ChrSA (Schmitt, 1999). When cellular iron levels drop, DtxR is not active and derepression of *hemO* occurs resulting in a low level of expression. ChrSA recognizes when extracellular heme is present and activates the now derepressed *hemO* gene. The sensor kinase encoded by *chrS* recognizes extracellular heme. Upon detection of heme, ChrS is predicted to, like other sensor kinases, undergo autophosphorylation. The phosphoryl group can then be transferred to a residue on ChrA, which in turn activates ChrA allowing it to activate transcription of the *hmuO* promoter. This mechanism ensures that efficient

transcription of *hemO* only occurs when cellular iron levels are low, and in the presence of extracellular heme.

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Figure 1.1 *Brucella* intracellular trafficking in the macrophage. The brucellae gain entry into macrophages through lipid rafts and the *Brucella* containing vacuoles (BCVs) enter into an intracellular trafficking pathway. The BCVs undergo transient interactions with lysosomes, resulting in their acidification. The BCVs then interact extensively with the endoplasmic reticulum and their intracellular pH rises to a level that allows for intracellular replication of the brucellae (Adapted from Roop et al., 2009).

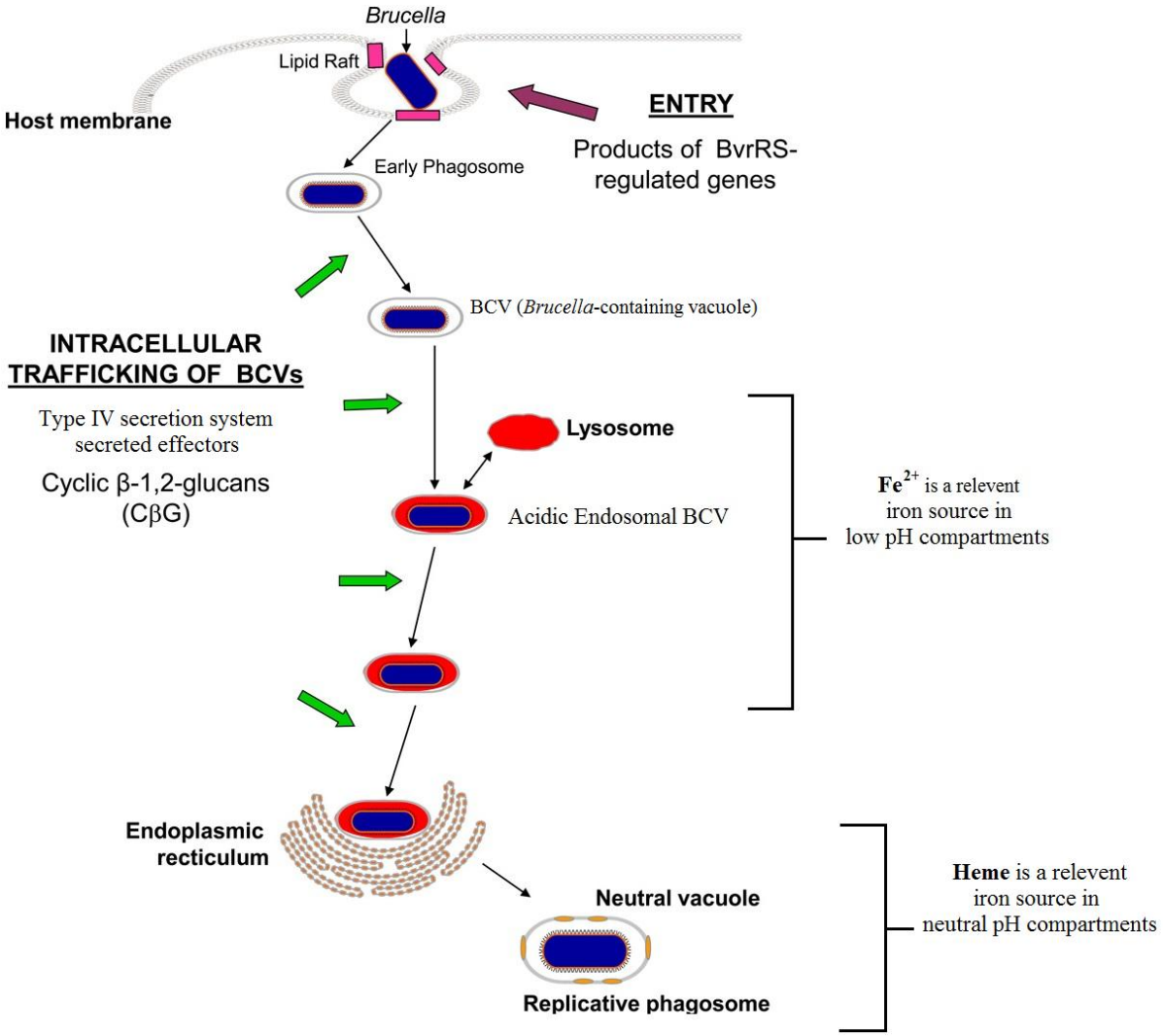
Brucella
Lipid Raft
Host membrane
ENTRY
Products of BvrRS-regulated genes
Early Phagosome
BCV (Brucella-containing vacuole)
INTRACELLULAR TRAFFICKING OF BCVs
Type IV secretion system secreted effectors
Cyclic β-1,2-glucans (CβG)
Lysosome
Acidic Endosomal BCV
Fe2+ is a relevent iron source in low pH compartments
Endoplasmic recticulum
Neutral vacuole
Replicative phagosome
Heme is a relevent iron source in neutral pH compartments

Figure 1.1

Figure 1.2 Iron acquisition systems in *Brucella*. The siderophores 2,3-DHBA and brucebactin are secreted into the extracellular environment where they bind to iron and are then transported back into the cell by either the CirA/FepB/FepCD or Fiu/FatB/FatCDE systems. Heme is transported through the outer membrane by BhuA and through the inner membrane by BhuT and BhuUV where iron is removed from heme by BhuQ to be utilized as an iron source. The FtrABCD system transports Fe^{2+} into the cell. The SfuBC-1 system is predicted to transport ferric citrate into the cell. A functional siderophore transport system is required for wild-type virulence in the cattle and goat model of acute infection, and functional heme and Fe^{2+} transport systems are required for wild-type virulence in the mouse model of chronic infection (Adapted from Roop et al., 2012).

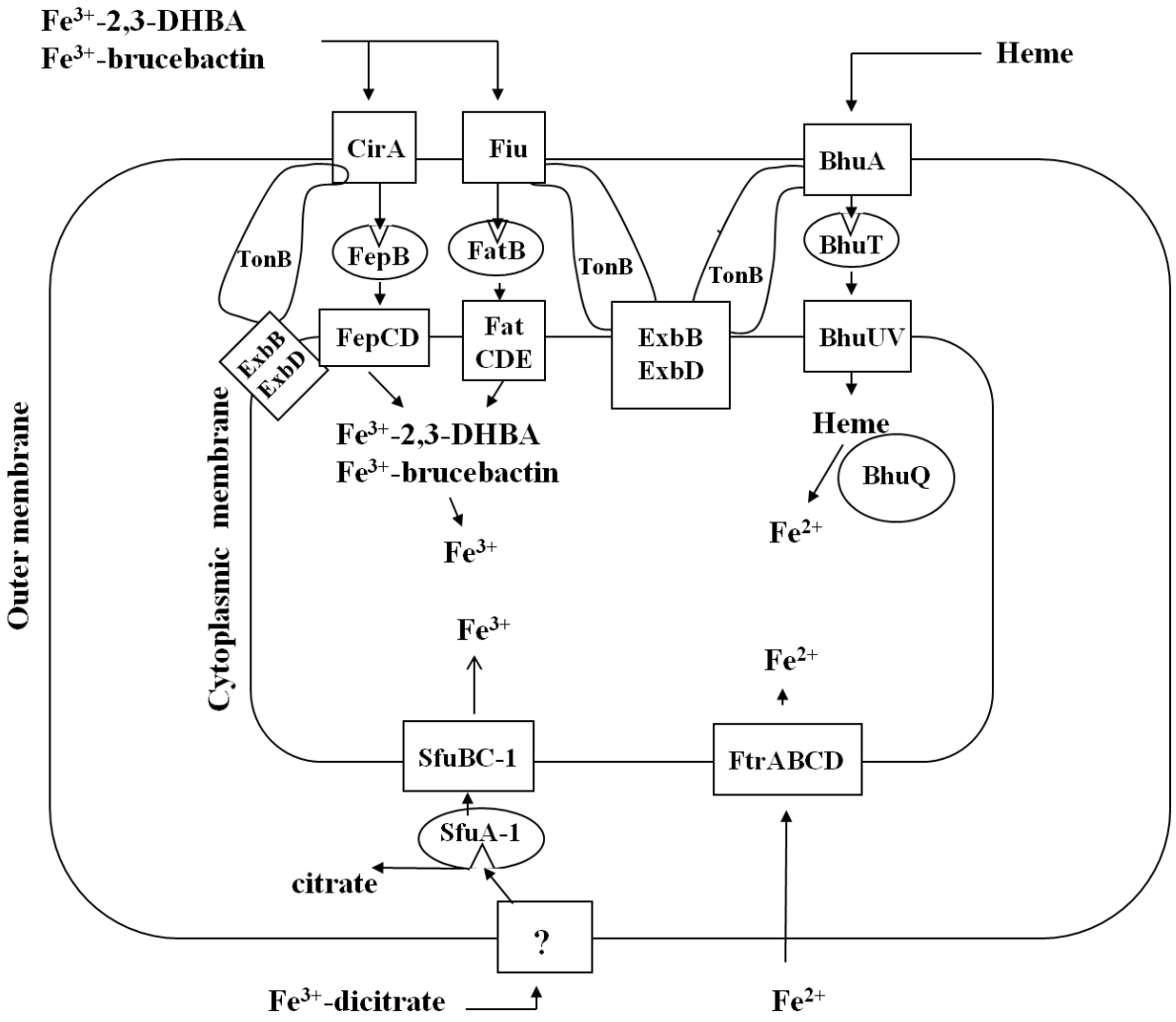
Fe3+-2,3-DHBA
Fe3+-brucebactin
Heme
CirA
Fiu
BhuA
TonB
FepB
FatB
TonB
TonB
BhuT
ExbB
ExbD
FepCD
Fat
CDE
ExbB
ExbD
BhuUV
Outer membrane
Cytoplasmic membrane
Fe3+-2,3-DHBA
Fe3+-brucebactin
Heme
BhuQ
Fe3+
Fe2+
Fe3+
Fe2+
SfuBC-1
FtrABCD
SfuA-1
citrate
?
Fe3+-dicitrate
Fe2+

Figure 1.2

Figure 1.3 Direct Fur repression and Fur activation of gene expression through the sRNA RyhB. Under iron replete conditions, Fur forms a homodimer and binds to the Fur binding sites in the promoter regions of genes coding for iron uptake systems and *rhyB* blocking access to the promoters by the DNA-dependent RNA polymerase resulting in repression of transcription. Under these conditions, *sodB* mRNA is stable, and the synthesis of SodB occurs. Under iron deplete conditions Fur is inactive, allowing for the depression of genes coding for iron uptake systems and *ryhB*. The RNA chaperone Hfq binds to RyhB and to the target mRNA of *sodB* resulting in RNase dependent cleavage and a reduction in the half life of *sodB* mRNA and consequently the repression of SodB synthesis.

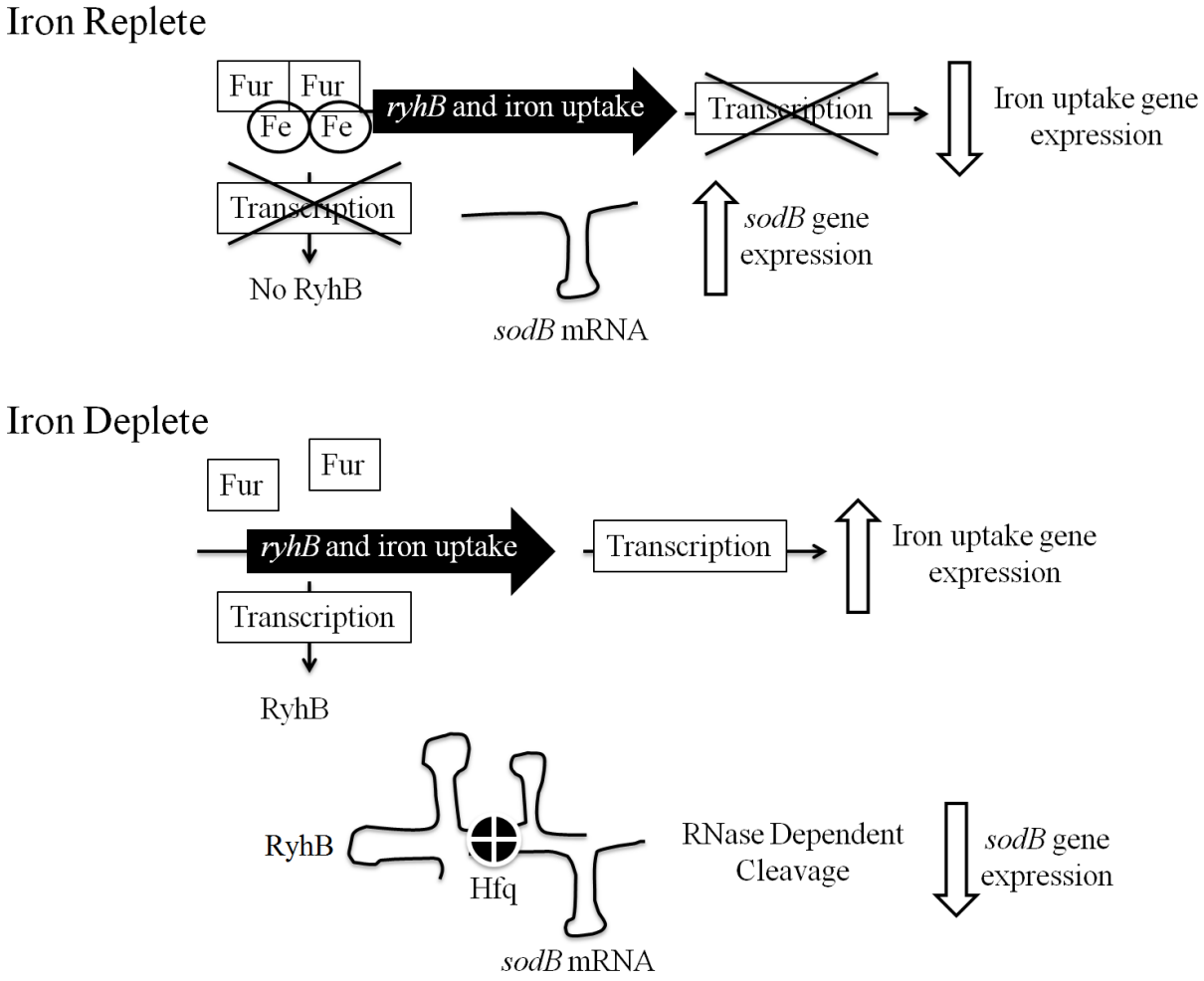
Iron Replete
Fur
Fur
Fe
Fe
ryhB and iron uptake
Transcription
Iron uptake gene expression
Transcription
No RyhB
sodB gene expression
sodB mRNA
Iron Deplete
Fur
Fur
ryhB and iron uptake
Transcription
Iron uptake gene expression
Transcription
RyhB
RyhB
Hfq
RNase Dependent Cleavage
sodB gene expression
sodB mRNA

Figure 1.3

Figure 1.4 Predicted ICE motif (iron control element) consensus sequence. A motif with palindromic symmetry predicted to be a conserved Irr binding site was identified by analyzing the upstream regions of genes involved in iron homeostasis in the *Rhizobiacease* family and *Mesorhizobium*, *Brucella*, and *Bartonella* genera derived from the program SignalX (adapted from Rodionov et al., 2006).

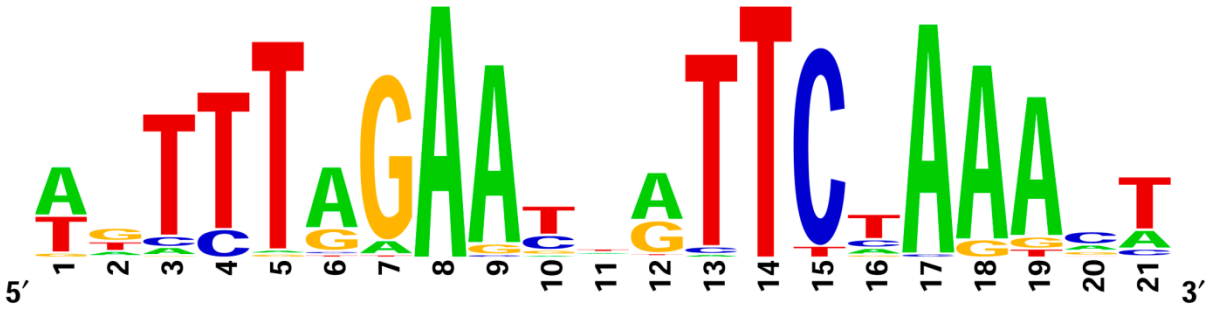

Figure 1.4

Figure 1.5 Model of iron-responsive gene regulation in *Bradyrhizobium japonicum* and *Rhizobium leguminosarum*. Irr is the sole iron-responsive transcriptional regulator in *B. japonicum*. When cellular iron levels are low, Irr activates the expression of genes coding for iron acquisition systems, and represses the heme biosynthesis pathway. When cellular iron levels are high, the enzymatic activity of ferrochelatase delivers heme to Irr resulting in its degradation. In *R. leguminosarum*, RirA is the predominant iron-responsive transcriptional regulator with Irr regulating a small set of genes. When cellular iron levels are low, RirA is not active and derepression of genes coding for iron acquisition systems occurs and Irr represses a small set of genes that encode products that require iron for their function. When cellular iron levels are high, Irr is inactive and derepression of its regulon occurs. RirA is active in these conditions where it represses the expression of genes that encode iron acquisition systems.

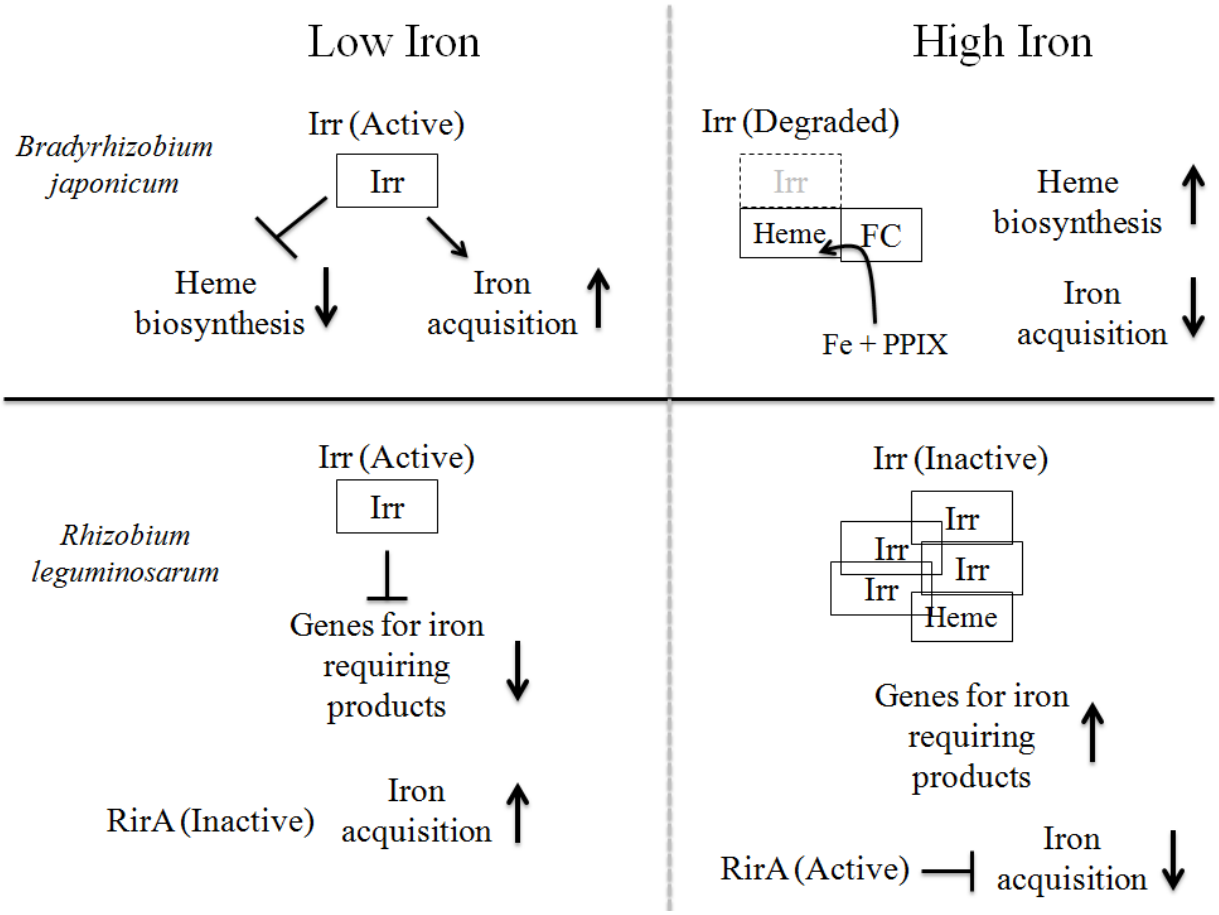
Low Iron
High Iron
Bradyrhizobium japonicum
Irr (Active)
Irr
Heme biosynthesis
Iron acquisition
Irr (Degraded)
Irr
Heme
FC
Fe + PPIX
Heme biosynthesis
Iron acquisition
Rhizobium leguminosarum
Irr (Active)
Irr
Genes for iron requiring products
RirA (Inactive)
Iron acquisition
Irr (Inactive)
Irr
Irr
Irr
Irr
Heme
Genes for iron requiring products
RirA (Active)
Iron acquisition

Figure 1.5

Figure 1.6 The activity of *Bradyrhizobium japonicum* Irr is controlled by ICE motif (iron control element) binding affinity, Irr inactivation and Irr degradation. When cellular iron levels are low, Irr levels are high. Irr binds to ICE motifs upstream of the promoter region of genes it activates and on the promoter regions of genes it represses. As cellular iron levels rise, so too does the biosynthesis of heme. As heme is made and delivered to Irr by ferrochelatase it partially inactivates Irr. Genes that were activated by Irr when cellular iron levels were low become deactivated. The genes repressed by Irr binding to high affinity ICE motifs stay repressed, whereas those with weak affinity are derepressed. When cellular iron levels reach a threshold level Irr is degraded and genes with high affinity ICE motifs are derepressed.

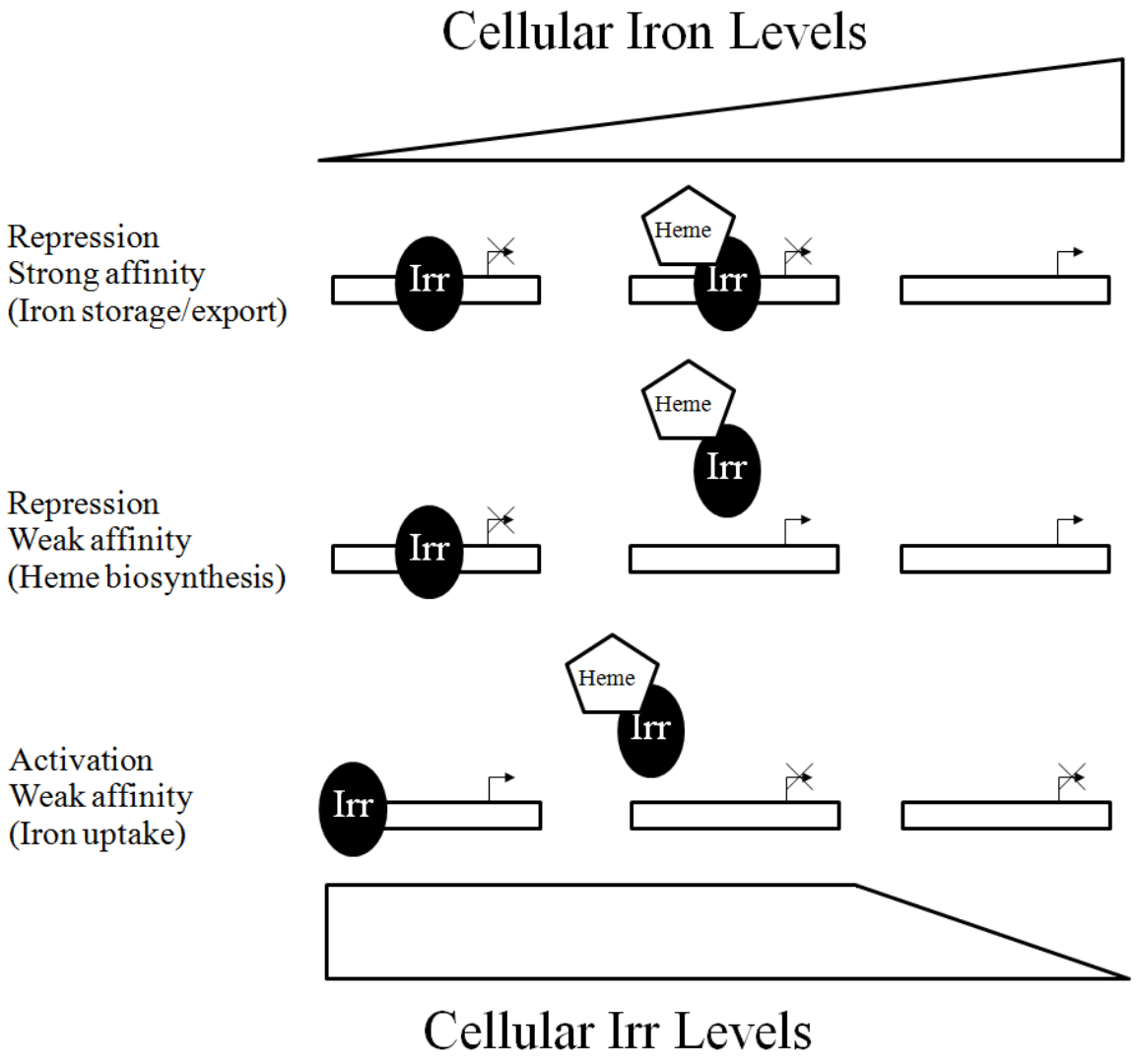
Cellular Iron Levels
Repression
Strong affinity
(Iron storage/export)
Heme
Irr
Irr
Repression
Weak affinity
(Heme biosynthesis)
Heme
Irr
Irr
Activation
Weak affinity
(Iron uptake)
Heme
Irr
Irr
Cellular Irr Levels

Figure 1.6

Statement of the problem

Brucella pathogenesis depends on the ability of the brucellae to survive and replicate within host phagocytic cells. Because of this intracellular lifestyle, the brucellae must obtain nutrients required for growth, such as iron, from the host. As part of the innate immune defense to limit the replication of invading pathogens, host cells restrict the availability of iron by keeping iron bound in iron sequestering proteins. Upon activation of host macrophages by INF-γ, iron restriction in phagosomal compartments become exacerbated though the reduced production of transferrin receptors on the surface of the macrophage and increased activity of Nramp1 on the surface of the phagosomal compartments that transports iron out of the phagosomes, further restricting the availability of iron to invading pathogens. To overcome iron restriction, intracellular pathogens utilize highly efficient iron acquisition systems to transport host derived iron into their cells. *Brucella* strains have the capacity to utilize siderophore mediated iron, ferrous iron, and heme transport systems to acquire sufficient iron for their physiological functions.

While iron is required for pathogens to establish and maintain infections in their hosts, excessive intracellular iron levels are detrimental to the bacteria due to the production of hydroxyl radicals that can damage biological molecules such as DNA, proteins and lipids. For this reason, the expression of genes involved in iron acquisition, use, and storage are tightly controlled. In the alpha-proteobacteria, Irr (iron-responsive regulator) and/or RirA (rhizobial iron regulator) are responsible for modulating gene expression in response to cellular iron levels. Two major questions about *Brucella* iron-responsive gene regulation were addressed in the work

described in this dissertation. First, we wanted to determine if Irr is the predominant iron-responsive transcriptional regulator in *Brucella* and its role as a virulence determinant because the expression of the genes coding for the *Brucella* siderophore biosynthesis enzymes have been previously been shown to be under the control of Irr. Second, there are two paradigms in the literature describing how the activity of Irr is controlled in different members of the alpha-proteobacteria. In both models, the ability of Irr to control iron homeostasis is tied to the cells capacity to synthesize heme. As cellular iron levels rise, so too does the production of heme. In the model characterized in *Bradyrhizobium japonicum*, heme is delivered to Irr through the enzymatic activity of ferrochelatase when cellular iron levels are high, resulting in the inactivation and degradation of the Irr protein. Alternatively, in the *Rhizobium leguminosarum* model, Irr is not degraded, but when cellular iron levels are high, heme interacts with the Irr protein resulting in its oligomerization and inactivation. The mechanism controlling Irr regulatory activity in *Brucella* was analyzed to determine which paradigm *Brucella* most closely follows.

Because the requirement for the synthesis of different *Brucella* iron transport systems varies depending on the cell type being infected, and because the intracellular lifestyle and resulting oxidative stress makes *Brucella* strains especially prone to iron toxicity, knowing how the pattern of gene expression in response to cellular iron levels is controlled is important for understanding *Brucella* pathogenesis.

Chapter 2: The iron-responsive regulator Irr is required for wild-type expression of the gene encoding the heme transporter BhuA in *Brucella abortus* 2308

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Journal of Bacteriology. 2011. **193**: 5359-5364

DOI: 10.1128/JB.00372-11

Martinson, DA Contribution to this Chapter

A major component of the study described in this paper was the analysis of the stability of Irr in response to cellular iron levels. There are two paradigms presented in the literature that describe how the activity of Irr is controlled in response to cellular iron levels. In the *Bradyrhizobium japonicum* model, Irr is degraded when cellular iron levels rise through its interaction with ferrochelatase and heme. *B. japonicum* Irr degradation is mediated through heme binding to Irr at a unique heme regulatory motif (HRM), and a second heme binding motif (HXH) that is conserved in Irr among the alpha-proteobacteria. Alternatively, in *Rhizobium leguminosarum*, which lacks an HRM, Irr is inactivated and multimerizes in response to rising cellular iron levels. Based on a comparison of an amino acid alignment of the *B. japonicum, R. leguminosarum*, and *Brucella* Irr proteins, it was predicted that the *Brucella* Irr protein would be stable because it also lacks the HRM found in *B. japonicum*. I found that detectable levels of Irr were found only in *Brucella* cells by Western blot analysis following growth under iron-limiting conditions, as shown in Figure 2.7. These findings were highly suggestive that *Brucella* Irr follows the *B. japonicum* Irr degradation model in response to cellular iron levels independent of an HRM. This finding countered the idea that the reason for *B. japonicum* Irr degradation and *R. leguminosarum* Irr stability in response to cellular iron levels was because of the presence or lack of an HRM, respectively. This set the stage for the rest of my project directed at analyzing the role of the conserved HXH heme binding motif in controlling the activity of the *Brucella* Irr protein through degradation in response to cellular iron levels.

Abstract

Irr and RirA, rather than Fur, serve as the major iron-responsive regulators in the α-proteobacteria. With only a few exceptions, however, the relative contributions of these transcriptional regulators to the differential expression of specific iron metabolism genes in *Brucella* strains is unclear. The gene encoding the outer membrane heme transporter BhuA exhibits maximum expression in *Brucella abortus* 2308 during growth under iron deprived conditions, and mutational studies indicate that this pattern of *bhuA* expression is mediated by the iron-responsive regulator Irr. Specifically, a *bhuA-lacZ* transcriptional fusion does not produce elevated levels of β-galactosidase in response to iron deprivation in the isogenic *irr* mutant BEA5, and unlike the parental strain, *B. abortus* BEA5 cannot utilize heme as an iron source in *in vitro* and is attenuated in mice. A derivative of the *bhuA-lacZ* transcriptional fusion lacking the predicted Irr binding site upstream of the *bhuA* promoter does not produce elevated levels of β-galactosidase in response to iron deprivation in the parental *B. abortus* 2308 strain, and a direct and specific interaction between a recombinant version of the *Brucella* Irr and the *bhuA* promoter region was observed in an electrophoretic mobility shift assay. Despite the fact that it lacks the heme regulatory element linked to the iron-responsive degradation of its counterpart in *Bradyrhizobium japonicum*, readily detectable levels of Irr were only found in *B. abortus* 2308 cells by Western blot analysis following growth under iron deprived conditions.

Introduction

Brucella abortus is a Gram-negative intracellular pathogen that causes abortion and infertility in its natural bovine host. Incidental infection in humans results in a prolonged illness known as undulant fever. As with most bacteria, iron is an essential micronutrient for *Brucella* strains (Evenson et al., 1995; Waring et al., 1953). This requirement for iron presents a particular challenge for the brucellae, as in nature these bacteria are found exclusively in association with mammalian hosts (Moreno & Moriyon, 2002; Roop et al., 2009). Iron not incorporated into host tissues is sequestered by host iron-binding proteins such as transferrin and lactoferrin in extracellular spaces and by ferritin within host cells (Griffiths, 1999). This tight sequestration of iron in mammals serves to prevent iron toxicity in the host, as well as limiting the availability of this nutrient to invading microbes. Indeed, iron restriction plays an important role in both the innate and acquired immune responses of the host (Cellier et al., 2007; Nemeth et al., 2004; Weinberg, 1995; Weiss, 2005).

Previous studies have shown that *B. abortus* 2308 can use heme as an iron source (Paulley et al., 2007). The TonB-dependent outer membrane transporter BhuA is required for heme utilization by this strain *in vitro*, and an isogenic *bhuA* mutant cannot maintain a chronic spleen infection in experimentally infected mice. Like most other bacterial genes that encode heme transporters, *bhuA* expression in *B. abortus* 2308 is maximal during growth under iron deprived conditions (Paulley et al., 2007), but the regulator responsible for the iron-responsive nature of *bhuA* expression is unknown. A logical candidate for this regulatory role is the iron-responsive regulator (Irr), a member of the Fur family of transcriptional regulators. Irr was initially identified in *Bradyrhizobium japonic*um as a repressor of *hemB*, a gene involved in heme biosynthesis (Hamza et al., 1998). The stability of the *B. japonicum* Irr is modulated by cellular

iron levels. When sufficient iron is available to allow heme biosynthesis, Irr is degraded and *hemB* repression is relieved. Iron-responsive degradation of Irr requires direct interaction with ferrochelatase (Qi & O'Brian, 1999), which allows Irr to coordinate the activity of the heme biosynthetic pathways with the cellular availability of iron (Small et al., 2009). Further studies have linked Irr to the regulation of numerous iron metabolism genes (including those involved in heme acquisition) in *B. japonicum* (Rudolph et al., 2006; Yang et al., 2006), *Rhizobium leguminosarum* (Todd et al., 2006), and *Bartonella quintana* (Parrow et al., 2009).

Experimental evidence indicates that Irr can serve as either a repressor or an activator (Rudolph et al., 2006; Yang et al., 2006). Since Irr is functional when cellular iron levels are low, Irr often serves as an activator of genes involved in iron acquisition in the α–proteobacteria (Small et al., 2009). In contrast, Irr generally represses genes in these bacteria that encode proteins that require iron for their activity, proteins that participate in cellular processes that require iron (e.g. heme biosynthesis), or iron storage proteins (Small et al., 2009). Recent studies have shown that Irr regulates siderophore and heme biosynthesis genes in *B. abortus* 2308 (Martinez et al., 2005; Martinez et al., 2006), and the studies described in this report were designed to investigate the role of this regulator in the iron-responsive expression of the gene encoding the heme transporter BhuA in this strain.

Materials and Methods

Culture media and growth conditions.

Routine cultivation of *Escherichia coli* strains was carried out in Luria-Bertani (LB) broth or on tryptic soy agar (TSA) plates with appropriate antibiotic supplementation as necessary. *Brucella* strains were routinely grown in brucella broth (Difco) at 37°C or on Schaedler agar (Difco) supplemented with 5% defibrinated bovine blood incubated at 37°C under 5% CO_2. Ampicillin, chloramphenicol and kanamycin were added to these culture media as needed at final concentrations of 100, 5 and 45 μg/ml, respectively. Low iron minimal medium was prepared as previously described (Lopez-Goni et al., 1992), and 50 μM $FeCl_3$ was added to this medium as a control for growth under iron-replete conditions.

Construction and genetic complementation of *B. abortus irr* mutants.

A 2051 bp fragment containing the *irr* open reading frame (BAB1_2175) and flanking sequences was amplified from *B. abortus* 2308 genomic DNA using *Taq* DNA polymerase (primers: Fwd 5'- ACCGGCTTTCGGATCAAG-3', Rev 5'-GCGGCCGCATGAAAACTC-3') and ligated into the SmaI site of pBlueScript KS+ (Stratagene). The resulting plasmid was linearized with NruI and ligated to a 987 bp SmaI/HincII fragment containing the chloramphenicol resistance gene (*cat*) gene from pBlue-Cm2 (Robertson et al., 2000), or a 1.4 kb SmaI fragment containing the kanamycin resistance gene (*aph3a*) from pKS-kan (Kovach et al., 1995). These plasmids were then used to construct isogenic *irr* mutants from *B. abortus* 2308 via gene replacement using previously described procedures (Elzer et al., 1994). The genotypes of the resulting *B. abortus irr* mutants (designated BEA2 [*irr*::cat] and KH3 [*irr*::*aph3a*]) were

confirmed by PCR amplification of the mutated *irr* loci and restriction enzyme digestion and DNA sequence analysis of the amplified DNA fragments. To facilitate genetic complementation studies, the same 2051 bp *irr*-containing PCR fragment described above was also cloned into pGEM-T Easy (Promega), excised from the resulting plasmid by EcoRI digestion, and ligated with EcoRI digested pBBR1MCS-4 (Kovach et al., 1995). This plasmid, pKHS2, was introduced into *B. abortus* BEA2 by electroporation (Elzer et al., 1994).

Determination of the transcriptional start site for *bhuA* by primer extension.

The transcriptional start for the *bhuA* gene was determined by primer extension analysis performed on total RNA preparations obtained from *B. abortus* 2308 cultures grown for 120 h in low iron minimal medium using the primer 5'- TGATTTGATTC CAAATGGTTCC -3' and the methods described by Robertson et al. (Robertson et al., 2000).

Construction of *bhuA-lacZ* transcriptional fusions and β-galactosidase assays.

The construction of pbhuA-lacZ, a pMR15-based plasmid containing a *bhuA-lacZ* transcriptional fusion, has been previously described (Paulley, 2007). Derivatives of this plasmid carrying truncated versions of the *bhuA-lacZ* fusion with 29 bp and 120 bp 5' deletions were constructed by amplifying *B. abortus* 2308 genomic DNA with the forward primers 6a.5aF: 5'-CGCCGTGCCGTTTTCGCATAAA-3' and 5aF: 5'- TAGTCATCTTTATATCCCTCCC-3' in conjunction with the same reverse primer (5'- GACGGTGGCAAGCAGAGA-3') used to construct pbhuA-lacZ by PCR with *Pfu* polymerase, and cloning the resulting PCR fragments into pMR15 (Bellaire et al., 2003). The resulting transcriptional reporters, designated pbhuA6.5A-lacZ and pbhuA5A-lacZ, respectively, were introduced into *B. abortus* 2308 by

electroporation. Derivatives of *B. abortus* 2308 carrying pbhuA-lacZ, pbhuA6.5A-lacZ, pbhuA5A-lacZ and pMR15 were grown in 50 ml low iron minimal medium (Lopez-Goni et al., 1992) or low iron minimal medium supplemented with 50μM $FeCl_3$ in 250 ml Erlenmeyer flasks at 37ºC with shaking at 175 rpm. β-galactosidase production by these cultures was measured using the procedures described by Miller (Miller, 1972).

Capacity of the *B. abortus* strains to use heme as an iron source.

Free iron was removed from the hemin used to make stock solutions by treatment with 10 mM HCl (Staggs et al., 1991). To test for the capacity of hemin to serve as an iron source, *B. abortus* strains were grown on SBA for 48 h at 37° C with 5% CO_2. Bacterial cells were harvested into PBS (pH 7.2) and adjusted to an OD_{600nm} of 0.15 (=10^9 CFU/ml). One hundred (100) μl portions of these bacterial cell suspensions were then added to 250 ml flasks containing 50 ml low iron minimal medium. These flasks were incubated at 37°C with shaking at 250 rpm. Following 96 h of growth the bacterial cultures were adjusted to an OD_{600nm} of 0.15, and 100 μl of the bacterial cell suspensions mixed with 3 ml tryptic soy broth (TSB) containing 0.7% agar and 300 μM EDDA. This mixture was overlaid onto 100 mm plates containing TSB with 1.5% agar and 300 μM EDDA. Seven (7) mm sterile filter paper (Whatman no. 3) disks were placed onto the plates and 10 μl of a 20 mM solution of hemin or a 50 mM solution of $FeCl_3$ was added to the filter disks and the plates incubated at 37˚ C with 5% CO_2. Following 96 h incubation, the diameter of the zone of bacterial growth around each filter disk was measured and recorded in millimeters.

Production of recombinant *Brucella* Irr and electrophoretic mobility shift assays with the *bhuA* promoter region.

A 444 bp fragment encompassing the *irr* coding region (BAB1_2175) was amplified from *B. abortus* 2308 genomic DNA by PCR (primers F:5'- CGCGCGGAATT CGTGAATATGCATTCTTCACATACCCACTC-3', R: 5'- TCAGCGGGCCTGACGGCGCAG ACGCACAATGATATCCACAT-3') using *Pfu* polymerase and directionally cloned into the EcoRI and EcoRV sites of pASK-IBA6 (IBA). The resulting plasmid, pStrep-irr, produces a chimeric version of Irr carrying an OmpA secretion signal and Streptactin affinity tag on its N-terminus. pStrep-irr was introduced into *E. coli* strain DH5-α. Following the manufacturer's directions, the recombinant Irr protein was isolated from the periplasmic compartment of recombinant *E. coli* cultures and purified by passage over a Strep-Tactin Sepharose column.

For electrophoretic mobility shift assays, a 153 bp fragment encompassing the *bhuA* promoter region (primers: F 5'-ATAAGGCGACCTTACCGAG-3', R 5'-GCGACCGATCCGGGAGGG-3') was amplified from *B. abortus* 2308 genomic DNA by PCR with *Pfu* polymerase and radiolabeled with ^{32}P dATP using *Taq* polymerase as described previously (Anderson et al., 2007). Radiolabeled probe was purified using a Qiaquick Nucleotide Removal Kit (Qiagen) and re-suspended in 30 µl ddH_2O. For a specific DNA competitor, an unlabeled version of the same fragment was used. A 175 bp NcoI/EcoRI restriction digestion fragment from pBlue-Cm2 was employed as a non-specific competitor. Binding reactions contained 75 ng radiolabeled *bhuA* promoter DNA, 2 µg sheared herring sperm DNA, 10 µg BSA, 12 µl of binding buffer (10 mM Tris-HCl, pH 8, 40 mM KCl, 1 mM $MgCl_2$, 1 mM dithiothreitol, 5% glycerol) and purified Irr protein at 0.25, 0.5, 0.75 or 1 µg. Mixtures were incubated for 30 minutes at 37ºC and then loaded onto a 5% non-denaturing acrylamide gel and

subjected to electrophoresis at 100 volts. The gel was physically transferred to a piece of Whatman 1M paper using a Bio-Rad Model 583 gel dryer and visualized by autoradiography.

Experimental infection of mice.

Previously described methods (Robertson et al., 1999) were used to evaluate the spleen colonization profiles of *B. abortus* 2308 and BEA2 in C57BL/6 mice obtained from Harlan Laboratories. Briefly, mice were infected with 5×10^4 brucellae via the intraperitoneal route, and at each sampling point post infection, the mice were euthanized, their spleens removed, and spleen homogenates serially diluted and plated on SBA to determine the number of viable brucellae present. These experiments were performed prior to receiving notification from the Centers for Disease Control and Prevention's Select Agent Program that the introduction of *B. melitensis*, *B. suis* and *B. abortus* strains carrying chloramphenicol resistance genes into experimentally infected animals is no longer permitted.

Immunoblot analysis.

Whole cell protein lysates were collected from *Brucella* cultures grown to the desired time points (i.e., 72, 96 or 120 hours) in low iron minimal medium and low iron minimal medium supplemented with 50 μM $FeCl_3$. Cells were collected by centrifugation **(**6800 × *g*, 10 min, room temperature) and the pellet was suspended in 1 ml of protein sample buffer (0.3% SDS, 200 mM DTT, 22 mM Tris-Base, and 28 mM Tris-HCl). The cells were then boiled for 1 hour followed by 10 cycles (20 seconds per cycle) of disintegration in with Lysing Matrix B (MP Biomedicals, Solon, OH) using a BIO101 FastPrep FP120 cell disruptor (Thermo Savant [Thermo Fisher Scientific Inc.], Waltham, MA). A Bradford assay with BSA standards was used

to determine protein concentrations. Eighty μg of total protein per culture was separated by a 13% SDS-PAGE gel. Proteins were transferred to a nitrocellulose membrane by electroblotting at 100 V for 2 hours. Membranes were blocked at room temperature in 5% skim milk in PBST (155 mM NaCl, 8 mM Na_2HPO_4, 2 mM NaH_2PO_4, 0.05% Tween-20). Primary antibodies (anti-Irr [1:10,000]) were incubated with the membranes at room temperature in 5% skim milk. Secondary antibodies (anti-rabbit IgG HRP conjugate) (Promega) were used at a 1:10,000 dilution. All washing steps were performed with PBST. Visualization of the HRP signal was performed using SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific). Anti-Irr serum was raised in New Zealand White rabbits using the recombinant *Brucella* Irr as the antigen and TiterMax® Gold (Sigma) as an adjuvant for the primary immunization.

Statistical analysis.

All statistical analyses were performed using the student two-tailed *t* test (Rosner, 2000). *P* values of ≤ 0.001 (* *) and ≤ 0.05 (*) were considered significant.

Results and Discussion

Irr regulates the expression of the genes encoding the BhuA homologs, HmuR and HutA, in two close phylogenetic relatives of the brucellae, *Bradyrhizobium japonicum* and *Bartonella quintana*, respectively (Parrow et al., 2009; Rudolph et al., 2006). In *B. japonicum*, Irr is required for the induction of *hmuR* in response to iron deprivation, and genetic and biochemical evidence suggest that Irr serves as a transcriptional activator of *hmuR* (Rudolph et al., 2006). In contrast, in *B. quintana*, overproduction of Irr has a negative impact on *hutA* expression, and Irr has been shown to bind upstream of the *hutA* promoter in an electrophoretic mobility shift assay (Parrow et al., 2009). As shown in Figure 2.1, transcription of *bhuA* is induced during stationary phase following growth in low iron minimal medium in *B. abortus* 2308, but not in the isogenic *irr* mutant BEA2. Moreover, introduction of a plasmid-borne copy of *irr* into BEA2 restores low-iron-responsive *bhuA* expression in this strain. These data indicate that Irr is required for the low iron-responsive induction of *bhuA* transcription in *B. abortus* 2308. Such a role is reminiscent of the one that Irr plays in the iron-responsive expression of *hmuR* and several other genes encoding TonB-dependent outer membrane iron transporters in *B. japonicum* where Irr serves as a transcriptional activator (Rudolph et al., 2006; Small et al., 2009). Consistent with its inability to induce *bhuA* expression in response to iron deprivation, the *B. abortus irr* mutant BEA2 is also unable to use hemin as an iron source in an *in vitro* assay (Figure 2.2A), while the parental 2308 strain and the complemented mutant (BEA2.C) grow equally well around disks containing hemin on plates containing the iron-specific chelator EDDA. In contrast, BEA2 produces a zone of growth similar to that of the parental 2308 strain around disks containing $FeCl_3$ (Figure 2.2B).

The capacity of *B. abortus* 2308 to use heme as an iron source has been linked to its ability to maintain chronic spleen infections in experimentally infected mice (Paulley et al., 2007). Thus, the accelerated clearance of the *B. abortus irr* mutant from the spleens of infected

mice compared to the parental strain (Figure 2.3) is consistent with the inefficient *bhuA* expression exhibited by the *irr* mutant and its inability to use heme as an iron source *in vitro*. The attenuation of the *B. abortus irr* mutant in mice observed in these studies appears at first to be in contrast to the results reported by Martínez et al (Martinez et al., 2006), who reported that a *B. abortus irr* mutant is not attenuated at 1 and 3 weeks post infection in BALB/c mice. However, it should be noted that no attenuation was observed for the *irr* mutant at 1 or 2 weeks post infection in mice in the present study (Figure 2.3), with attenuation being observed starting at weeks 4 post infection. In addition, C57BL/6 mice were used in the study reported here, while BALB/c mice were employed in the study described by Martínez et al. (Martinez et al., 2006). From a regulatory standpoint, it is important to point out that the mouse infections described in this report were performed prior to notification from the Centers for Disease Control and Prevention's Select Agent Program that the introduction of *B. melitensis*, *B. suis* and *B. abortus* strains carrying chloramphenicol resistance genes into experimentally infected animals is no longer permitted.

To begin to define the *cis*-acting regulatory elements that control the iron-responsive expression of *bhuA* in *B. abortus* 2308, primer extension was employed to determine the transcriptional start site for this gene. A guanine residue (G) 141 nucleotides upstream of the predicted *bhuA* start codon was identified as the +1 start site for the *bhuA* transcript (Figure 2.4). This transcript would include the open reading frame (ORF) annotated as BAB2_1151 in the *B. abortus* 2308 genome sequence which is predicted to encode a hypothetical protein. Whether or not the BAB2_1151 ORF is translated is unknown and currently under investigation. Notably, 156 nucleotides upstream of the transcriptional site for *bhuA* is a consensus Irr binding site (also known as an "ICE" box) (Figure 2.4). The spacing of this putative Irr binding site relative to the

bhuA transcriptional start site is similar to that found in the *blr4504* gene (121 nt) in *B. japonicum*, which requires Irr for its induction in response to iron deprivation (Small et al., 2009).

To evaluate the importance of the ICE box for iron-responsive expression of *bhuA* in *B. abortus* 2308, β-galactosidase production by derivatives of this strain carrying pbhuA-lacZ (a pMR15-based plasmid carrying a *bhuA-lacZ* transcriptional fusion [Paulley et al., 2007]) and truncated versions of this plasmid was evaluated. Plasmid pbhuA6.5a-lacZ contains a *lacZ* fusion containing 179 bp upstream of the *bhuA* transcriptional start site that includes the putative Irr binding site (Figures 2.4 and 2.5). Plasmid pbhu5a-lacZ, on the other hand, contains a *lacZ* fusion containing 88 bp upstream of the *bhuA* transcriptional start site lacking an Irr binding site. As shown in Figure 2.4, both of the *bhuA-lacZ* transcriptional fusions (pbhuA-lacZ and pbhuA6.5a-lacZ) containing the putative Irr binding site exhibit enhanced expression in *B. abortus* 2308 following growth in low iron minimal medium, but basal expression when this strain is grown under iron-replete conditions. In contrast, the *bhuA-lacZ* transcriptional fusion lacking the Irr binding site (pbhuA5a-lacZ) displays basal levels of expression in both low iron medium and low iron medium supplemented with 50 μM $FeCl_3$. These experimental findings verify that the putative Irr-binding site upstream of *bhuA* is required for the low iron-responsive induction of this gene in *B. abortus* 2308, and moreover, suggest that Irr is serving as a transcriptional activator. This proposition is further supported by the observation that Irr directly binds to the *bhuA* promoter region in a specific manner in an EMSA (Figure 2.6).

Considering the essential role that Irr plays in regulating the iron-responsive expression of *bhuA* in *B. abortus* 2308, and important question to address is how Irr activity is modulated in this strain. As noted previously, the stability of the Irr protein in *B. japonicum* is controlled by

cellular iron levels (Qi et al., 2002). When iron levels are sufficient to allow heme biosynthesis, Irr is degraded. The studies described by Martínez et al. (Martinez et al., 2005) suggest that a similar relationship between cellular iron levels and Irr stability may also exist in *B. abortus* 2308. However, recent studies with another α–proteobacterium, *Rhizobium leguminosarum*, suggest that Irr activity in this bacterium is modulated by heme, and Irr is inactivated, but not degraded, when cellular iron levels increase (Singleton et al., 2010). Instead, heme binding to the *R. leguminosarum* Irr inhibits its DNA binding capacity. Distinguishing between these two models for regulating Irr function is important because the *Brucella* Irr, like the *R. leguminosarum* Irr, does not possess the heme regulatory element (GCPWAD) in its N-terminus that is required for the iron-responsive degradation of Irr in *B. japonicum* (Qi et al., 1999; Singleton et al., 2010). As shown in Figure 2.7, Western blot analysis of cell lysates from *B. abortus* 2308 cultures grown in low iron minimal medium and this medium supplemented with $FeCl_3$ suggests that Irr is degraded in this strain in response to increasing intracellular levels of iron. Thus, despite its lack of a conserved GCPWAD domain, it appears that the activity of the *Brucella* Irr is being modulated by iron-responsive degradation in a manner similar to that proposed for the *Bradyrhizobium* Irr (Qi et a., 2002; Small et al., 2009).

The experimental findings presented here build upon those presented by Martínez et al. (Martinez et al., 2005; Martinez et al., 2006) and demonstrate that Irr is a major iron-responsive regulator of gene expression in *B. abortus* 2308. Indeed, the presence of Irr appears to be essential for the wild-type expression of *bhuA* which encodes a major virulence determinant (Paulley et al., 2007). Because the brucellae must carefully balance cellular iron levels to avoid the enhancement of endogenous oxidative stress arising from their respiratory metabolism and exogenous oxidative stress derived from the NADPH and iNOS activity of host macrophages

(Roop et al., 2009), it will be important to obtain further insight into the nature of the Irr regulon in *Brucella*. In addition to Irr, *Brucella* strains also possess a homolog of the rhizobial iron regulator RirA, another important regulator of iron metabolism genes in the α–proteobacteria (Todd et al., 2002), and preliminary studies indicate that RirA also plays an important role in controlling the expression of iron metabolism genes in *B. abortus* 2308 including *bhuA* (Paulley, 2007). Thus, it will also be important to determine how Irr and RirA work together to regulate iron metabolism in this strain.

Acknowledgements

This work was supported by a grant from NIAID (AI-63516) to R. M. R. II.

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Figure 2.1 Transcriptional activity of a *bhuA-lacZ* reporter in *B. abortus* 2308, BEA2 (2308 Δ*irr*) and BEA2.C [BEA2 (pKHS2)]. Gray bars show β–galactosidase production in Miller units (Miller et al., 1972) by *B. abortus* strains carrying pbhuA-lacZ following 96 h growth in low iron minimal medium (low iron). The cross-hatched bars show β–galactosidase levels produced by these strains following 96 h growth in low iron medium supplemented with 50 μM $FeCl_3$ (high iron). The results presented are means and standard deviations from a single experiment that is representative of multiple (≥ 3) experiments performed from which equivalent results were obtained. The average β–galactosidase levels produced by the parental plasmid pMR15 in *B. abortus* 2308 during growth in low iron minimal medium or this medium supplemented with 50 μM $FeCl_3$ was subtracted from each data set to account for background activity.

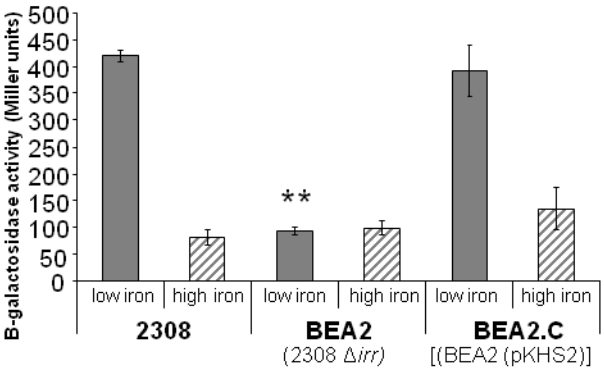

B-galactosidase activity (Miller units)
500
450
400
350
300
250
200
150
100
50
0
**
low iron
high iron
low iron
high iron
low iron
high iron
2308
BEA2
(2308 Δirr)
BEA2.C
[(BEA2 (pKHS2)]

Figure 2.1

Figure 2.2 Capacity of *B. abortus* 2308, HR1703 (2308 Δ*bhuA*), BEA2 (2308 Δ*irr*) and BEA2.C [BEA2 (pKHS2)] to use (A) hemin or (B) $FeCl_3$ as an iron source in a disk diffusion assay. The results presented are from a single experiment that is representative of multiple (≥ 3) experiments performed from which equivalent results were obtained.

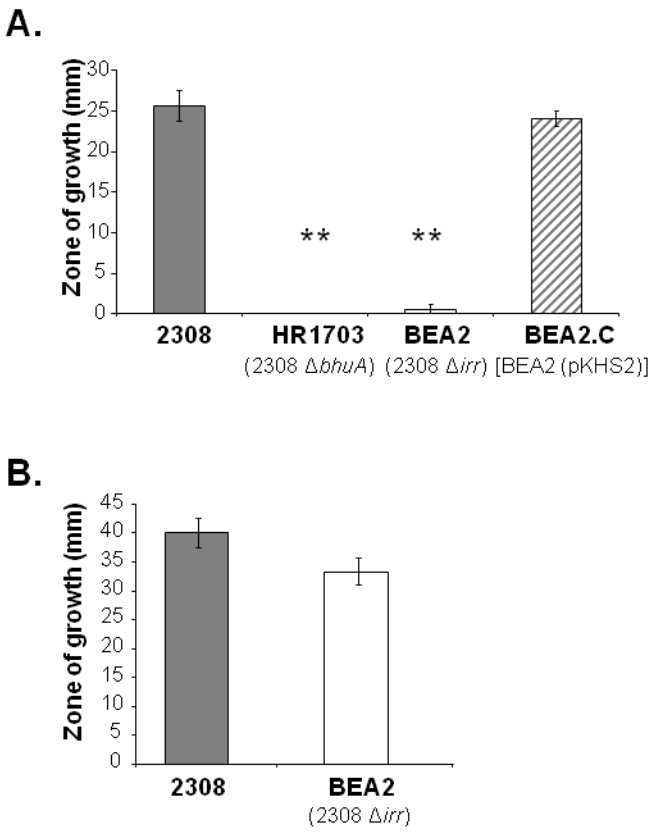

A.
Zone of growth (mm)
30
25
20
15
10
5
0
**
**
2308
HR1703
(2308 ΔbhuA)
BEA2
(2308 Δirr)
BEA2.C
[BEA2 (pKHS2)]
B.
Zone of growth (mm)
45
40
35
30
25
20
15
10
5
0
2308
BEA2
(2308 Δirr)

Figure 2.2

Figure 2.3 Spleen colonization profiles of *B. abortus* 2308 and BEA2 (2308 Δ*irr*) in C57BL/6 mice. The data are means and standard deviations for the number of brucellae detected in the spleens of five mice infected with each strain at each experimental time point in a single experiment. A single asterisk (*) indicates that the *P* value is ≤ 0.01 for comparisons of the data obtained with 2308 and BEA2. The double daggers (††) indicate that no brucellae were detected in the spleens of 2 of the mice infected with the *B. abortus irr* mutant at 8 weeks post infection.

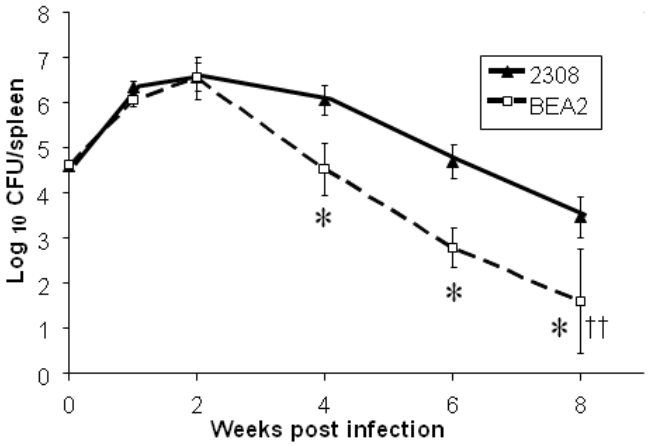

Log 10 CFU/spleen
Weeks post infection
2308
BEA2
0
1
2
3
4
5
6
7
8
0
2
4
6
8
*
*
*
††

Figure 2.3

Figure 2.4 Genetic organization of the *dhbR-bhuA* locus in *B. abortus* 2308. The transcriptional start site for the *bhuA* gene (which would also include the hypothetical gene designated BAB2_1151) is denoted by +1 and the corresponding guanine residue (G) is shown in boldface and highlighted. The nucleotides comprising the putative Irr binding site upstream of *bhuA* are enclosed in a box labeled as the "ICE box". The oligonucleotide primers used to construct the truncated versions of the plasmid-borne *bhuA-lacZ* fusion are indicated by the arrows labeled 7AF, 6.5AF and 5AF. The DNA region used for EMSA is indicated by a dashed line.

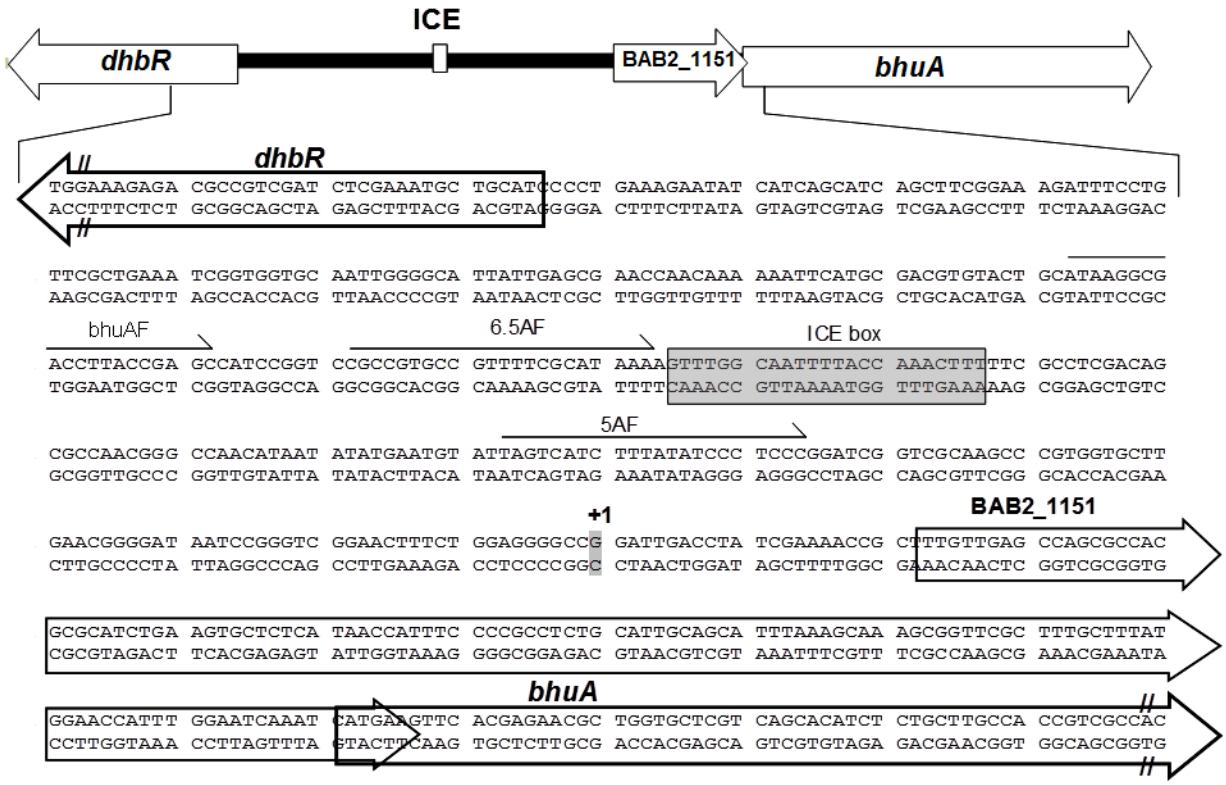
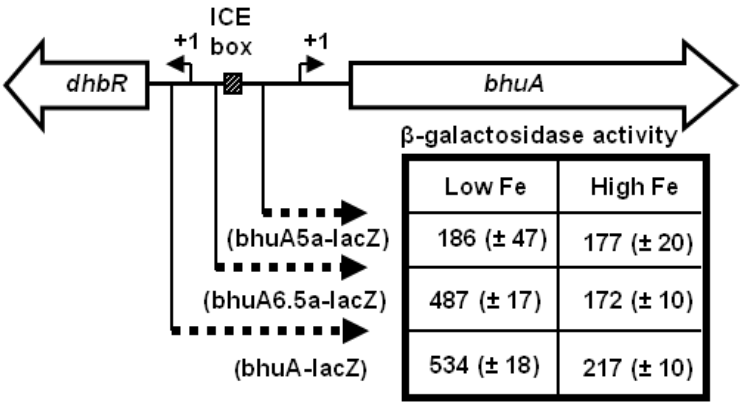

ICE
dhbR
BAB2_1151
bhuA
dhbR
TGGAAAGAGA CGCCGTCGAT CTCGAAATGC TGCATCCCCT GAAAGAATAT CATCAGCATC AGCTTCGGAA AGATTTCCTG
ACCTTTCTCT GCGGCAGCTA GAGCTTTACG ACGTAGGGGA CTTTCTTATA GTAGTCGTAG TCGAAGCCTT TCTAAAGGAC
TTCGCTGAAA TCGGTGGTGC AATTGGGGCA TTATTGAGCG AACCAACAAA AAATTCATGC GACGTGTACT GCATAAGGCG
AAGCGACTTT AGCCACCACG TTAACCCCGT AATAACTCGC TTGGTTGTTT TTTAAGTACG CTGCACATGA CGTATTCCGC
bhuAF
6.5AF
ICE box
ACCTTACCGA GCCATCCGGT CCGCCGTGCC GTTTTCGCAT AAAAGTTTGG CAATTTTACC AAACTTTTTC GCCTCGACAG
TGGAATGGCT CGGTAGGCCA GGCGGCACGG CAAAAGCGTA TTTTCAAACC GTTAAAATGG TTTGAAAAAG CGGAGCTGTC
5AF
CGCCAACGGG CCAACATAAT ATATGAATGT ATTAGTCATC TTTATATCCC TCCCGGATCG GTCGCAAGCC CGTGGTGCTT
GCGGTTGCCC GGTTGTATTA TATACTTACA TAATCAGTAG AAATATAGGG AGGGCCTAGC CAGCGTTCGG GCACCACGAA
+1
BAB2_1151
GAACGGGGAT AATCCGGGTC GGAACTTTCT GGAGGGGCCG GATTGACCTA TCGAAAACCG CTTTGTTGAG CCAGCGCCAC
CTTGCCCCTA TTAGGCCCAG CCTTGAAAGA CCTCCCCGGC CTAACTGGAT AGCTTTTGGC GAAACAACTC GGTCGCGGTG
GCGCATCTGA AGTGCTCTCA TAACCATTTC CCCGCCTCTG CATTGCAGCA TTTAAAGCAA AGCGGTTCGC TTTGCTTTAT
CGCGTAGACT TCACGAGAGT ATTGGTAAAG GGGCGGAGAC GTAACGTCGT AAATTTCGTT TCGCCAAGCG AAACGAAATA
bhuA
GGAACCATTT GGAATCAAAT CATGAAGTTC ACGAGAACGC TGGTGCTCGT CAGCACATCT CTGCTTGCCA CCGTCGCCAC
CCTTGGTAAA CCTTAGTTTA GTACTTCAAG TGCTCTTGCG ACCACGAGCA GTCGTGTAGA GACGAACGGT GGCAGCGGTG

Figure 2.4

Figure 2.5 Identification of the iron-responsive *bhuA* promoter in *B. abortus* 2308 by deletion analysis. β–galactosidase production by *B. abortus* 2308 carrying the plasmid-borne *bhuA-lacZ* transcriptional fusions following 120 h growth in low iron minimal medium (low Fe) or low iron minimal medium supplemented with 50 μM $FeCl_3$ (high Fe) is shown. The results presented are from a single experiment that is representative of multiple (≥ 3) experiments performed from which equivalent results were obtained. The regions of the *dhbR-bhuA* locus included in the *bhuA-lacZ* fusions are shown as dashed arrows, and the location of the putative Irr binding motif is denoted as the ICE box.



β-galactosidase activity

	Low Fe	High Fe
(bhuA5a-lacZ)	186 (± 47)	177 (± 20)
(bhuA6.5a-lacZ)	487 (± 17)	172 (± 10)
(bhuA-lacZ)	534 (± 18)	217 (± 10)

Figure 2.5

Figure 2.6 Irr interacts directly with the *bhuA* promoter region in an EMSA. Lane 1, 75 ng ^{32}P-labeled *bhuA* promoter-specific DNA fragment; lanes 2 through 5, 75 ng ^{32}P-labeled *bhuA* promoter-specific DNA fragment plus increasing concentrations (0.25, 0.5, 0.75 and 1 μg, respectively) of the Irr protein; lane 6, 75 ng ^{32}P-labeled *bhuA* promoter-specific DNA fragment plus 1 μg Irr plus 750 ng un-labeled *bhuA* promoter-specific DNA fragment (specific inhibitor); lane 7, 75 ng ^{32}P-labeled *bhuA* promoter-specific DNA fragment plus 1 μg Irr plus 750 ng un-labeled pBlue-Cm2 specific DNA fragment (non-specific inhibitor). The results presented are from a single experiment that is representative of multiple (≥ 3) experiments performed from which equivalent results were obtained. (Martinson, DA contributed to the work presented in this figure).

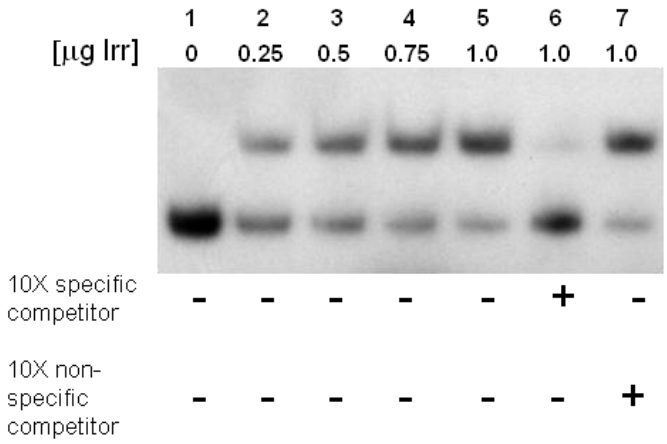

1 2 3 4 5 6 7
[μg Irr] 0 0.25 0.5 0.75 1.0 1.0 1.0
10X specific competitor - - - - - + -
10X non-specific competitor - - - - - - +

Figure 2.6

Figure 2.7 Analysis of Irr protein levels in *B. abortus* 2308 during growth under low and high iron conditions. Irr-specific antiserum was used in Western blot analysis to detect Irr levels in cell lysates from *B. abortus* 2308 cultures (lanes 1 thru 6) grown in low iron minimal medium or low iron minimal media supplemented with 50 μM $FeCl_3$ as indicated for 72 hours (lanes 1 and 2), 96 hours (lanes 3 and 4), or 120 hours (lanes 5 and 6). Lane 7 shows a cell lysate from *B. abortus* KH3 (2308 *irr*) grown for 96 hours in low iron minimal medium. Antiserum specific for the *Brucella* GroEL was used as a protein loading control. (Martinson, DA contributed to the work presented in this figure).

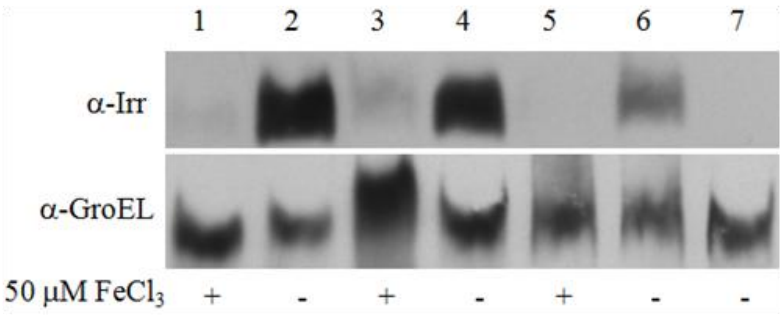


Figure 2.7

Chapter 3: The *bhuQ* gene encodes a heme oxygenase that contributes to the ability of *Brucella abortus* 2308 to use heme as an iron source and is regulated by Irr

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Journal of Bacteriology. 2012. **194**: 4052-4058.

DOI: 10.1128/JB.00368-12

Martinson, DA contribution to this Chapter

The major component of the study described in this paper was BhuQ and its function as a heme oxygenase in *Brucella*. During the course of the work, it became apparent that *bhuQ* and *rirA* are cotranscribed as an operon, and that Irr might be regulating the expression of these genes in *Brucella*. RirA is a known repressor of genes coding for iron acquisition systems in *Agrobacterium tumefaciens*, so determining if there is a regulatory link between Irr and *rirA* was necessary to provide insight into how the regulatory activities of Irr and RirA might be coordinated in *Brucella*. I helped to identify the location of a putative Irr binding motif (ICE box) in the promoter region of the *rirA* gene, as shown in Figure 3.4. I performed replicates for the data presented in Figure 3.5, which shows that there is Irr mediated repression of the *rirA-bhuQ* operon when cellular iron levels are low. I purified recombinant Irr and contributed to the DNase I footprint analysis presented in Figure 3.6. I also played a role in the development of the model of describing iron-responsive gene regulation shown in Figure 3.7. These findings support the proposition that Irr is the dominant iron-responsive transcriptional regulator when cellular iron levels are low, and RirA activity is limited as an iron-responsive transcriptional regulator to when cellular iron levels are high. Irr repression of *rirA* expression ensures that genes coding for iron acquisition systems are expressed when cellular iron levels are low in order for the brucellae to maintain physiologically relevant cellular iron levels.

Abstract

The *Brucella* BhuQ protein is a homolog of the *Bradyrhizobium japonicum* heme oxygenases HmuD and HmuQ. To determine if this protein plays a role in the ability of *Brucella abortus* 2308 to use heme as an iron source, an isogenic *bhuQ* mutant was constructed and its phenotype evaluated. Although the *B. abortus bhuQ* mutant DCO1 did not exhibit a defect in its capacity to use heme as an iron source nor evidence of increased heme toxicity *in vitro*, this mutant produced increased levels of siderophore in response to iron deprivation compared to 2308. Introduction of a *bhuQ* mutation into the *B. abortus dhbC* mutant BHB2 (which cannot produce siderophores) resulted in a severe growth defect in the *dhbC bhuQ* double mutant JFO1 during cultivation under iron restricted conditions, which could be rescued by the addition of $FeCl_3$, but not heme, to the growth medium. The *bhuQ* gene is co-transcribed with the gene encoding the iron-responsive regulator RirA, and both of these genes are repressed by the other major iron-responsive regulator in the α–proteobacteria, Irr. The results of these studies suggest that *B. abortus* 2308 has at least one other heme oxygenase that works in concert with BhuQ to allow this strain to efficiently use heme as an iron source. The genetic organization of the *rirA-bhuQ* operon also provides the basis for the proposition that BhuQ may perform a previously unrecognized function by allowing the transcriptional regulator RirA to recognize heme as an iron source.

Introduction

Iron represents an essential micronutrient for *Brucella* strains (Roop et al., 2011). Acquiring sufficient iron to meet their physiological needs is particularly challenging for the brucellae because these bacteria are found in nature almost exclusively in mammalian hosts, an environment where the iron restriction faced by pathogenic microbes is well documented (Schaible & Kaufmann, 2004). *Brucella* strains can use heme as an iron source *in vitro*, and studies with an isogenic mutant have shown that the presence of the TonB-dependent outer membrane heme transporter BhuA is required for the wild-type virulence of *B. abortus* 2308 in experimentally infected mice (Paulley et al., 2007), suggesting that heme is a biologically relevant source of iron for the brucellae during infection.

Heme oxygenases catalyze the release of iron from heme, and these enzymes contribute to the ability of a variety of bacteria to utilize heme as an iron source (Schmitt, 1997; Zhu et al., 2000; Ratliff et al., 2001). The product of the gene designated BMEII0706 in the *B. melitensis* 16M genome sequence shares 58 and 50% amino acid identity with the heme oxygenases HmuD and HmuQ, respectively, from *Bradyrhizobium japonicum*, and this *Brucella* protein exhibits heme oxygenase activity in an *in vitro* assay (Puri & O'Brian, 2006). Based on its documented biochemical activity and its homology to the IsdG/HmuQ family of heme oxygenases, we have given this protein the designation BhuQ (*Brucella* heme utilization oxygenase Q). The purpose of the experiments described in this report was to determine if the homologous protein in *B. abortus* 2308 (which is encoded by BAB2_0677) plays a role in the capacity of this strain to use heme as an iron source.

Materials and Methods

Bacterial strains, media, and growth conditions.

Brucella abortus strains (Table 3.1) were routinely grown in brucella broth at 37°C with aeration, or on Schaedler agar supplemented with 5% bovine blood (SBA) at 37°C under 5% CO_2. Kanamycin (45 μg/ml) (Sigma) and/or ampicillin (25 μg/ml) (Sigma) were added to these media as appropriate for the selection of strains with antibiotic resistance markers. *Escherichia coli* strain DH5α was used for the propagation of plasmids for procedures involving recombinant DNA, and this strain was cultivated at 37°C in LB broth or on LB agar plates containing either 100 μg/ml ampicillin, or 45 μg/ml kanamycin when appropriate. Gerhardt's minimal medium (GMM) (Gerhardt et al., 1950) and low iron minimal medium (Lopez-Goni et al., 1992) were prepared as previously described.

Construction of a *B. abortus bhuQ* mutant and a *dhbC bhuQ* double mutant.

Polymerase chain reaction (PCR) utilizing Taq polymerase (Invitrogen) with the oligonucleotide primers *bhuQ*-1F and *bhuQ*-1R (Table 3.2) was used to amplify a 1605 bp fragment encompassing the *bhuQ* gene (BAB2_0677) from *B. abortus* 2308 genomic DNA. This fragment was then cloned into pGEM®-T Easy (Promega). Inverse PCR employing AccuPrime *Pfx* supermix (Invitrogen) with this plasmid as a template and the primers *bhuQ*-2F and *bhuQ*-2R (Table 3.2) was then employed to generate a blunt-ended linear fragment from which 70 bp internal to the *bhuQ* coding region had been removed. This fragment was ligated with a 1345 bp fragment containing the *aph3a* gene from pKS-Kn (Kovach et al., 1995). The resulting construct, pGEMΔ*bhuQ*, was introduced into *B. abortus* strain 2308 by electroporation and transformants were selected on SBA supplemented with 45 μg/ml kanamycin. Putative *B. abortus bhuQ*

deletion mutants were identified based on their resistance to kanamycin and sensitivity to ampicillin, and their genotypes confirmed by PCR analysis and DNA sequence analysis. Chromosomal DNA preparations from putative deletion mutants and strain 2308 were harvested and oligonucleotides *bhuQ* F1 and Kan R (Table 3.2) were used to determine the presence of the *aph3a*-based gene in the proper orientation, the absence of the ampicillin resistance gene from pGEM®-T Easy (Promega) (*amp* F and *amp* R), and the absence of the 70 bp in the middle of *bhuQ (bhuQ* F1 and *bhuQ* R1). One confirmed *B. abortus bhuQ* mutant was selected for further phenotypic evaluation and given the designation DCO1.

The approach described in the previous paragraph was also used to introduce a *bhuQ* mutation into *B. abortus* BHB2 (Bellaire et al., 2003). BHB2 has an unmarked, in frame deletion in its *dhbC* gene, which renders it unable to produce either of the two siderophores produced by *Brucella* strains – 2,3-dihydroxybenzoic acid (Lopez-Goni et al., 1992) or brucebactin (Gonzalez Carrero et al., 2002). The *B. abortus dhbC bhuQ* double mutant constructed in this fashion was given the designation JFO1.

Reconstruction of the *bhuQ* loci in the *B. abortus bhuQ* and *dhbC bhuQ* mutants.

Because the *bhuQ* gene is the terminal gene in an operon and lies downstream of a transcriptional regulator (Figure 3.4), reconstruction of the mutated *bhuQ* genes in DCO1 and JFO1 was chosen as a strategy for verifying the link between genotype and the phenotypes exhibited by these strains, rather than genetic complementation with a plasmid-borne *bhuQ* gene. A 920 bp fragment encompassing the *bhuQ* gene from *B. abortus* strain 2308 genomic DNA was amplified by PCR using the primers *bhuQ*-3F and *bhuQ*-3F (Table 3.2) and cloned into the BamHI and SalI sites of pNPTS138Ap (Table 3.1), an ampicillin–resistant derivative of the

sacB-containing ColE1-based vector pNPTS138 (Spratt et al., 1986). The resulting plasmid, designated pNPTS138*bhuQ* was introduced into *B. abortus* DCO1 and JFO1 by electroporation, and a previously described *sacB*-based counterselection strategy (Bellaire et al., 2003) was used to select for derivatives of these mutants in which the mutated *bhuQ* genes had been replaced by the parental *bhuQ* gene. The genotypes of the resulting *B. abortus* strains, designated DCO1RC and JFO1RC were confirmed by PCR amplification and DNA sequence analysis.

Measurement of siderophore production by *Brucella* strains.

Following growth of the *B. abortus* strains in low iron minimal medium (Lopez-Goni et al., 1992) at 37°C with shaking (165 rpm), bacterial cells from 1.5 ml portions of the cultures were pelleted by centrifugation (15,550 x *g*, 1 min, room temperature), 1 ml of the resulting supernatant removed to a fresh tube (10 ml), and the level of catechol siderophore present measured using the Arnow assay (Arnow, 1937).

Determination of the growth characteristics of *B. abortus* strains in an iron limited culture medium.

B. abortus strains were grown on SBA plates at 37°C with 5% CO_2 for 48 h and harvested into PBS. The resulting cell suspensions were used to inoculate 25 ml low iron minimal medium in 125 ml Erlenmeyer flasks at a final concentration of approximately 10^6 CFU/ml. When applicable, the medium was supplemented with either 50 μM $FeCl_3$ or 25μM deferrated hemin (Spratt et al., 1986). Cultures were incubated at 37°C with shaking at 165 rpm, and at 24 hour time points post inoculation these cultures were serially diluted in PBS and plated on SBA, followed by incubation at 37°C under 5% CO_2.

Relative quantification of *bhuQ* transcript levels using real-time RT-PCR.

B. abortus 2308 and BEA2 (2308 *irr*) (Anderson et al., 2011) were grown in low-iron minimal medium and low-iron minimal medium supplemented with 50 µM $FeCl_3$, or Gerhardt's Minimal Medium with or without 25 µM deferrated hemin. Total cellular RNA was isolated from these cultures using a previously described procedure (Caswell et al., 2012). The RNA was treated with RQ1 DNase (Ambion) following the manufacturer's instructions to remove residual contaminating DNA. The absence of DNA from the RNA preparations was confirmed via PCR analysis and lack of an amplified product as visualized on an agarose gel confirmed that the RNA sample was free of DNA contamination. Concentrations of RNA in the samples were determined by measuring the absorbance at 260 nm using a NanoDrop ND-1000 Spectrophotometer.

The SuperScript III First-Strand Synthesis System for RT-PCR kit (Invitrogen) was used to convert 1 µg of RNA from these preparations into cDNA for each preparation following the manufacturer's instructions and using the random hexamers supplied with the kit. The cDNA preparations were then used as the templates in real-time RT-PCR analysis along with FastStart SYBR Green Master 2X (Roche) to evaluate the relative levels of gene-specific mRNA transcripts in the total cellular RNA preparations. Gene-specific oligonucleotide primers were utilized for the following genes 16S, *dhbC, bhuA*, and *bhuQ* (see Table 3.2). The differences in the levels of the *bhuQ*-, *bhuA*-, and *dhbC* -specific transcripts present were calculated using methods described by Pfaffl (Pfaffl, 2001), using the 16S gene as an internal standard. This gene encodes the ribosomal 16S protein, and its expression is constitutive in *B. abortus* 2308 under the experimental conditions used here.

Determination of the operonic organization of the *ybaK, rirA, bhuQ,* and *bfr* genes in *B. abortus* 2308.

Reverse transcriptase PCR was performed using cDNA prepared from clean RNA harvested from *B. abortus* 2308 grown for 72 h in low iron minimal medium. Primers that span the intragenic regions between *ybaK* (BAB2_0679), *rirA* (BAB2_0678), *bhuQ* (BAB2_0677), and *bfr* (BAB2_0676) (Table 3.2) were used in order to verify the presence or absence of a continuous transcript containing these genes. The resulting PCR products were separated by electrophoresis in a 0.7% agarose gel and visualized by staining the gel with ethidium bromide.

Determination of the transcriptional start site for the *rirA-bhuQ* operon.

The *bhuQ* gene is co-transcribed with the upstream gene, *rirA*. In order to determine a transcriptional start site for this operon, 5' RNA ligase mediated rapid amplification of the cDNA end (5' RLM-RACE) was performed using a primer (Rev) anchored in the reverse orientation within the *rirA* ORF following the manufacturer's instructions (FirstChoice RLM-RACE Kit, Ambion, AM1700). The PCR product generated from this reaction was cloned into pCR2.1 (Invitrogen), and the authenticity of the PCR fragment verified by DNA sequence analysis.

Identification of the Irr binding site in the *rirA* promoter region.

A recombinant version of the *Brucella* Irr was purified, and used in a DNase I footprint analyis with the *rirA* promoter region from *B. abortus* 2308 using previously described methods

(Anderson et al., 2011; Menscher et al., 2012). Briefly, the oligonucleotide primers *rirA* F and *rirA* R (Table 3.2) were individually labeled with [γ-^{32}P]ATP (Perkin Elmer) using the T_4 polynucleotide kinase reaction (Promega, Madison, WI) prior to their use in PCR reactions with Pfx polymerase to generate 300-bp DNA fragments representing the *rirA* promoter and transcriptional start site. The resulting PCR products were subjected to agarose gel electrophoresis and purified by gel extraction (Fermentas, Glen Burnie, MD). DNA probes corresponding to 8,000 cpm of the forward labeled and reverse labeled templates were incubated separately in EMSA binding buffer (10 mM Tris-HCl, pH 8, 40 mM KCl, 1 mM MgCl2, 1 mM dithiothreitol [DTT], 5% glycerol) supplemented with 100 ng/ml bovine serum albumin (BSA) and 50 ng/ml salmon sperm DNA (nonspecific competitor) in the presence of 100 μM $MnCl_2$ and increasing concentrations of the recombinant *Brucella* Irr protein. The reaction mixtures were incubated at room temperature for 30 min prior to treatment with 0.08 U of DNase I freshly diluted in 10× DNase I buffer (400 mM Tris-HCl [pH 8.0], 100 mM $MgSO_4$, 10 mM $CaCl_2$) for 4 min. Reactions were stopped by the addition of 5 mM EDTA and heating at 65°C for 10 min. Reaction mixtures were ethanol precipitated and resuspended in 4 μl of formamide loading buffer (98% formamide, 10 mM EDTA [pH 8.0], 1 mg/ml xylene cyanol FF, 1 mg/ml bromophenol blue). Digested DNA fragments were separated on a denaturing 6% (wt/vol) acrylamide and 7 M urea sequencing gel in glycerol-tolerant buffer (Menscher et al., 2012). Gels were dried under vacuum and subjected to autoradiography. The sequence protected by Irr was determined by comparing the nucleotide sequences generated for a 100-bp region of the *rirA* promoter region using the SequiTherm Excel II DNA sequencing kit (Epicentre, Madison, WI) and *B. abortus* 2308 DNA preparations exposed to DNase I treatment with and without recombinant Irr as templates.

Statistical analysis.

All statistical analysis was performed using the Student's two-tailed t-test. *P* values of less than 0.05 were considered significant (Rosner, 2000).

Results and Discussion

Pfam analysis of the *Brucella* BhuQ protein indicates that it belongs to the antibiotic monooxygenase (ABM) family of heme oxygenases along with the HmuD and HmuQ proteins from *B. japonicum* (Puri & O'Brian, 2006) and the IsdI and IsdG proteins from *Staphylococcus aureus* (Skaar, 2004). Moreover, BhuQ contains the conserved Asn 7, Trp 67 and His 77 residues shown experimentally to be important for the heme oxygenase activity of IsdG (Wu et al., 2005) (Figure 3.1), and BhuQ has been shown to bind and degrade heme *in vitro* (Puri & O'Brian, 2006). Despite the documuented heme oxygenase activity of BhuQ, however, an isogenic *bhuQ* mutant (DCO1) constructed from *B. abortus* 2308 exhibited a comparable growth pattern in low iron minimal medium and an equivalent resistance to the iron specific chlelator ethylenediamine-N,N'-bis(2-hydroxyphenylacetic acid) (EDDHA) to that displayed by the parental strain. More importantly, both strains were also able to use heme as an iron source with equivalent efficiency in a chelator based disk assay on a solid growth medium (data not shown).

A distinctive characteristic of the *B. abortus bhuQ* mutant that was noticed during its phenotypic characterization, however, was that this mutant consistently and reproducibly produces more siderophore during growth in low iron minimal medium than the parental 2308 strain (Figure 3.2A). This increase in siderophore production is accompanied by a corresponding increase in transcription of *dhbC* in the *bhuQ* mutant in comparison to the parental strain (2308) following growth under iron limiting conditions (Figure 3.2B). This phenotype suggests that the *B. abortus bhuQ* mutant is experiencing a greater degree of iron deprivation in the low iron minimal medium than 2308, and in turn increasing its siderophore production to compensate. Enhanced siderophore production could explain why the *B. abortus bhuQ* mutant does not exhibit a readily detectable iron acquisition defect in *in vitro* assays such as growth in low iron

minimal medium or sensitivity to EDDHA. The ability of this mutant to use heme as an iron source in the disk diffusion assays, however, indicates that *B. abortus* 2308 possesses one or more additional heme oxygenases that can compensate for the loss of BhuQ and allow the *bhuQ* mutant to use heme as an iron source. The identity of these other heme oxygenases is currently unknown.

Brucella strains have the capacity to use multiple iron sources during *in vitro* growth (Roop et al., 2011), and it is not usual for bacterial strains with mutations affecting single iron transporters to exhibit little or no defect in iron utilization assays due to compensation by other iron transport systems. Indeed, the fact that the *B. abortus bhuQ* mutant exhibits increased siderophore production in response to iron deprivation compared to the parental strain provided an experimental avenue for assessing the role of BhuQ in iron metabolism. Specifically, if the loss of BhuQ from *B. abortus* DCO1 is leading to an increased demand for iron, and siderophore production is being increased in this mutant to meet this demand, then a derivative of this strain that cannot produce siderophore (e.g. a *B. abortus dhbC bhuQ* double mutant) would be expected to show an enhanced iron deprivation phenotype compared to *B. abortus* 2308 or the *bhuQ* mutant when grown under iron limiting conditions. This is in fact the relationship that was observed. As shown in Figure 3A, the *dhbC bhuQ* double mutant JFO1 showed a greatly enhanced growth defect compared to the parental BHB2 (*dhbC* mutant) strain when they were cultivated in low iron minimal medium, a phenotype that was not observed when these strains were grown in iron-replete medium (Figure 3.3B). More importantly, the enhanced growth defect exhibited by the *B. abortus dhbC bhuQ* double mutant during cultivation in low iron minimal medium could be rescued by the addition of $FeCl_3$ (Figure 3.3C), but not heme (Figure 3.3D), to the growth medium 48 hours after inoculation of the bacterial cultures. In contrast,

either $FeCl_3$ (Figure 3.3C) or heme (Figure 3.3D) was able to rescue the growth defect exhibited by the *dhbC* mutant BHB2 during growth under low iron conditions, although in both cases the alleviation of the growth restriction occurred more rapidly when $FeCl_3$ was added. To verify the link between the *bhuQ* mutations in *B. abortus* DCO1 and JFO1 and the phenotypes exhibited by these strains, a *sacB*-based counterselection strategy (Bellaire et al., 2003) was used to reconstruct the *bhuQ* genes in these mutants. The resulting strains, designated DCO1RC and JFO1RC, exhibited the expected parental phenotype with regard to their production of siderophore in response to iron deprivation (Figure 3.2), sensitivities to iron deprivation (Figures 3.3A and 3.3B) and their abilities to use $FeCl_3$ and heme as iron sources (Figures 3.3C and 3.3D). Although these experimental findings establish a role for BhuQ in the capacity of the *B. abortus dhbC* mutant to use heme as an iron source, this activity appears to be masked in *B. abortus* 2308 by the activity of other heme oxygenases when alternative iron sources are readily available in the growth medium. The capacity of the *B. abortus bhuQ* mutant to compensate for the loss of one heme oxygenase was also observed in experimental models of infection, as this strain exhibited wild-type virulence in cultured murine macrophages and experimentally infected BALB/c mice (data not shown).

RT-PCR analysis indicates that *bhuQ* is the second gene in an operon transcribed as *rirA* (BAB2_0678) - *bhuQ* (BAB2_0677) in *B. abortus* 2308 (Figure 3.4). RirA is a well-characterized regulator of iron metabolism genes in several of the other α–proteobacteria (Todd et al., 2002; Chao et al., 2005; Ngok-Ngam et al., 2009; Hibbing & Fuqua, 2011). A predicted iron control element (ICE) motif is located in the -10 region of the promoter of the *rirA-bhuQ* operon (Figure 3.4), suggesting that the iron response regulator Irr regulates the expression of these genes in response to cellular iron levels (Martinez et al., 2006). In fact, when the

expression patterns of the *rirA* and *bhuQ* genes in *B. abortus* 2308 were independently evaluated by real time PCR, both genes exhibited a modest induction in response to iron deprivation (Figure 3.5). In contrast, the expression of both of these genes was elevated >50 fold in the *B. abortus irr* mutant BEA2 when this strain was grown under iron limiting conditions. Thus, it appears as if Irr represses the expression of *rirA* in *Brucella* strains during periods of iron deprivation in much the same manner as it does in the related α–proteobacterium *Agrobacterium tumefaciens* (Hibbing & Fuqua, 2011). DNase I footprint analysis indicated that Irr directly binds to the *rirA* promoter (Figure 3.6), protecting a 28 nucleotide sequence, 5'-CATATATTTTAAGAATGATTCTAAAGTG-3'. Notably, this sequence includes the conserved ICE motif in the promoter region of *rirA* (underlined) predicted by Rodionov et al. (Rodionov et al., 2006).

Genetic studies suggest that RirA functions as an iron-responsive repressor of iron acquisition genes in the α–proteobacteria (Todd et al., 2002), in much the same fashion as Fur does in other bacteria. The potential benefit of Irr repressing the expression of an iron-responsive repressor when the bacterial cell is experiencing iron-deprivation is not difficult to envision. But such a regulatory link would appear to be counterproductive for the *bhuQ* gene if its product is solely dedicated the utilization of heme as an iron source. One scenario that might explain a possible benefit of co-regulation of *bhuQ* and *rirA* in *B. abortus* 2308 is that BhuQ production leads to an increased level of heme oxygenase activity which enhances the capacity of RirA to recognize exogenous heme being utilized as an iron source and represses the cell's iron acquisition systems accordingly (Figure 3.7).

In addition to their ability to provide iron from heme, some bacterial heme oxygenases also function to protect the cell against heme toxicity (Anzaldi & Skaar, 2010). Heme has a high

redox potential, and too much heme inside a bacterial cell can be toxic (Nir, 1991). Neither *B. abortus* 2308 nor the isogenic *bhuQ* mutant display a growth defect in low iron medium supplemented with up to 200 µM deferrated hemin. It is possible, however, that any role that BhuQ might be playing in the detoxification of heme is masked by the activity of another heme oxygenase. Another factor that may affect this observed lack of heme toxicity is that *Brucella* strains possess multiple homologs of the outer membrane heme-binding proteins (Hbps) that have been proposed to play a role in capturing heme and preventing heme toxicity in *Bartonella* (Minnick et al., 2009). In *Brucella*, these proteins are known as the Omp25/31 family of proteins (Cloeckaert et al., 1996). Heme export systems have also been proposed as a means by which bacteria protect themselves from heme toxicity (Anzaldi & Skaar, 2010). Although a heme exporter has not been identified in *Brucella* strains, genes that potentially encode orthologs of proteins linked to porphyrin (Tatsumi & Wachi, 2008) and heme (Rasmussen et al., 2005) export in other bacteria can be found in the genome sequence of *B. abortus* 2308.

In order to fully understand the role of BhuQ in iron and heme metabolism in *Brucella* strains, it will be imperative to identify the other heme oxygenase(s) present in these bacteria. Phenotypic evaluation of mutants lacking combinations of these enzymes can then be used to assess the relative contributions of the heme oxygenases to iron and heme metabolism, as well as their potential role in modulating the regulatory capacity of RirA. Such studies may also provide an added practical benefit, as prokaryotic heme oxygenases have been proposed to be targets for the development of antimicrobial agents (Furci et al., 2007). Brucellosis in humans is notoriously difficult to treat, requiring a combination of antibiotics for a prolonged period (Ariza et al., 2007). Hence, the development of improved chemotherapeutic regimens for treating this disease would of great benefit to the medical community.

Acknowledgements

This work was funded by a grant (AI 63516) from the National Institute of Allergy and Infectious Diseases to R.M.R.

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TABLE 3.1 Bacterial strains used in this study

Strain or plasmid	Genotype or description	Reference or source
Strains		
Escherichia coli		
DH5α	F^- φ80*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(rK^- mK^+) *phoA supE44 thi-1 gyrA96 relA1* λ^-	Invitrogen
Brucella abortus		
2308	Virulent challenge strain	Laboratory stock
DCO1	70 bp gene deletion of *bhuQ* (BAB2_0677) with an *ahpA3* kanamycin resistance gene insertion	This study
DCO1RC	DCO1 with *bhuQ* (BAB2_0677) reconstructed onto the chromosome using pNPTS138	This study
BHB2	Nonpolar, in frame deletion of *dhbC*	Bellaire et al., 1999, this study
JFO1	*ΔdhbC/ΔbhuQ*	This study
JFO1RC	*ΔdhbC/ΔbhuQ* with *bhuQ* (BAB2_0677) reconstructed onto the chromosome using pNPTS138	This study
BEA2	*Δ*irr	Anderson et al., 2011, this study
Plasmids		
pGEM-T Easy	ColE1-based cloning vector; Ap^R	Promega
pKS + Kan	794-bp *aph3A* gene from Tn*phoA* cloned into SalI-HindIII-digested pBluescript II KS+	Kovach et al., 1995
pNPTS138	*sacB*-containing counterselection vector; Amp^R	Spratt et al., 1986

TABLE 3.2 Primers used for this study

bhuQ-1F	5’-GACATTTCGCTGAAGACATA -3’
bhuQ-1R	5’-GAGCTTACTTTACCGTTGGC-3’
bhuQ-2F	5’-TCTATGCATCCCACACAGTC-3’
bhuQ-2R	5’-GAGCTGGGAGTCACGGTTCT-3’
amp - F	5’-TATTTCGTTCATCCATA-3’
amp - R	5’-GTTTTCCAATGATGAGC-3’
Kan R	5’-CCACTCCAGCATGAGAT-3’
bhuQ-3F	5’-ACTGGATCCCGGAGCTGTTTCTATTC-3’
bhuQ-3F	5’-ACTGTCGACTTCTAAATATGAGTTAA-3’
16S Fwd	5’-TCTCACGACACGAGCTGACG-3’
16S Rev	5’-CGCAGAACCTTACCAGCCCT-3’
dhbC Fwd	5’-GTGCCAAGCTTGGTCTGTACTTC-3’
dhbC Rev	5’-CGTGGATTGTTTACCGGC-3’
bhuQ Fwd	5’-CGGTAGCGAAACCGATTTTGAGAC-3’
bhuQ Rev	5’-ATGCGCATGGCGAAACTGTT-3’
rirA F	5’-GCACCGCAGAAAATGGCCGA-3’
rirA R	5’-TGGAACATTCCCGAACCGGA-3’
Rev	5’-GAAGGCATTGAGTGCTTCGC-3’
Ybak/rirA F	5’-TAAAGGTGAGAAAAGGTTTC-3’
Ybak/rirA R	5’-GACGCATGATTATTCCTTTT-3’
rirA/bhuQ F	5’-GGATAGCTGTGGCCTGAATG-3’
rirA/bhuQ R	5’-GAGCTGGGAGTCACGGTTCT-3’
bhuQ/bfr F	5’-GCTTCCGTATCGTCACTTTT-3’
bhuQ/bfr R	5’-GAGCTTACTTTACCGTTGGC-3’
rirA F	5’-GCACCGCAGAAAATGGCCGA-3’
rirA R	5’-TGGAACATTCCCGAACCGGA-3’

Figure 3.1 The *B. abortus* BhuQ protein shares amino acid homology with HmuD/Q and IsdG/I. The *Brucella abortus* (Ba) BhuQ protein shares amino acid homology with the heme oxygenases HmuD and HmuQ from *Bradyrhizobium japonicum* (Bj) and IsdG and IsdI from *Staphylococcus aureus* (Sa) and contains the conserved Asn 7, Trp 67 and His 77 residues (shown in larger, bold font) shown experimentally to be important for the heme oxygenase activity of IsdG (Skaar et al., 2004).

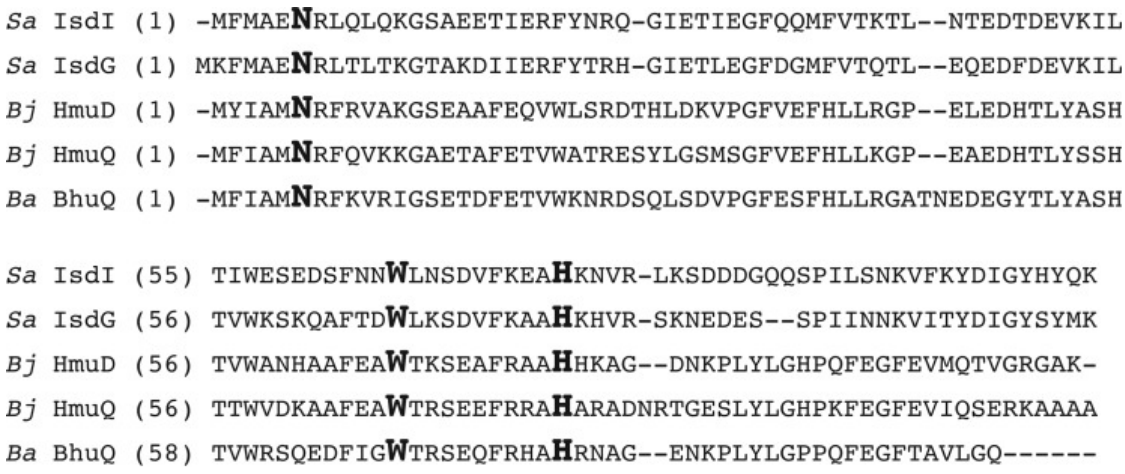
Sa IsdI (1) -MFMAENRLQLQKGSAEETIERFYNRQ-GIETIEGFQQMFVTKTL--NTEDTDEVKIL
Sa IsdG (1) MKFMAENRLTLTKGTAKDIIERFYTRH-GIETLEGFDGMFVTQTL--EQEDFDEVKIL
Bj HmuD (1) -MYIAMNRFRVAKGSEAAFEQVWLSRDTHLDKVPGFVEFHLLRGP--ELEDHTLYASH
Bj HmuQ (1) -MFIAMNRFQVKKGAETAFETVWATRESYLGSMSGFVEFHLLKGP--EAEDHTLYSSH
Ba BhuQ (1) -MFIAMNRFKVRIGSETDFETVWKNRDSQLSDVPGFESFHLLRGATNEDEGYTLYASH
Sa IsdI (55) TIWESEDSFNNWLNSDVFKEAHKNVR-LKSDDDGQQSPILSNKVFKYDIGYHYQK
Sa IsdG (56) TVWKSKQAFTDWLKSDVFKAAHKHVR-SKNEDES--SPIINNKVITYDIGYSYMK
Bj HmuD (56) TVWANHAAFEAWTKSEAFRAAHHKAG--DNKPLYLGHPQFEGFEVMQTVGRGAK-
Bj HmuQ (56) TTWVDKAAFEAWTRSEEFRRAHARADNRTGESLYLGHPKFEGFEVIQSERKAAAA
Ba BhuQ (58) TVWRSQEDFIGWTRSEQFRHAHRNAG--ENKPLYLGPPQFEGFTAVLGQ------

Figure 3.1

Figure 3.2 *B. abortus* DC01 (2308 Δ*bhuQ*) produces significantly more siderophore than *B. abortus* 2308 in response to iron deprivation. (A) Siderophore production by *B. abortus* 2308, DCO1 (Δ*bhuQ*) and DCO1RC (DCO1 *bhuQ*$^{+}$) following 72 h growth in low iron minimal medium. The values on the Y axis represent the levels of catechol siderophore detected by Arnow assay (Arnow, 1937). ** = $P < 0.01$ for comparisons of the data obtained for these strains in the Student's two-tailed t-test (Rosner, 2000). (B) Iron-responsive expression of *dhbC* in *B. abortus* 2308 and DCO1 (2308 *bhuQ*). 'Fold induction' on the Y axis represents the difference between the levels of *dhbC* transcripts detected in RNA preparations from *B. abortus* 2308 and DCO1 cultures after 72 h of growth in low iron minimal medium compared to RNA preparations from these cultures after growth in low iron minimal medium supplemented with 50 μM $FeCl_3$.

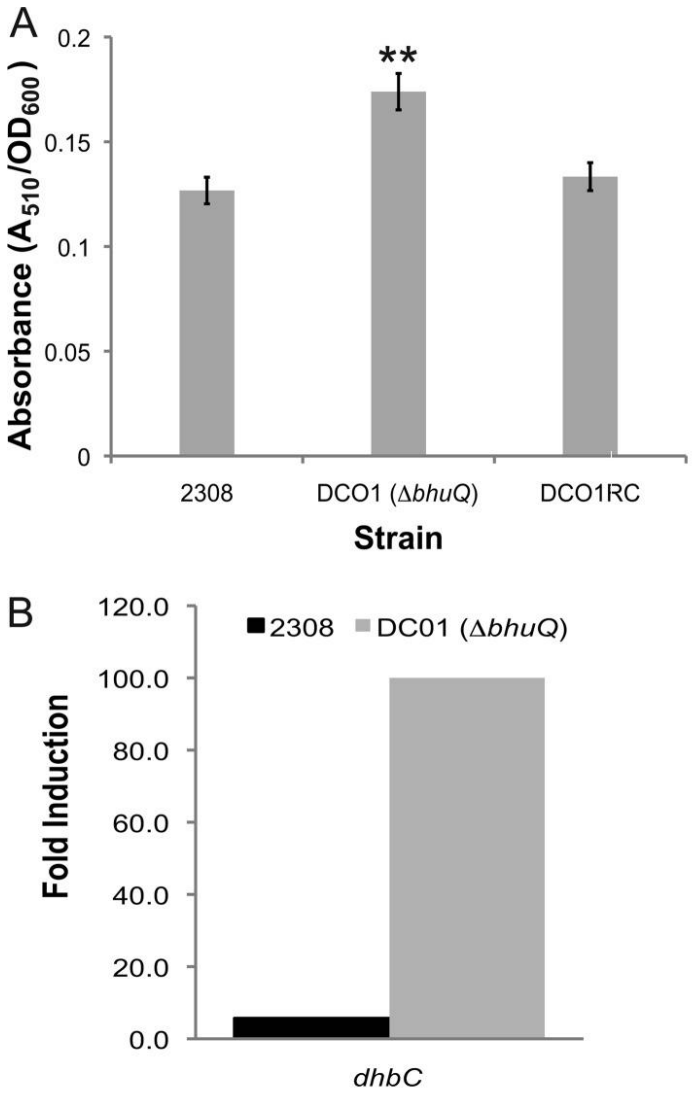
A
0.2
0.15
0.1
0.05
0
Absorbance (A_{510}/OD_{600})
**
2308
DCO1 (ΔbhuQ)
DCO1RC
Strain
B
120.0
100.0
80.0
60.0
40.0
20.0
0.0
Fold Induction
2308
DC01 (ΔbhuQ)
dhbC

Figure 3.2

Figure 3.3 *B. abortus* JF01 (2308 Δ*dhbC*Δ*bhuQ*) has a growth defect in the presence of heme as the sole iron source. Growth of *B. abortus* 2308 (X in squares), *B. abortus* DCO1 (2308 Δ*bhuQ)* (squares), BHB2 (2308 Δ*dhbC)* (circles), JFO1 (2308 Δ*dhbC/*Δ*bhuQ)* (triangles), and JFO1RC (JFO1 *bhuQ*+) (diamonds) in (A) low iron minimal medium, (B) low iron minimal medium containing 50μM $FeCl_3$, (C) low iron minimal medium supplemented with 50μM $FeCl_3$ at 48 hours post-inoculation, and (D) low iron minimal medium supplemented with deferrated 25μM hemin at 48 hours post inoculation. *B. abortus* 2308 wild type strain was not included in this figure for simplicity's sake and has a growth profile similar to that of DCO1. The data presented are from one experiment, but representative of multiple experiments (>3) from which similar trends were observed. ** = $P < 0.01$ and *** = $P < 0.001$ for comparisons of the data obtained for these strains in the Student's two-tailed t-test (Rosner, 2000).

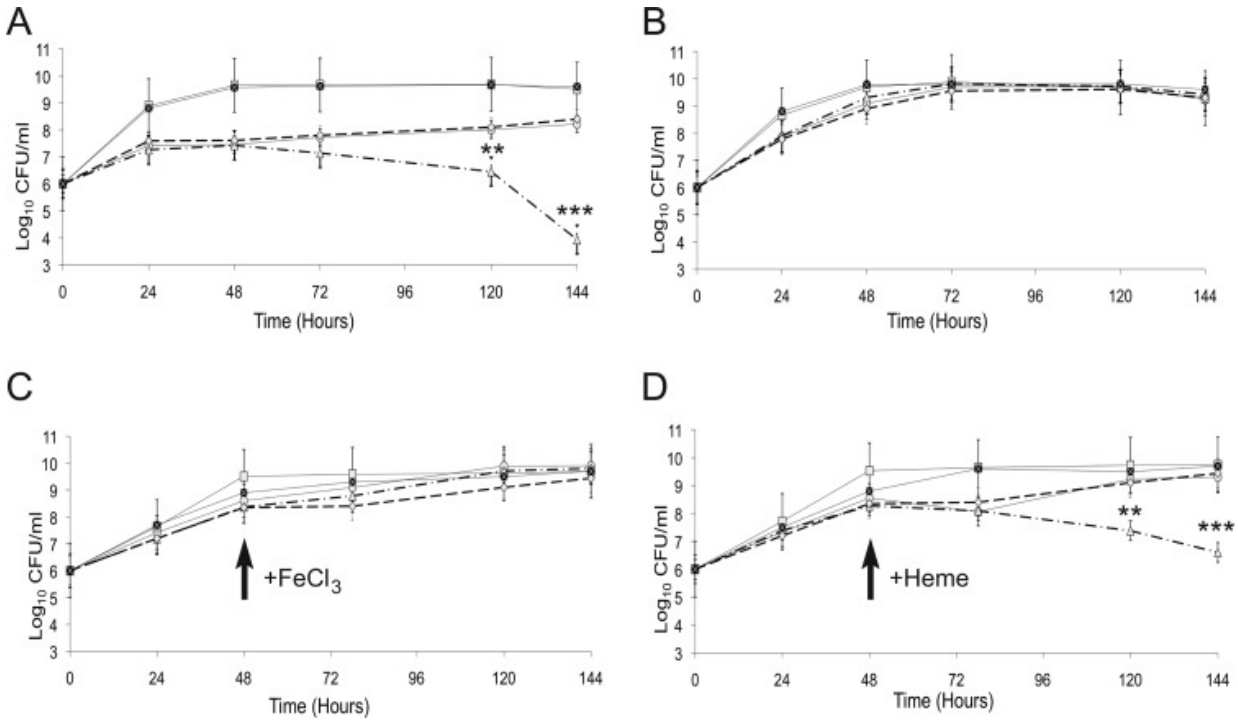

Figure 3.3

Figure 3.4 *rirA* and *bhuQ* are cotranscribed as an operon in *B. abortus* 2308. The start site (+1) for the *rir-bhuQ* transcript is located 134 base pairs upstream of the *rirA* ORF (bold, underlined), and the location of an Irr binding motif predicted by Rodionov *et al*. is denoted as a 'ICE Box' and shown in larger, bold-face type (Rodionov et al., 2006), while the nucleotide sequence protected by Irr is shown in a box. Primer sets 1, 2, and 3 denote the intragenic regions used to define the transcript and the corresponding lanes are marked on the agarose gel picture below it. (Martinson, DA contributed to the work presented in this figure).

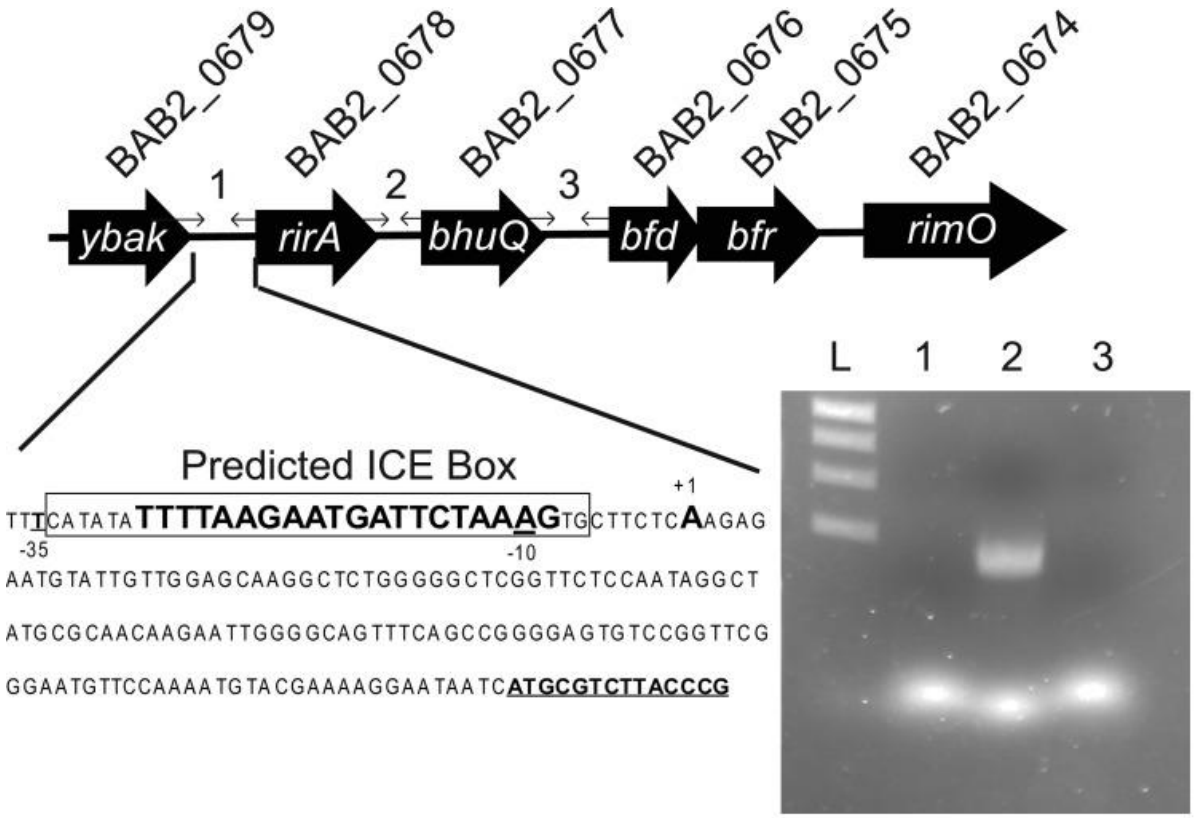
BAB2_0679
BAB2_0678
BAB2_0677
BAB2_0676
BAB2_0675
BAB2_0674
1
2
3
ybak
rirA
bhuQ
bfd
bfr
rimO
Predicted ICE Box
+1
TTTCATATATTTTAAGAATGATTCTAAAGTGCTTCTCAAGAG
-35
-10
AATGTATTGTTGGAGCAAGGCTCTGGGGGCTCGGTTCTCCAATAGGCT
ATGCGCAACAAGAATTGGGGCAGTTTCAGCCGGGGAGTGTCCGGTTCG
GGAATGTTCCAAAATGTACGAAAAGGAATAATCATGCGTCTTACCCG
L
1
2
3

Figure 3.4

Figure 3.5 *rirA* and *bhuQ* expression in response to iron deprivation in *B. abortus* 2308 and the isogenic *irr* mutant BEA2. 'Fold induction' on the Y axis represents the difference between the levels of *rirA, bhuQ* and *bhuA* transcripts detected in RNA preparations from *B. abortus* 2308 and BEA2 after 72 h of growth in low iron minimal medium compared to RNA preparations from these cultures after growth in low iron minimal medium supplemented with 50 μM $FeCl_3$. The pattern of *bhuA* transcription was included in this figure because this gene exhibits elevated expression in response to iron deprivation in *B. abortus* 2308, and this low iron-responsive induction is dependent upon the presence of Irr (Anderson et al., 2011). (Martinson, DA contributed to the work presented in this figure).

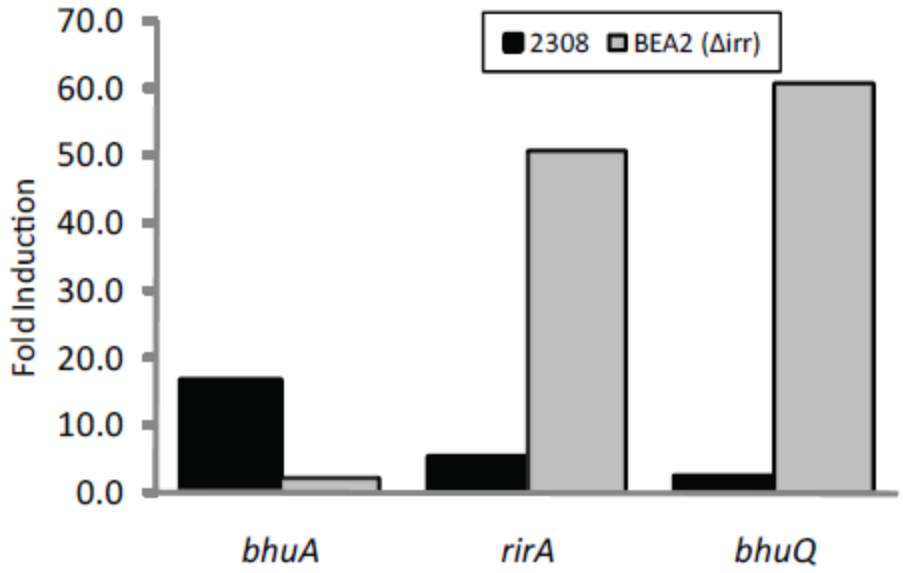
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Fold Induction
2308
BEA2 (Δirr)
bhuA
rirA
bhuQ

Figure 3.5

Figure 3.6 Irr binds directly to the *rirA* promoter in *B. abortus* 2308 and protects a 28 nucleotide sequence in a DNase I footprint analysis. The triangle above the lanes indicates that the corresponding reaction mixtures contain increasing concentrations (700 ng, 1.4 µg, 2.1 µg, and 3.5 µg) of recombinant *Brucella* Irr, and the nucleotide sequences shown to the right of the gel photos denote the nucleotides protected from DNaseI digestion in the forward and reverse strands of the *rirA* promoter sequence. (Martinson, DA contributed to the work presented in this figure).

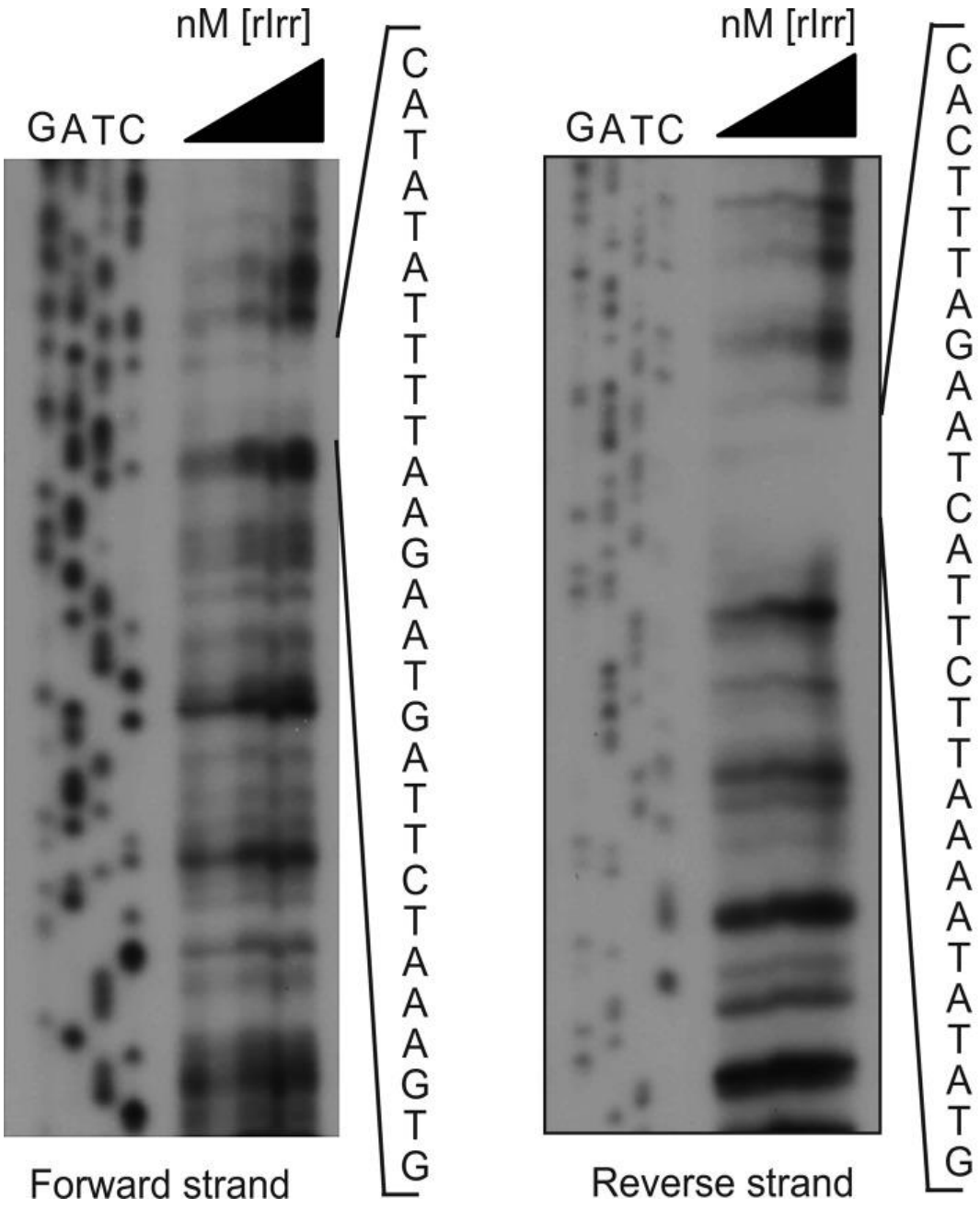
nM [rIrr]
GATC
CATATATTTTTAAGAATGATTCTAAAGTG
Forward strand
nM [rIrr]
GATC
CACTTTTAGAATCATTCTTAAAATATATG
Reverse strand

Figure 3.6

Figure 3.7 Model showing the proposed role of BhuQ in allowing the transcriptional regulator RirA to recognize heme as an iron source in *Brucella*. Under iron-deprived conditions, Irr is stable and drives the expression of genes involved in iron and heme transport. As intracellular iron levels increase, Irr is degraded, which allows expression of the *rirA-bhuQ* operon. BhuQ degrades the heme being transported into the cell, releasing Fe^{2+}. When cellular iron levels reach a threshold, RirA is postulated to repress the expression of the iron and heme acquisition genes. (Martinson, DA contributed to the work presented in this figure).

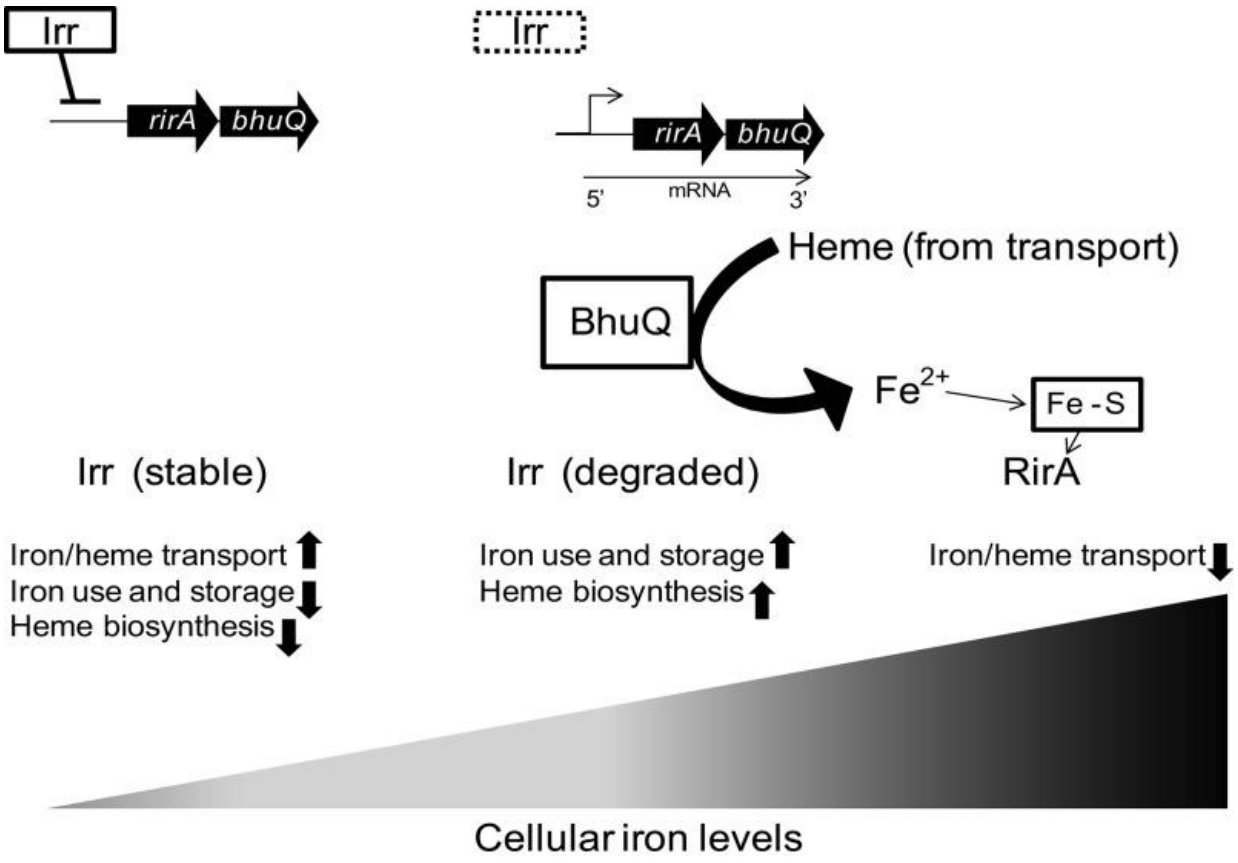
Irr
rirA
bhuQ
Irr
rirA
bhuQ
5'
mRNA
3'
Heme (from transport)
BhuQ
Fe2+
Fe -S
RirA
Irr (stable)
Irr (degraded)
Iron/heme transport
Iron use and storage
Heme biosynthesis
Iron use and storage
Heme biosynthesis
Iron/heme transport
Cellular iron levels

Figure 3.7

Chapter 4: The activity of Irr as an iron-responsive transcriptional regulator in *Brucella* is controlled by inactivation and degradation through its interaction with heme in response to cellular iron levels

Abstract

In *Brucella*, the Irr protein (iron-responsive regulator) is the main iron-responsive transcriptional regulator. The genes coding for the ferrous iron acquisition system FtrABCD are positively regulated by Irr, and the gene coding for the iron exporter MbfA is repressed by Irr. The *ftrABCD* operon has a predicted ICE motif (iron control element) located upstream of its promoter region and *mbfA* has a predicted ICE motif that overlaps its promoter region. We confirmed the location of these ICE motifs by binding recombinant Irr to DNA probes containing the predicted ICE motifs. The location of the ICE motif in relation to the promoter region dictates whether Irr will activate or repress these two target genes. *Brucella* Irr displays iron-responsive degradation although it lacks the HRM needed for iron-responsive degradation in the *B. japonicum* Irr protein. *Brucella* Irr has the ability to bind to heme, and a derivative of Irr with a mutated HXH heme binding motif is not degraded in response to rising cellular iron levels, suggesting that this motif is important for heme dependent *Brucella* Irr turn-over via a mechanism that is independent of an HRM. We have also found that the degradation of the *Brucella* Irr protein requires the enzymatic activity of ferrochelatase. By receiving heme as it is synthesized by ferrochelatase, heme can act as a signaling molecule to control Irr activity in response to cellular iron levels without the need for excessive heme or iron levels to accumulate in the cell. The stable version of Irr does not have the ability to activate the expression of the

ftrABCD operon, and has a generalized defect in iron acquisition, but retains partial repressor activity on *mbfA* expression when cellular iron levels are low. Controlling the activity of Irr through inactivation and degradation allows for a greater sensitivity in response to cellular iron levels than by degradation alone ensuring physiologically relevant iron levels are maintained to sustain bacterial replication.

Introduction

Brucella abortus is an intracellular pathogen that causes abortion and infertility in its natural ruminant host (Olsen and Tatum, 2010), and undulant fever in humans (Pappas et al., 2006). Successful *Brucella* infections depend on the ability to these bacteria survive for long periods of time in the phagosomal compartments of host macrophages (Baldwin & Roop, 1999). Upon entry into the host cells, the brucellae reside in *Brucella* containing vacuoles (BCVs) that interact with components of the endocytic pathway. The events that occur beginning with *Brucella* entry into the macrophage and ending with *Brucella* gaining access to their replicative niche in a vacuole within the endoplasmic reticulum are known as *Brucella* trafficking. Immediately after internalization, the BCVs undergo very transient interactions with early endosomes (Celli et al., 2003). BCV acidification is an essential step for proper *Brucella* trafficking and intracellular survival (Starr et al., 2008; Boschiroli et al., 2002). Acidification of the BCV induces the production of the Type IV secretion system that secretes effector molecules to control the trafficking of the BCV (Celli et al., 2003). Following transient fusion events with the endocytic pathway and acidification, the BCVs fuse with the endoplasmic reticulum and return to a neutral pH, resulting in a safe niche for intracellular persistence of the brucellae (Celli et al., 2005).

Iron is an essential micronutrient which is required for *Brucella* replication (Evenson & Gerhardt, 1955). The natural environment of *Brucella* is in association with mammalian host cells which presents a problem for these bacteria in regards to acquiring enough iron for their physiological needs. Iron sequestration by mammals serves as an important innate immune response by limiting iron availability to invading organisms (Nairz et al., 2010). Iron that is not incorporated into host molecular components is tightly bound to host iron-proteins such as

transferrin, lactoferrin and ferritin (Griffiths, et al., 1999). By compartmentalizing iron into iron storage proteins the host prevents iron toxicity for itself, along with limiting the availability of iron to invading microbes. Upon activation of host macrophages by INF-γ, iron restriction in the phagosomal compartment becomes exacerbated. There is a reduced flux of iron through these phagocytes because of the reduced production of transferrin receptors on the surface of activated macrophages (Byrd and Horwitz, 1989). INF-γ also stimulates of the activity of the natural resistance-associated macrophage protein 1 (Nramp1), which pumps divalent cations such as Fe^{2+} out of the phagosomal compartment (Cellier et al., 2007). In order to overcome iron deprivation while within the host, *Brucella* have a functional siderophore system to acquire iron (Lopez-Goni et al., 1992; Bellaire et al., 2003), can use heme as an iron source (Paulley et al., 2007), and have an efficient ferrous iron transport system (Elhassanny et al., 2013).

While iron is required for *Brucella* survival in host cells, it can also act as a partner with oxygen and reduced oxygen species in the Fenton reaction yielding hydroxyl radicals that can damage biological molecules such as DNA, proteins and lipids (Touati, 2000). A balance must therefore be achieved between efficiently scavenging iron to acquire adequate levels for physiological processes, and preventing the excessive accumulation of cellular iron to protect against iron toxicity. To sustain physiologically relevant intracellular iron levels, bacteria typically rely on iron-responsive transcriptional regulators, such as the ferric uptake regulator (Fur) found in *Escherichia coli*, to control the expression of genes coding for iron acquisition systems in response to cellular iron levels.

In many of the alpha-proteobacteria the predominant iron-responsive transcriptional regulator is Irr (iron-responsive regulator). Irr is a member of the Fur family of metalloregulators, but unlike Fur, which is typically active when cellular iron levels are high, Irr

is active when cellular iron levels are low (Hamza et al., 2000). Another important characteristic of Irr that differs from Fur, which is generally thought of as a repressor of transcription, is the ability of Irr to directly activate or repress the expression of genes depending on where it binds relative to the promoter regions of its target genes (Rudolph et al., 2006). In *Brucella*, the Irr protein has the capacity to positively and negatively regulate target genes in response to cellular iron levels and the regulatory function of Irr is required for wild type virulence in experimentally infected mice (Anderson et al., 2012). Irr regulates the expression of the genes coding for siderophore biosynthesis enzymes (Martinez, et al., 2006), the outer membrane heme transporter BhuA, (Anderson et al., 2012), and the ferrous iron transport system FtrABCD (Elhassanny et al., 2013) in response to iron deprivation, all of which are required for virulence in animals. *Brucella* Irr has also been shown to repress the *rirA-bhuQ* operon that encodes another putative iron-responsive regulator and a heme oxygenase (Ojeda et al., 2012). The cumulative body of work analyzing the Irr regulon has led to the proposition that Irr is the predominant iron-responsive transcriptional regulator in *Brucella*. *Brucella* are exposed to a great deal of oxidative stress in host cells, and the activity of Irr ensures that the expression of genes coding for iron acquisition systems is only activated when cellular iron levels are low, preventing excessive cellular iron from building up and exacerbating the oxidative stress experienced by these bacteria.

Because Irr controls the expression of *Brucella* iron acquisition systems that are required for wild-type virulence in the animal model of infection, knowing the mechanism through which Irr activity is controlled in response to cellular iron levels is an important aspect to understanding *Brucella* pathogenesis. Control of Irr activity has been most comprehensively characterized in another alpha-proteobacterium *Bradyrhizobium japonicum*. In *B. japonicum*, Irr is a

conditionally stable protein that is active when cellular iron levels are low and is inactivated and degraded as cellular iron levels rise (Hamza et al., 1998; Jaggavarapu & O'Brian, 2014). Irr activity is not directly controlled by iron, but rather through the biosynthesis of heme, which serves as an indicator of cellular iron levels. As iron levels rise from the uptake of extracellular iron, so too does the production of heme. Irr interacts directly with ferrochelatase, the enzyme that catalyzes the final step in heme biosynthesis, inserting iron into the heme precursor protoporphyrin IX (Qi et al., 2002). By interacting with ferrochelatase, Irr is in close proximity to heme as it is synthesized allowing for heme to act as a signal that cellular iron levels are rising without the need for cellular iron or heme to accumulate in the bacterial cell.

The *B. japonicum* Irr protein has a unique heme regulatory motif (HRM) at the N-terminus of that binds to oxidized heme, and an internal HXH motif that binds to reduced heme and is highly conserved among alpha-proteobacteria Irr proteins (Qi et al., 1999; Yang et al., 2004). The ability of the *B. japonicum* Irr protein to interact with both the oxidized and reduced forms of heme is required for efficient Irr degradation, suggesting that the redox activity of heme may be important for controlling Irr activity and turnover (Yang et al., 2006). An alternative model to explain how the activity of Irr is controlled in other alpha-proteobacteria has been described in *Rhizobium leguminosarum*, where Irr is a stable protein in the presence of intracellular iron or heme and its regulatory activity is controlled through the loss of DNA binding activity upon heme binding (Singleton et al., 2010). Inactivation of the *R. leguminosarum* Irr protein is dependent on heme binding to the conserved internal HXH heme binding motif (Singleton et al., 2010) and a second heme binding site that is involved in the oligomerization of Irr (White et al., 2011) that is also conserved in the *Brucella* Irr protein.

Figure 1.5 presents a comparative model depicting how the activity of Irr is modulated in *B. japonicum* and *R. leguminosarum*.

The *Brucella* Irr protein has the conserved internal HXH heme binding motif (Singleton et al., 2010), the ability to bind to heme (Martinez et al., 2005), and is degraded as cellular iron levels rise (Anderson et al., 2011) suggesting that the activity of *Brucella* Irr is controlled by a mechanism similar to that observed in *B. japonicum*. Because Irr regulatory activity is required for wild type virulence of *Brucella* (Anderson et al., 2011) and Irr is the predominant iron-responsive transcriptional regulator in these bacteria, it is clear that controlling Irr activity in response to cellular iron levels is an important virulence determinant. In the current study, we address the mechanism used by *Brucella* to respond to cellular iron levels and the potential benefits to these bacteria as a result of how the activity of Irr is controlled are discussed.

Materials and Methods

Bacterial strains and growth conditions.

Brucella abortus 2308 and derivative strains were grown on Schaedler agar containing 5% defibrinated bovine blood, in brucella broth, or in the low-iron minimal medium described previously (Lopez-Goni et al., 1992). *E. coli* strain DH5α was used for cloning and was grown on tryptic soy agar or in Luria-Bertani broth. When needed for plasmid selection, media were supplemented with carbenicillin (100 μg/mL).

Site directed mutagenesis of the *Brucella* Irr protein.

Primers Irr HXH mut For and Irr HXH mut Rev (Table 4.1) were used to amplify a 2.4 kb fragment of *B. abortus* 2308 genomic DNA with the Irr coding region centrally located using *Pfx* DNA polymerase. This fragment was ligated into the multiple cloning site of pGEMT-Easy to generate the plasmid pGEMT-Easy-Irr. This plasmid was used as a template in a site directed mutagenesis protocol. Mutagenesis primers Irr HXH to AAA For, Irr HXH to AAA Rev (Table 4.1) were designed using the web-based QuikChange Primer Design Program available at www.agilent.com/genomics/qcpd. The Agilent QuikChange II XL Site-Directed Mutagenesis Kit was used to carry out the mutagenesis strategy according to the manufacturer's instructions. Codons 97, 98, and 99 of Irr were changed from HQH to AAA. The nucleotide sequence of the resulting derivative, pGEMT-Easy-Irr^{AAA} was determined to confirm the site-directed mutation. pGEMT-Easy-Irr^{AAA} was used as a template with the primers Irr Heme mut For, Irr Heme mut Rev (Table 4.1) to amplify the mutated *irr* locus using *Pfx* DNA polymerase. The amplified PCR fragment was ligated into the EcoRI site of pBBR1MCS4 (Kovach et al., 1995), resulting in the

plasmid p1MCS4-Irr^{AAA} which was electroporated into the *irr* mutant derivative of *B. abortus* KHS3 (Anderson et al., 2011).

Relative quantification of transcript levels using real-time RT-PCR.

Total RNA isolated from cultures of *B. abortus* 2308, the *irr* mutant KHS3 (Δ*irr*) (Anderson et al., 2012) and from KHS3 complemented with a gene coding for wild type Irr (Irr^{WT}) (Anderson et al., 2012) or with the mutated version of Irr (Irr^{AAA}) following growth in low-iron minimal medium and low-iron minimal medium supplemented with 50 μM $FeCl_3$ until early logarithmic phase. RNA was isolated from the bacterial cells using previously described procedures (Caswell et al., 2012). cDNA was generated from the final RNA preparation using the iScript Reverse Transcriptase Synthesis System (Promega) by following the manufacturer's protocol. The cDNA preparations were then used as the templates for real-time RT-PCR with SYBR green PCR Supermix (Promega) to evaluate the relative levels of gene-specific mRNA transcripts in the total cellular RNA preparations. The gene specific oligonucleotide primers for *mbfA* and *ftrA* are listed in Table 4.1 and *gap* gene that codes for glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was used as a control gene. Parameters for PCR included a single denaturing step for 5 min at 95°C, followed by 40 cycles (denature for 15 s at 95°C, anneal for 15 s at 50°C, and extend for 15 s at 72°C) of amplification. Fluorescence from SYBR green incorporation into double-stranded DNA was measured with a Bio-Rad CFX96 Thermocycler.

Production of recombinant *Brucella* Irr protein.

The *irr* gene from *B. abortus* 2308 genomic DNA was amplified by PCR with Pfx Supermix and the primers rIrr For and rIrr Rev (Table 4.1) and was cloned into the pETM-11

vector (Jaggavarapu & O'Brian, 2014). Cells were inoculated from a 5 mL overnight culture grown in Luria-Bertani media containing 45 μg/mL kanamycin into 500 mL of 2x YT medium (8 g tryptone, 5 g yeast extract, 2.5 g NaCl, 100 μM $MnCl_2$, pH=7.5). Production of the recombinant protein was induced in cells at the mid log phase by the addition of 1mM isopropyl-1-thio-β-D galactopyranoside (IPTG) at 37^{o}C for 4 hours with shaking. Cells were pelleted by centrifugation and resuspended in 40 mL of lysis buffer (50 mM Tris-HCl, 100 μM $MnCl_2$, pH=8.0). Cells were disrupted by passage through a French pressure cell. The insoluble material was pelleted by centrifugation and washed in lysis buffer with 4% NP-40 and again with lysis buffer with 2 M urea. Inclusion bodies were solubilized in lysis buffer with 8 M urea. The soluble material was dialyzed against 20 mM Tris-HCl, 100 μM $MnCl_2$, pH=7.5.

Electrophoretic mobility shift assays (EMSAs).

The test DNA probes were purchased from Eurofins as single stranded oligonucleotides and were 45 bp in length with an ATGATC 5' overhang on one strand and contained the predicted ICE motifs from the *B. abortus* 2308 *mbfA* and *ftrA* promoter regions in the center (Table 4.1). Complementary probes were annealed together by slowly cooling to room temperature after boiling. DNA Polymerase I, Large (Klenow) Fragment (Thermo Scientific) was used to fill in the 5' overhang with α-[^{32}P] dCTP. The resulting probes were incubated for thirty minutes at room temperature with increasing concentrations of recombinant Irr in 1x EMSA binding buffer (10 mM MES Buffer, pH=7.5, 1 mM $MgCl_2$, 40 mM KCl, 5% glycerol, 0.1% NP-40, 1 mM DTT, 100 μM $MnCl_2$). The reactions were then loaded onto a 5% acrylamide gel (5% acrylamide, 20 mM MES Buffer, 20 mM Tris Base, 100 μM $MnCl_2$, pH=7.45) and subjected to electrophoresis at 100V in running buffer (20 mM MES Buffer, 20

mM Tris Base, 100 μM $MnCl_2$, pH=7.45). The gels were transferred to Whatman 1 M paper using a Bio-Rad model 583 gel dryer and visualized by autoradiography.

N-Methyl protoporphyrin IX sensitivity assay and Western blotting.

B. abortus 2308 was cultivated in low iron minimal medium (Lopez-Goni et al., 1992) to late exponential growth phase. N-Methyl protoporphyrin IX (NMPP) (Frontier Scientific) was dissolved in dimethyl sulfoxide. Cultures were treated with 20 μM NMPP or the equivalent volume of dimethyl sulfoxide for 30 minutes at 37^{o}C in a shaking incubator followed by the addition of $FeCl_3$ to a final concentration of 50 μM. At the specified time points, portions of the cultures were collected by centrifugation and the pellets were suspended in 50:50 ethanol: acetone. Cells were again collected by centrifugation and the pellets were suspended in 1 mL of protein sample buffer (0.3% SDS, 200 mM DTT, 22 mM Tris-Base, and 28 mM Tris-HCl). The cells were then boiled for 1 hour followed by 10 cycles (20 seconds per cycle) of disintegration with Lysing Matrix B (MP Biomedicals, Solon, OH) using a BIO101 FastPrep FP120 cell disruptor (Thermo Savant [Thermo Fisher Scientific Inc.], Waltham, MA). A Bradford assay with BSA standards was used to determine protein concentrations. Ten μg of total protein per culture was separated on a 13% SDS-PAGE gel. Proteins were transferred to a nitrocellulose membrane by electroblotting at 100 V for 2 hours. Membranes were blocked at room temperature in 5% skim milk in PBST (phosphate buffered saline, 155 mM NaCl, 8 mM Na_2HPO_4, 2 mM NaH_2PO_4, 0.05% Tween-20). Primary antibodies (anti-Irr [1:10,000]) (Anderson et al., 2012) were incubated with the membranes at room temperature in 5% skim milk. Secondary antibodies (anti-rabbit IgG HRP conjugate) (Promega) were used at a 1:10,000

dilution. All washing steps were performed with PBST. Visualization of the HRP signal was performed using SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific).

Measurement of siderophore production.

Following growth of *B. abortus* 2308, the *irr* mutant KHS3 (Δ*irr*) (Anderson et al., 2012) and KHS3 complemented with a gene coding for wild type Irr (Irr^{WT}) (Anderson et al., 2012) or with the mutated version of Irr (Irr^{AAA}) in low-iron minimal medium (Lopez-Goni et al., 1992), portions of the cultures were pelleted by centrifugation and the resulting supernatant was removed to a fresh tube. The levels of catechol siderophore present was measured using the Arnow assay (Arnow, 1937).

Streptonigrin sensitivity assay.

B. abortus 2308, the *irr* mutant KHS3 (Δ*irr*) (Anderson et al., 2012), KHS3 complemented with a gene coding for wild type Irr (Irr^{WT}) (Anderson et al., 2012) or with the mutated version of Irr (Irr^{AAA}) were cultivated in low iron minimal medium until late exponential phase. Samples were adjusted to an optical density of OD_{600} of 0.15 ($1.0x10^9$ CFU/mL) into 0.5 mL of low iron minimal medium supplemented with 50 μM $FeCl_3$. Streptonigrin (SNG) solution was prepared in DMSO at 10 mg/mL. 100 μg/mL SNG or the equivalent volume of DMSO was added to the samples which were cultivated for 4 hours at 37°C while shaking. Samples were serially diluted and plated on SBA to determine the number of viable brucellae present.

Statistical analysis.

All statistical analyses were performed using the Student's two-tailed *t* test (Rosner, 2000). *P* values of $\leq$0.001 (**) and $\leq$0.01 (*) for the indicated comparisons were considered significant.

Results

The ability of *B. abortus* Irr to activate *ftrA* or repress *mbfA* is dependent upon where it binds in the promoter regions of these genes.

We chose to analyze the ability of Irr to regulate the genes designated *bab1_1691* and *ftrABCD* in an iron-responsive manner because these two genes are among the most highly iron-responsive genes in a microarray analysis (Martinson, unpublished). BAB1_1691 is a close homolog to MbfA, which has been characterized as an iron exporter in *Bradyrhizobium japonicum* (Sankari & O'Brian, 2014) and *Agrobacterium tumefaciens* (Bhubhanil et al., 2014). The *ftrABCD* operon encodes a ferrous iron transport system that is required for wild type virulence of *Brucella* in the mouse model of infection (Elhassanny et al., 2013). *B. abortus* 2308, the *irr* mutant KHS3 (Δ*irr*), and *irr* mutant complemented with a gene coding for wild type Irr (Irr^{WT}) were grown under iron deplete and iron replete conditions and the expression levels of the *mbfA* and *ftrA* genes were analyzed by real-time RT-PCR. Expression of the *ftrA* gene is significantly higher in the wild type and the *irr* mutant complemented with a gene coding for Irr^{WT} strains when compared to the level of expression in the *irr* mutant during growth in iron limiting conditions (Figure 4.1A). Alternatively, the expression of the *mbfA* gene is significantly higher in the *irr* mutant when compared to the level of expression in the wild type or *irr* mutant complemented with a gene coding for Irr^{WT} strains during growth in iron limiting conditions (Figure 4.1B). These results indicate that Irr is simultaneously repressing *mbfA* and activating *ftrABCD* during growth in iron deplete conditions.

The location of the ICE motif is the determining factor as to whether Irr will act as an activator or repressor in *B. japonicum* (Rudolph et al., 2006). In *Brucella*, the gene coding for the outer membrane heme transporter BhuA is activated by Irr and binds to the upstream region of

the *bhuA* gene (Anderson et al., 2011). The *Brucella rirA-bhuQ* operon is repressed by Irr, where it binds in the promoter region near the transcriptional start site (Ojeda et al., 2012). Computational analysis predicts the presence of *Brucella* ICE motifs in the promoter regions of *mbfA* and the *ftrABCD* operon (Rodionov et al., 2006). The putative ICE motif is located just upstream of the translational start site of *mbfA*, which Irr represses, and is located 59 bp upstream of the *ftrA* transcriptional start site, which Irr activates (Elhassanny et al., 2013) (Figure 4.1C). The ICE motif sequences for *ftrA* and *mbfA* have been aligned to show conservation of nucleotide residues among these two genes. Underlined sequences indicate 100% ICE motif homology between those nucleotide residues.

Probes were designed with the predicted *mbfA* and *ftrA* ICE motifs located in the center and the ability of *Brucella* Irr to bind to these specific regions of DNA was analyzed through electrophoretic mobility shift assays (EMSAs). Recombinant *Brucella* Irr binds to the predicted ICE motifs in the upstream promoter regions of *ftrA* and *mbfA* indicating that Irr can act directly as a activator and a repressor on these genes (Figure 4.1D, E). The ability of the *Brucella* Irr protein to activate or repress target genes when cellular iron levels are low has also been observed in other *Brucella* Irr regulated genes (Anderson et al., 2011; Ojeda et al., 2012) and parallel regulation has been observed in *B. japonicum* Irr regulated genes (Rudolph et al., 2006).

The *B. abortus* 2308 Irr protein is degraded in response to high cellular iron levels.

In *B. japonicum,* Irr is degraded when cellular iron levels are high (Hamza et al., 1998). Iron-dependent degradation of *B. japonicum* Irr involves direct binding of heme to Irr at a unique amino-terminal heme regulatory motif (HRM) (Qi et al., 1999) and an internal HXH heme binding motif that is highly conserved in alpha-proteobacteria Irr proteins (Yang et al., 2004). *B.*

abortus Irr lacks the *B. japonicum* HRM, but has the conserved HXH heme binding motif that is important for controlling the activity of Irr in *B. japonicum*, *R. leguminosarum* and *Agrobacterium tumefaciens* (Figure 4.2) (Yang et al., 2004; Singleton et al., 2010; Bhubhanil et al., 2012). Previous studies have shown that *B. abortus* Irr binds to heme *in vitro* (Martinez et al., 2005) and is degraded during growth in iron replete medium (Anderson et al., 2012). In order to determine if the conserved HXH heme binding motif is required for the degradation of Irr in response to intracellular iron levels, we constructed a plasmid coding for a version of Irr that has the residues 97, 98, and 99 mutated from HQH to AAA (Irr^{AAA}) using site directed mutagenesis. After growth in low iron minimal medium for 72 hours, an isogenic *irr* mutant (KHS3) expressing a gene coding for either Irr^{WT} or Irr^{AAA} have similar levels of total Irr protein present, however, after exposure to 50 μM iron, Irr^{WT} is quickly degraded, whereas Irr^{AAA} is completely stable (Figure 4.3). These findings are important for two reasons. First, we have confirmed that the *Brucella* Irr protein is degraded quickly in response to rising cellular iron levels. Second, the conserved HXH heme binding motif is required for the iron-responsive degradation of the *Brucella* Irr protein through an HRM independent mechanism.

Degradation of *B. japonicum* Irr is dependent on the enzymatic activity of ferrochelatase to deliver heme to the protein in response to cellular iron levels (Qi & O'Brian, 2002). We wanted to know if *Brucella* Irr degradation was also dependent on ferrochelatase, but were unsuccessful in multiple attempts to construct a *B. abortus* ferrochelatase mutant, which would be a heme auxotroph, so we used the ferrochelatase specific inhibitor N-Methyl protoporphyrin IX (NMPP) (Dailey & Fleming, 1983) as an alternative approach. When *B. abortus* was pre-treated with NMPP after growth in low iron minimal medium and exposed to 50 μM $FeCl_3$, Irr degradation was inhibited, whereas Irr was degraded in the absence of NMPP (Figure 4.4). The

data support the proposition that the enzymatic activity of ferrochelatase is required for the heme dependent, iron-responsive degradation of *B. abortus* Irr. *B. japonicum* Irr binds directly to ferrochelatase putting Irr in close proximity to heme as it is being synthesized (Qi & O'Brian, 2002). This mechanism prevents the need for heme levels to build in order to act as signaling molecules and appears to be what happens in *Brucella* as well. The ability to respond to heme quickly and at low concentrations is advantageous for *Brucella*, because heme can react with oxygen to form reactive oxygen species that can damage biological molecules (Aft & Mueller, 1984), so keeping heme levels at a minimum reduces the risk of oxidative damage mediated by heme or iron.

The regulatory activity of *B. abortus* 2308 Irr^{AAA} is altered compared to the wild type version of the protein.

Because Irr is a global iron regulator in *Brucella* and Irr^{AAA} is a stable protein, a *Brucella* derivative producing this protein is likely to exhibit a defect in its ability to regulate iron response genes. At first glance, it seems that one possibility is that this stable version of Irr would be constitutively active resulting in the unregulated expression of genes coding for iron acquisition regardless of cellular iron levels. However, mutations in the conserved HXH heme binding motif in the plant pathogen *Agrobacterium tumefaciens* results in an Irr protein that has greatly diminished repressor activity (Bhubhanil et al., 2012). We therefore analyzed the ability of a *B. abortus irr* mutant complemented with a gene coding for either Irr^{WT} or Irr^{AAA} to respond to cellular iron deprivation. As shown in Figure 4.5A, wild type *B. abortus* and the *B. abortus irr* mutant complemented with a gene coding for Irr^{WT} produce significantly more siderophore than the *irr* mutant strain and the *irr* mutant strain complemented with a gene coding for Irr^{AAA}. We

also looked at resistance to the iron activated antibiotic streptonigrin in these *B. abortus* derivatives to check for a more global defect in iron uptake. Results presented in Figure 4.5B show that the *B. abortus irr* mutant, and the mutant complemented with a gene coding for Irr^{AAA} are resistant to streptonigrin, while the wild type strain and the *irr* mutant complemented with a gene coding for Irr^{WT} are significantly less resistant to streptonigrin than without treatment. These results were mirrored at the genetic level when *ftrA* expression was analyzed by real-time RT-PCR in response to iron deprivation in these *B. abortus* derivatives. Figure 4.5C shows that *ftrA* expression is significantly high during growth under low iron conditions in the *B. abortus irr* mutant complemented with a gene coding for Irr^{WT}, compared expression levels in the *irr* mutant, while the *irr* mutant and the *irr* mutant complemented with a gene coding for Irr^{AAA} exhibit similar *ftrA* expression levels. These data indicate that although a derivative producing Irr^{AAA} has consistently high levels of Irr^{AAA} present, Irr^{AAA} has a general defect in responding to iron deprivation and lacks the ability to activate the expression of genes involved in iron acquisition. We also tested the expression of *mbfA* in response to iron limitation in these *B. abortus* derivatives to see if Irr^{AAA} repressor activity was affected. Figure 4.5D shows that *mbfA* expression is high in a *B. abortus irr* mutant compared to the mutant complemented with a gene coding for Irr^{WT}, which is consistent with Irr dependent repression during growth in low iron conditions. Expression of *mbfA* in a *B. abortus irr* mutant complemented with a gene coding for Irr^{AAA} is at an intermediate level, indicating that Irr^{AAA} retains some repressor activity. Taken together, results from Figure 4.5 show that Irr^{AAA} has no activator activity, but does retain an intermediate level of repressor activity. This suggests that the *Brucella* HXH heme binding motif plays a more important role in controlling the activator function of Irr than it does the repressor activity.

Discussion

Successful *Brucella* infections depend on the ability of the brucellae to survive and replicate within host phagocytic cells (Roop et al., 2009). Because of this intracellular lifestyle, *Brucella* must obtain nutrients required for growth, such as iron, from the host. As part of the innate immune defense to limit the replication of pathogens, host cells restrict the availability of iron by keeping iron bound in iron sequestering proteins such as transferrin and lactoferrin in the extracellular environment and ferritin which is found intracellularly (Griffiths et al., 1999). Upon activation of host macrophages by INF-γ, the flux of iron through these phagocytes is reduced due to the decreased production of transferrin receptors on their surface (Byrd & Horwitz, 1989) and iron restriction in the phagosomal compartment becomes exacerbated as a result of the activity of the natural resistance-associated macrophage protein 1 (Nramp1), which pumps iron out of the phagosomal compartment (Cellier et al., 2007). IFN-γ is important for the clearance of *Brucella* during macrophage infections (Zhan & Cheers, 1993; Pasquali et al., 2001), making the resulting iron restriction relevant to understanding the biology of *Brucella* infections. *Brucella* rely on efficient iron transport systems to overcome IFN-γ mediated iron restriction in the phagosome. *Brucella* derivatives that lack the ability to synthesize siderophore molecules, or to transport heme or ferrous iron exhibit attenuation in animals (Bellaire et al., 2003; Paulley et al., 2007; Elhassanny et al., 2013). This furthers the argument that responding to iron deprivation is an important component of *Brucella* pathogenesis.

While iron acquisition is important for *Brucella* to establish and maintain an infection in the host, excessive intracellular iron levels can be detrimental to the brucellae due to the production of hydroxyl radicals that can damage biological molecules such as DNA, proteins and

lipids (Touati, 2000). For this reason, the expression of genes involved in iron metabolism and transport are tightly controlled in bacteria. In the alpha-proteobacteria, Irr (iron-responsive regulator) and/or RirA (rhizobial iron regulator) are responsible for modulating gene expression in response to cellular iron levels. In *B. japonicum*, Irr is the primary iron-responsive transcriptional regulator, where when cellular iron levels are low, it activates the expression of genes coding for iron acquisition systems and represses the expression of genes that encode products that require iron for their activity (Hamza et al., 2000; Johnston et al., 2007). In *R. leguminosarum*, RirA is the primary iron-responsive transcriptional regulator, with Irr having a limited role in responding to cellular iron levels. When *R. leguminosarum* cellular iron levels are high, RirA represses the expression of genes coding for iron acquisition systems (Todd et al., 2002; Todd et al., 2005). When *R. leguminosarum* cellular iron levels are low, RirA is inactive, and derepression of genes coding for iron acquisition systems occurs, whereas Irr is active and represses the expression of genes coding for products that require iron for their function, while having no effect on the expression of genes coding for iron acquisition systems (Todd et al., 2006). In *Brucella*, Irr directly regulates the expression of genes coding for siderophore biosynthesis (Martinez et al., 2006), heme transport (Anderson et al., 2011; Ojeda et al., 2012) and ferrous iron transport (Figure 4.1) (Elhassanny et al., 2013) which have all been linked to virulence in mammals, suggesting that Irr is the primary iron-responsive transcriptional regulator in *Brucella*.

The literature presents two paradigms explaining how the activity of Irr is controlled (see Figure 1.5). In. *B. japonicum*, Irr activity is controlled by inactivation and degradation through a direct interaction with heme (Jaggavarapu & O'Brian, 2014; Hamza et al., 1998). Degradation of *B. japonicum* Irr is dependent on the ability of the protein to bind to the reduced form of heme at

a unique N-terminal heme regulatory motif (HRM) and to the oxidized form of heme at an internal HXH heme binding motif that is highly conserved among the alpha-proteobacteria (Yang et al., 2004). Alternatively, in *R. leguminosarum*, as cellular iron levels rise, Irr becomes inactivated and oligomerizes, losing its ability to bind to DNA, as cellular iron levels rise (Singleton et al., 2010; White et al., 2011). *R. leguminosarum* Irr heme dependent inactivation relies on the ability of the protein to bind to reduced heme at the conserved HXH motif and a second, low-affinity heme binding motif (White et al., 2011) that is also conserved in the *Brucella* Irr protein. The *Brucella* Irr protein has the capacity to bind to heme (Martinez et al., 2005) and is degraded when cellular iron levels are high (Anderson et al., 2011), suggesting that this protein follows the *B. japonicum* Irr degradation paradigm. While the *Brucella* Irr protein lacks the HRM found in *B. japonicum*, it has the HXH heme binding motif that is highly conserved among the alpha-proteobacteria (Figure 4.2). A *Brucella* Irr derivative that has the HXH heme binding motif mutated (Irr^{AAA}) is stable regardless of cellular iron levels, whereas the wild type Irr (Irr^{WT}) is quickly degraded as cellular iron levels rise (Figure 4.3). The stability of the Irr^{AAA} derivative indicates that this motif is important for heme dependent degradation in *Brucella*. In *B. japonicum*, Irr degradation is enhanced when heme is delivered by the ferrochelatase enzyme (Qi & O'Brian, 2002). The enzymatic activity of ferrochelatase is to insert iron into the heme precursor, protoporphyrin IX, resulting in the synthesis of a heme molecule. Ferrochelatase binds directly to Irr, so when the heme molecule is made, it is in close proximity to Irr (Qi & O'Brian, 2002). While we were unable to construct a *Brucella* ferrochelatase mutant, we found that the ferrochelatase inhibitor N-Methyl protoporphyrin IX interferes with the iron-responsive degradation of Irr in *Brucella* (Figure 4.4). An advantage to delivering heme to Irr as it is synthesized is that heme can act as a signaling molecule immediately preventing the need for

cellular heme levels to build to modulate the activity of Irr. This is important because heme can react with oxygen to form reactive oxygen species that damage biological molecules. Keeping cellular heme levels low helps to protect *Brucella* from heme mediated oxidative stress which is advantageous for these bacteria during an intracellular infection where they are already exposed to high levels of oxidative stress.

We know from the work in this, and previous studies, that Irr regulates the expression of genes in an iron-responsive manner. Here we have found that the *Brucella* Irr^{AAA} derivative is stable regardless of cellular iron levels. These findings led to the prediction that a *Brucella* Irr^{AAA} derivative would have a defect in its ability to act as a transcriptional regulator in an iron-responsive manner. In the alpha-proteobacterium, *Agrobacterium tumefaciens*, mutation of the HXH heme binding motif results in an Irr protein with no repressor activity (Bhubhanil et al., 2012). The *Brucella* Irr^{AAA} derivative is not able to complement the siderophore production defect or streptonigrin resistance phenotypes observed in the *Brucella irr* mutant. (Figure 4.5A,B). Furthermore, expression levels of the *ftrA* gene are the same in the *Brucella* Irr^{AAA} derivative as observed in the *Brucella irr* mutant during growth under low iron conditions (Figure 4.5C). The *Brucella* Irr^{AAA} derivative exhibits intermediate repressor activity on the *mbfA* gene during growth in the same conditions. This suggests that the HXH heme binding motif is more important for controlling Irr activator function than it is for repressor function, at least for the genes tested. One possibility is that the stable, mutated version of Irr has a reduced DNA binding activity, either through improper protein folding or the inability to diamerize effectively, preventing it from binding to lower affinity promoters, but still retaining the ability to bind to higher affinity promoters. This may reflect the environment *Brucella* is exposed to during an infection. There is considerable oxidative stress experienced by *Brucella* during

growth in the phagosomal compartment, which makes controlling cellular iron levels important to prevent additional iron related oxidative toxicity. Because the heme binding motif is more important for controlling Irr activation function than repressor function, activation of genes that encode products involved in iron acquisition is tapered quickly as cellular iron levels rise, preventing the excessive and potentially dangerous build up iron.

Figure 4.6 depicts our working model describing how *Brucella* Irr activity is controlled at different cellular iron levels. When cellular iron levels are low, Irr is active and promotes the expression of genes that encode products that are involved in iron acquisition while repressing genes involved in iron utilization or export. As cellular iron levels rise, heme is synthesized by ferrochelatase and delivered to the Irr protein inhibiting its activator activity, but only intermediately inhibiting its repressor activity. This is likely due to the DNA binding capacity of low (iron acquisition) vs. high (iron utilization or export) affinity promoters observed in *B. japonicum* (Jaggavarapu & O'Brian, 2014). As Irr becomes partially inactivated, it loses the capacity to bind to low affinity promoter regions, while retaining the ability to bind to the high affinity promoter regions. Finally, when cellular iron levels rise to a threshold level, Irr is degraded and full derepression of the genes coding for iron utilization and export occurs. The ability of *Brucella* to differentially modulate the expression of genes coding for iron uptake systems and iron export at different iron levels ensures that physiologically relevant cellular iron levels are maintained.

Some of the alpha-proteobacteria rely on both Irr and RirA to control the expression of genes in response to cellular iron levels. In *R. leguminosarum*, RirA is the predominant iron-responsive regulator, with Irr regulating only a small number of genes (Todd et al., 2002; Todd et al., 2005; Todd et al., 2006). In *A. tumefaciens*, however, the Irr and RirA regulons overlap

and are controlled in an antiparallel fashion (Hibbing & Fuqua, 2011). *A. tumefaciens* Irr is active when cellular iron levels are low, activating the expression of genes that encode products involved in iron acquisition (Hibbing & Fuqua, 2011). When cellular iron levels are high, RirA is active and represses the expression of genes that encode products involved in iron acquisition (Hibbing & Fuqua, 2011). Preliminary data suggest that the *Brucella* RirA and Irr regulons overlap in a manner similar to that observed in *A. tumefaciens* (Ojeda et al., 2012). The ability to ensure that expression of iron uptake genes occurs when cellular iron levels are low through Irr activity, while repressing the same genes when cellular iron levels are high through RirA activity provides these bacteria with a very precise control mechanism to modulate intracellular iron concentrations to physiological levels. The natural environment of *Brucella* exposes them to a number of stresses, including a high level of oxidative stress inside the phagosome of a macrophage that could be enhanced by excessive intracellular iron levels. Carefully monitoring and modulating intracellular iron levels may be a mechanism to help protect intracellular *Brucella* from certain killing mechanisms employed by macrophages. Thus, having both Irr and RirA to regulate cellular iron levels is beneficial for *Brucella* survival in the macrophage.

Acknowledgments

This work was funded by a grant (AI 63516) from the National Institute of Allergy and Infectious Disease to R.M.R.

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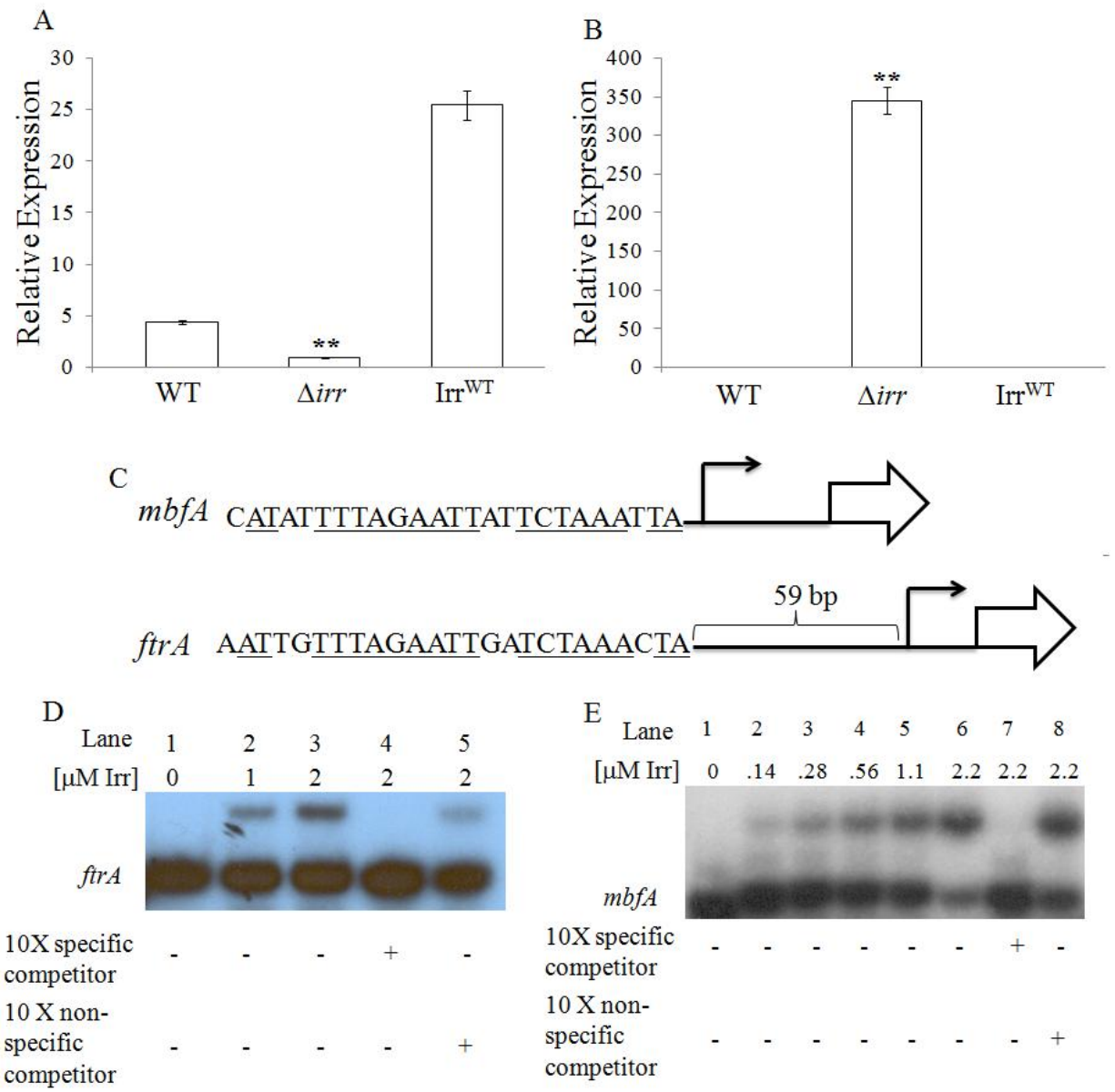
Table 4.1 Primers used in this study.

Primer Name	Primer Sequence
gap For	GCAGTTCGCGTCGCAAT
gap Rev	GAAGATGTGCGTTGGTTTCG
mbfA RT For	GCGAGCATGTCAGTGGCTA
mbfA RT Rev	GTTTGCGCGTGTCTGCATC
ftrA RT For	GCATGTTGATTGGCCGAC
ftrA RT Rev	GGTCGTTTCCTATGAGCT
dhbC RT For	TCCACAAGGACAAGGGAAAG
dhbC RT Rev	AGGCGATGTTCGTAATGGTC
rIrr For	GTTTCTTCCATGGCGGTGAATATGCATTC
rIrr Rev	GTTTCTTGAGCTCTCAGCGGGCCTGACG
mbfA EMSA For	ATGATCCCGCCGAGGCATATTTTAGAATTATTCTAAATTAATGGATTTAA
mbfA EMSA Rev	TTAAATCCATTAATTTAGAATAATTCTAAAATATGCCTCGGCGGG
ftrA EMSA For	ATGATCGGCGGGACAAAAATTGTTTAGAATTGATCTAAACTATTGTTTTTCTT
ftrA EMSA Rev	AAGAAAAACAATAGTTTAGATCAATTCTAAACAATTTTTGTCCCGCC
Irr HXH mut For	GAAGCCCAGTTCCGCATATTC
Irr HXH mut Rev	CCTTCACCTCGTCGCTGTGAT
Irr HXH to AAA For	CCTATTTCGACACCAATATCTCGATCACCAGCACTTCTTCCTCGAAGGGGGAAAATGTGG
Irr HXH to AAA Rev	CCACATTTTCCCCTTCGAGGAAGAAGTGCTGGTGATCGGAGATATTGGTGTCGAAATAGG

Figure 4.1 Irr directly regulates the expression of *ftrABCD* and *mbfA* in *B. abortus* 2308. Real-time RT-PCR analysis of *ftrA* (A) and *mbfA* (B) expression during exponential growth in iron deplete media. The results presented are means and standard deviations from a single experiment that is representative of at least three experiments performed from which equivalent results were obtained. (**) indicates a *P* value of $\leq$0.001. (C) Schematic depicting ICE motif nucleotide sequences and location in relation to transcriptional start sites. Underlined nucleotide sequence represents 100% conserved ICE Box residues between the two promoter regions. Closed black arrow indicates the location of the transcriptional start site and the open arrows represent the coding region of the gene. (D) Lane 1, 2 nM ^{32}-P-labeled *ftrABCD* promoter-specific DNA fragment; lanes 2 and 3, 2 nM ^{32}-P-labeled *ftrABCD* promoter-specific DNA fragment plus increasing concentrations (1, 2 μM) of the Irr protein; lane 4, 2 nM ^{32}-P-labeled *ftrABCD* promoter-specific DNA fragment plus 2 μM Irr and 200 nM un-labeled *ftrABCD* promoter-specific DNA fragment (specific inhibitor); lane 5, 2 nM ^{32}-P-labeled *ftrABCD* promoter-specific DNA fragment plus 2 μM Irr and 200 nM of a 45 bp fragment of DNA from the coding region of *mbfA* (non-specific inhibitor). (E) Lane 1, 2 nM ^{32}-P-labeled *mbfA* promoter-specific DNA fragment; lanes 2 through 6, 2 nM ^{32}-P-labeled *mbfA* promoter-specific DNA fragment plus increasing concentrations (0.14, 0.28, 0.56, 1.1, 2.2 μM) of the Irr protein; lane 7, 2 nM ^{32}-P-labeled *mbfA* promoter-specific DNA fragment plus 2 μM Irr and 200 nM un-labeled *mbfA* promoter-specific DNA fragment (specific inhibitor); lane 8, 2 nM ^{32}-P-labeled *mbfA* promoter-specific DNA fragment plus 2 μM Irr and 200 nM of a 45 bp fragment of DNA from the coding region of *mbfA* (non-specific inhibitor).


A
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B
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50
0
**
WT
Δirr
IrrWT
C
mbfA CATATTTTAGAATTATTCTAAATTA
59 bp
ftrA AATTGTTTAGAATTGATCTAAACTA
D
Lane 1 2 3 4 5
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ftrA
10X specific competitor - - - + -
10 X non-specific competitor - - - - +
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Lane 1 2 3 4 5 6 7 8
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mbfA
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10 X non-specific competitor - - - - - - - +

Figure 4.1

Figure 4.2 The *B. abortus* 2308 Irr protein has the HXH heme binding motif that is highly conserved among the alpha-proteobacteria. Alignment of Irr proteins from *B. japonicum*, *R. leguminosarum*, *A. tumefaciens* and *B. abortus*. Conserved amino acid residues are highlighted in black. The HRM unique to the *B. japonicum* Irr protein is boxed in blue. The conserved HXH motif is boxed in black. The alignment was created using VectorNTI.

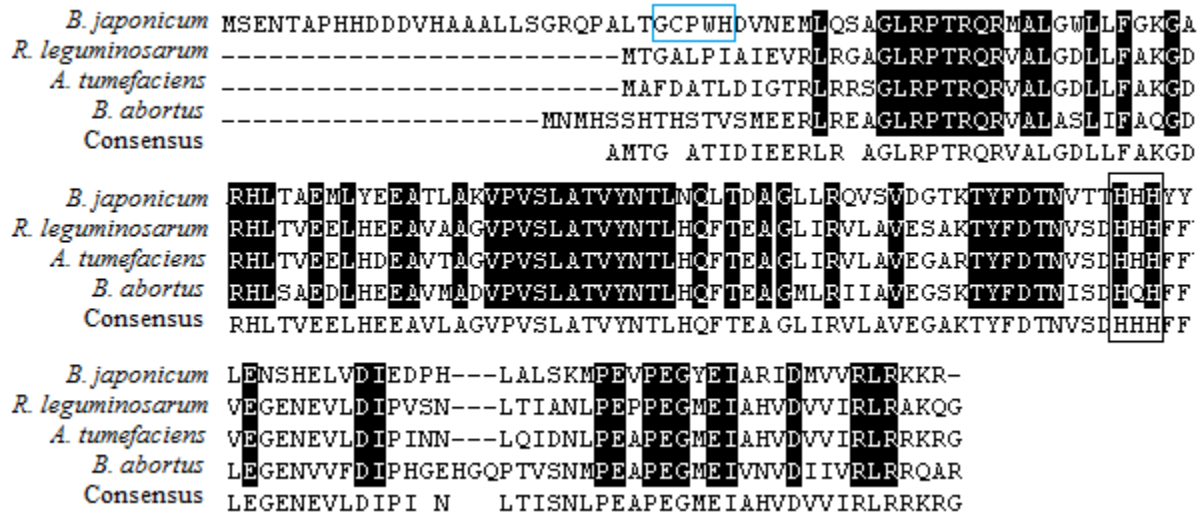

B. japonicum MSENTAPHHDDDVHAAALLSGRQPALTGCPWHDVNEMLQSAGLRPTRQRMALGWLLFGKGA
R. leguminosarum ----------MTGALPIAIEVRLRGAGLRPTRQRVALGDLLFAKGD
A. tumefaciens ----------MAFDATLDIGTRLRRSGLRPTRQRVALGDLLFAKGD
B. abortus ----------MNMHSSHTHSTVSMEERLREAGLRPTRQRVALASLIFAQGD
Consensus AMTG ATIDIEERLR AGLRPTRQRVALGDLLFAKGD
B. japonicum RHLTAEMLYEEATLAKVPVSLATVYNTLNQLTDAGLLRQVSVDGTKTYFDTNVTTHHHYY
R. leguminosarum RHLTVEELHEEAVAAGVPVSLATVYNTLHQFTEAGLIRVLAVESAKTYFDTNVSDHHHFF
A. tumefaciens RHLTVEELHDEAVTAGVPVSLATVYNTLHQFTEAGLIRVLAVEGARTYFDTNVSDHHHFF
B. abortus RHLSAEDLHEEAVMADVPVSLATVYNTLHQFTEAGMLRIIAVEGSKTYFDTNISDHQHFF
Consensus RHLTVEELHEEAVLAGVPVSLATVYNTLHQFTEAGLIRVLAVEGAKTYFDTNVSDHHHFF
B. japonicum LENSHELVDIEDPH---LALSKMPEVPEGYEIARIDMVVRLRKKR-
R. leguminosarum VEGENEVLDIPVSN---LTIANLPEPPEGMEIAHVDVVIRLRAKQG
A. tumefaciens VEGENEVLDIPINN---LQIDNLPEAPEGMEIAHVDVVIRLRRKRG
B. abortus LEGENVVFDIPHGEHGQPTVSNMPEAPEGMEIVNVDIIVRLRRQAR
Consensus LEGENEVLDIPI N LTISNLPEAPEGMEIAHVDVVIRLRRKRG

Figure 4.2

Figure 4.3 A derivative of Irr with a mutated heme binding motif does not display iron-responsive degradation in *B. abortus* 2308. *B. abortus* Δ*irr* complemented with a gene coding for wild type Irr (Irr^{WT}) and *B. abortus* Δ*irr* complemented with a gene coding for the mutated version of Irr (Irr^{AAA}) were cultivated in low iron minimal medium until late exponential phase. The cultures were exposed to 50 μM $FeCl_3$ for the indicated amount of time and protein lysates were analyzed by Western blot with α-Irr antibodies or with α-GroEL antibodies as a loading control (Anderson et al., 2011). The results presented are from a single experiment that is representative of at least three experiments performed from which equivalent results were obtained.

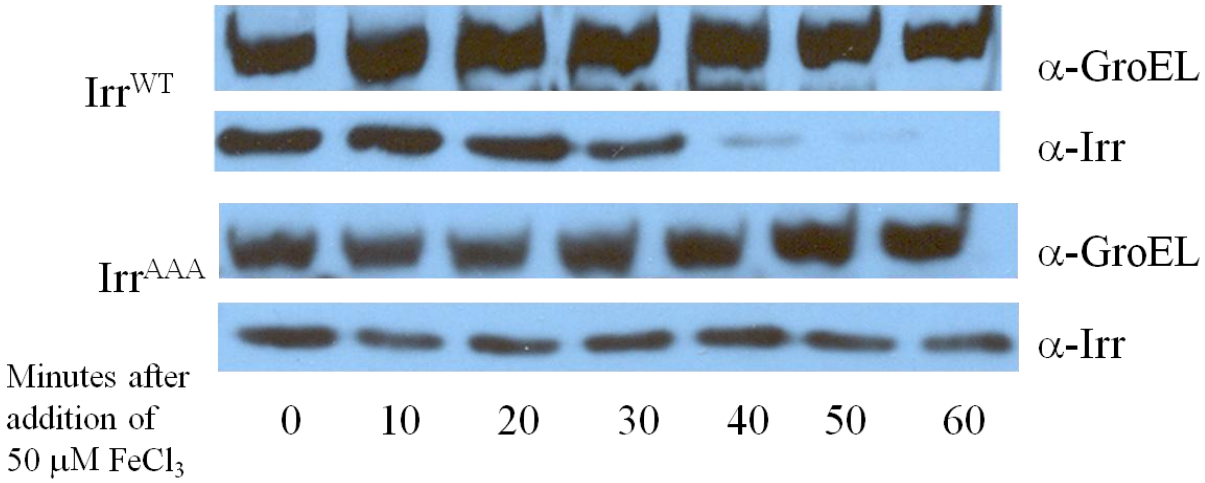

Irr^WT
α-GroEL
α-Irr
Irr^AAA
α-GroEL
α-Irr
Minutes after addition of 50 μM FeCl3
0
10
20
30
40
50
60

Figure 4.3

Figure 4.4 The ferrochelatase inhibitor N-Methyl protoporphyrin IX (NMPP) interferes with the iron-responsive degradation of Irr in *B. abortus* 2308. *B. abortus* 2308 was cultivated in low iron minimal medium until late exponential phase. Cultures were treated with 20 μM NMPP in DMSO or the equivalent volume of DMSO, followed by 50 μM $FeCl_3$. Protein lysates from the cells were obtained by disintegration with Lysing Matrix B using a cell disruptor at various time points after exposure to $FeCl_3$ and Western blot analysis was used to monitor Irr stability after exposure to NMPP and iron. The band labeled control is a constitutively expressed protein that the Irr antibody binds to. As an antibody control, lane 6 contains lysate from the *B. abortus irr* mutant. The results presented are from a single experiment that is representative of at least three experiments performed from which equivalent results were obtained.

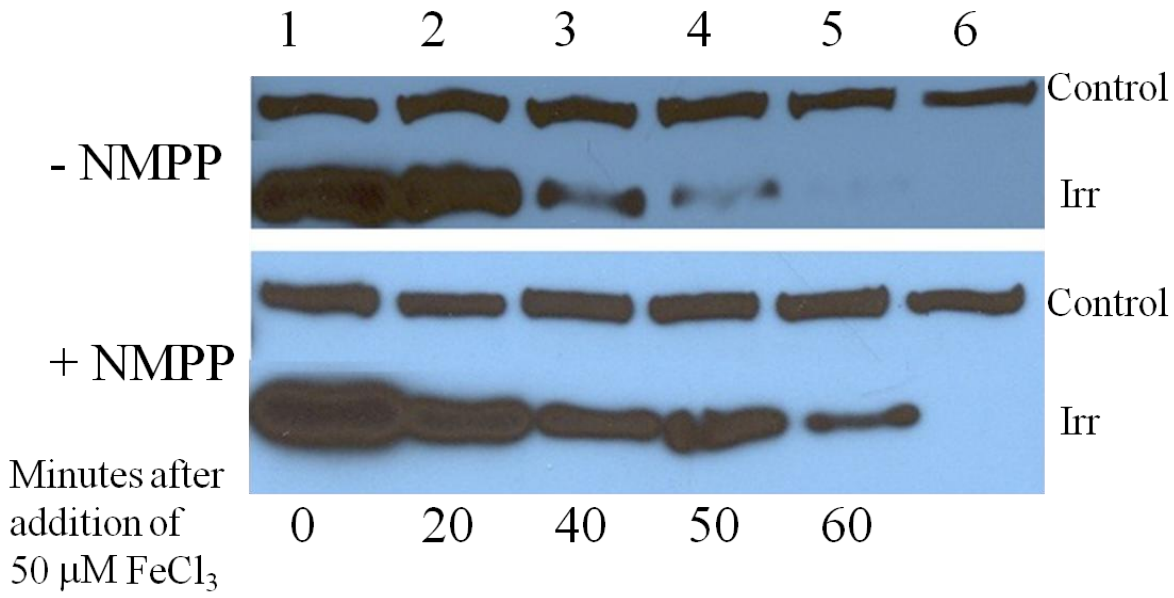
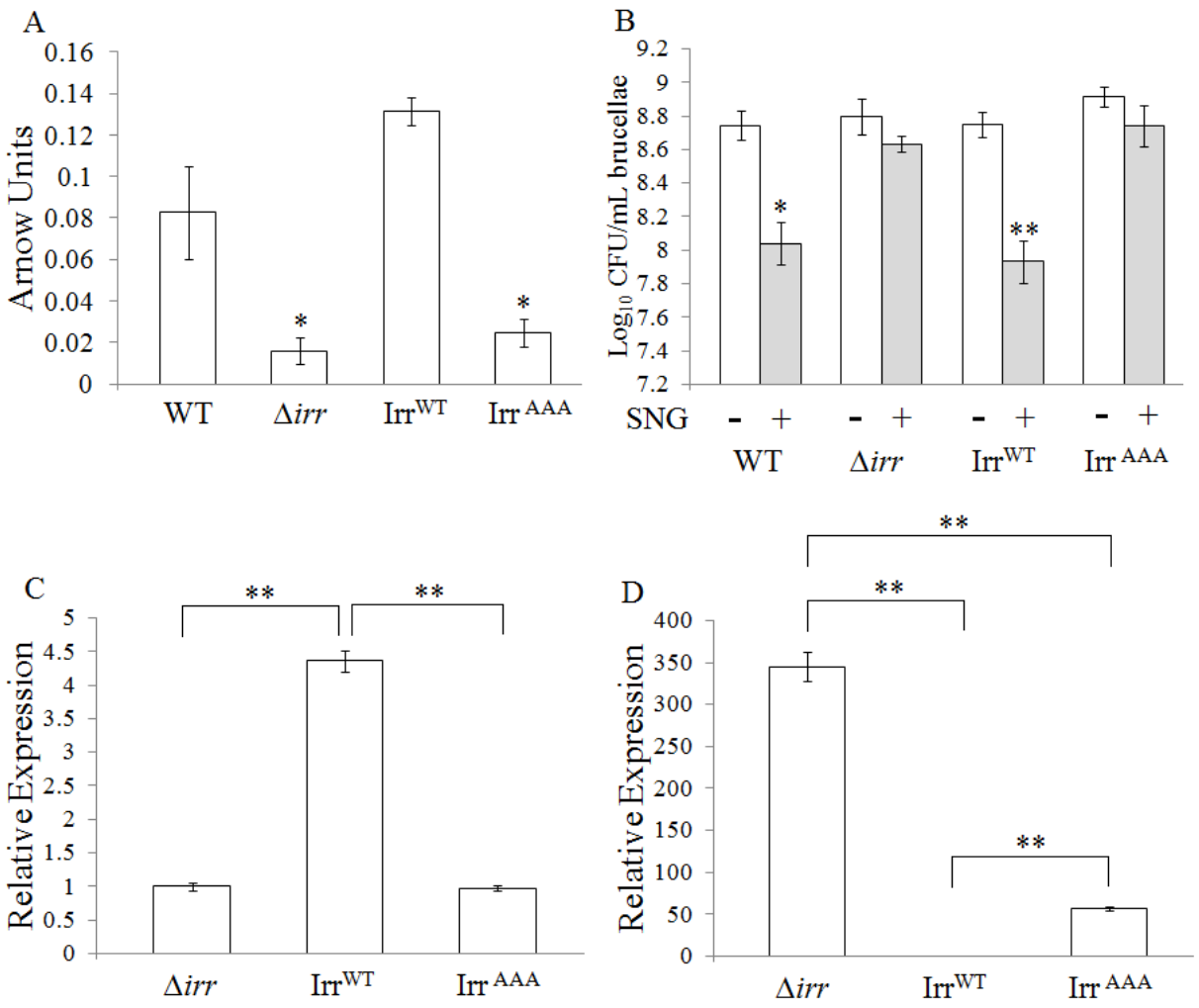

1
2
3
4
5
6
- NMPP
Control
Irr
+ NMPP
Control
Irr
Minutes after addition of 50 µM FeCl3
0
20
40
50
60

Figure 4.4

Figure 4.5 A derivative of the *B. abortus* 2308 Irr protein with a mutated heme binding motif exhibits altered regulatory capacity compared to the wild type version. (A) Catechol siderophore production by *B. abortus* 2308, an isogenic *irr* mutant (Δ*irr*), the *irr* mutant expressing a gene coding for wild type Irr (Irr^{WT}) or a mutated version of Irr (Irr^{AAA}), following growth in low iron minimal medium. Siderophore production was measured by the Arnow Asssay (Arnow, 1937). (B) Sensitivity of *B. abortus* 2308, an isogenic *irr* mutant (Δ*irr*), the *irr* mutant expressing a gene coding for wild type Irr (Irr^{WT}) or a mutated version of Irr (Irr^{AAA}) to killing by streptonigrin, an antibiotic whose killing efficiency is dependent on cellular iron levels. The streptonigrin assay was performed after growth in low iron minimal medium. Strains were exposed to DMSO (white bars), or to streptonigrin (gray bars). Real-time RT-PCR analysis of the expression of *ftrABCD* (C), and *mbfA* (D) during growth in exponential phase in low iron media. The results presented are from a single experiment that is representative of at least three experiments performed from which equivalent results were obtained. (**) indicates a *P* value of $\leq$0.001 and (*) indicates a *P* value of $\leq$0.01.


A
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Relative Expression
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50
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**
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Δirr
IrrWT
Irr AAA

Figure 4.5

Figure 4.6 Model showing the proposed mechanism through which the iron-responsive regulatory activity of Irr is controlled in *B. abortus* 2308. When cellular iron levels are low Irr is active and promotes the expression of genes coding for iron and heme transport systems while repressing the gene coding for the iron exporter MbfA and the *rirA-bhuQ* operon. As iron levels begin to rise from the import of extracellular iron, ferrochelatase (FC) incorporates iron into protoporphyrin IX (PPIX) making heme, which binds to and partially inactivates Irr stopping the induction of expression of genes coding for iron and heme transport systems. The capacity to serve as an activator ceases at a lower cellular iron level than the capacity to serve as a repressor. When cellular iron levels reach a high level Irr is degraded and the expression of *mbfA* and *rirA-bhuQ* are de-repressed.

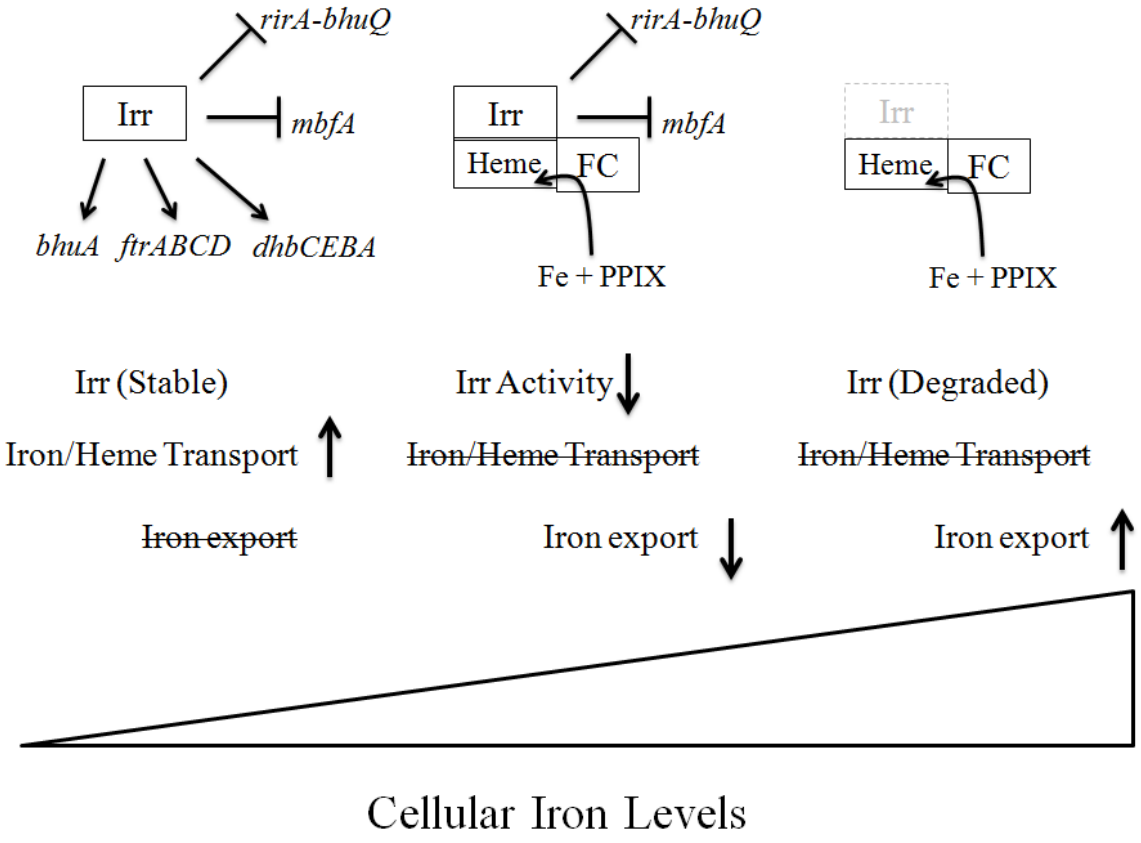
rirA-bhuQ
Irr
mbfA
bhuA
ftrABCD
dhbCEBA
rirA-bhuQ
Irr
mbfA
Heme
FC
Fe + PPIX
Irr
Heme
FC
Fe + PPIX
Irr (Stable)
Iron/Heme Transport
Iron export
Irr Activity
Iron/Heme Transport
Iron export
Irr (Degraded)
Iron/Heme Transport
Iron export
Cellular Iron Levels

Figure 4.6

Chapter 5: Summary and Future Directions

Brucella strains have the ability to utilize various host iron sources

Brucella strains are intracellular pathogens and their ability to cause disease in humans and animals is directly related to the ability of the brucellae to persist for long periods of time within host cells (Martirosyan et al., 2011). As intracellular pathogens, the brucellae are exposed to host mediated stresses, such as oxidative stress, low pH, and nutrient deprivation. Iron is an essential micronutrient required for growth by *Brucella* (Evenson & Gerhardt, 1995). Brucellae are found exclusively in association with mammalian hosts which presents a challenge to these bacteria in maintaining physiologically sufficient cellular iron levels (Moreno & Moriyon, 2002; Roop et al., 2009). The bioavailability of iron for brucellae in the host is low because iron is tightly sequestered by iron binding proteins such as transferrin and lactoferrin in extracellular spaces and by ferritin within host cells (Griffiths, 1999). In addition, IFN-γ activation of host macrophages by intracellular pathogens further exacerbates iron limitation by stimulating the activity of Nramp1 that pumps iron out of phagosomal compartments and by reducing the production of transferrin receptors on the surface of activated macrophages limiting the amount of iron coming into the macrophage (Cellier et al., 1995; Cellier et al., 2007; Hackam, et al., 1998; Byrd & Horwitz, 1989). Free cellular iron levels are kept low by this tight sequestration, serving to prevent host iron toxicity, and limiting the availability of iron to invading pathogens, making it an important part of the innate immune response (Hood & Skaar, 2010). IFN-γ is important for the clearance of *Brucella* during macrophage infections (Zhan & Cheers, 1993; Pasquali et al., 2001) suggesting that host sequestration is a mechanism used to control *Brucella* replication by limiting iron availability for these bacteria.

Brucella strains utilize efficient iron acquisition systems to obtain iron from their mammalian hosts in order to overcome host mediated iron sequestration (see Figure 1.2). One mechanism used by *Brucella* to access host iron is mediated by a siderophore iron acquisition system (Lopez-Goni et al., 1992; Gonzalez-Carrero et al., 2002) that is required for wild type virulence of *Brucella* in pregnant cattle (Bellaire et al., 2003a). In this model of infection, brucellae replicate to a high density in the placental trophoblasts which leads to a breakdown in the integrity of the placenta and eventual abortion (Smith et al., 1962). The characteristic abortion is important for the spread of infection throughout animal herds. The ruminant reproductive tract is rich in erythritol (Smith et al., 1962), which is a favored carbon source for *Brucella*. The metabolism of erythritol within placental trophoblasts results in rapid proliferation of brucellae which leads to the abortion. *Brucella* requires more iron when metabolizing erythritol than when using other carbon sources (Bellaire et al., 2003b) leading to the proposition that siderophore mediated iron uptake is required for the brucellae to maintain physiologically relevant cellular iron levels to carry out the metabolism of erythritol in the placental trophoblast. Trophoblast cells in bovine and small ruminants (goat and sheep) phagocytose erythrocytes where iron is liberated from hemoglobin as a mechanism of iron absorption for the fetus (Myagkaya et al., 1984). This mechanism provides a possible iron reservoir that *Brucella* can take advantage of through the action of siderophore mediated iron acquisition which is important for *Brucella* replication because of the high demand for iron during growth on trophoblast produced erythritol. High concentrations of erythritol are found in cattle, sheep, goats and pigs, whereas lower concentrations are found in humans (Williams et al., 1962) which is one factor explaining why *Brucella* does not localize to human placentas and why abortion is not a characteristic of human *Brucella* infections.

Once the brucellae are internalized by a macrophage, they reside in an acidic compartment called the *Brucella* containing vacuole (BCV) (see Figure 1.1) (Starr et al., 2012). Ferrous iron could serve as a biologically relevant iron source in these acidic conditions because the equilibrium between ferrous and ferric will be more in favor of the ferrous form (Roop et al., 2011). In fact, the ferrous iron transport system is required for wild type *Brucella* survival in the mouse model of infection (Elhassanny et al., 2013). This suggests that the ability to acquire ferrous iron is required for *Brucella* to establish chronic infections in animals which is important for disease maintenance in animal reservoirs.

Heme may also be a relevant source of iron for the brucellae in the macrophage. A central role of macrophages is to recycle host derived heme, meaning there is a considerable flux of heme through these phagocytes (Chrichton et al., 2001). Heme is transported to the endoplasmic reticulum (Taketani et al., 2005) which happens to be where brucellae make their replicative intracellular niche (see Figure 1.1) (Roop et al., 2009). The ability to transport heme into the bacterial cell is required for wild type *Brucella* persistence in the mouse model of infection, suggesting that heme does in fact serve as a relevant iron source during infection of the mammalian macrophage (Paulley et al., 2007). These findings are important because they suggest that, like ferrous iron acquisition, heme acquisition is an important virulence determinant for the prolonged replication in macrophages which is a requirement for maintaining an infection reservoir in animal herds.

Brucella pathogenesis is dependent upon the ability of these bacteria to replicate and survival in mammalian host cells. The evolution of different iron transport systems is most likely reflective of the environments the brucellae are exposed to during an infection. During an acute *Brucella* infection, extensive replication occurs in placental trophoblast cells which phagocytose

erythrocytes and liberate iron from hemoglobin, providing a ferric iron reservoir for *Brucella* to acquire via siderophore mediated iron transport. Alternatively, during a chronic infection, where the brucellae replicate in macrophages, there are different growth requirements and host iron sources available. Early *Brucella* phagosomes are acidic in nature, and iron in this environment will be mostly in the ferrous form, which serves as an iron substrate for the FtrABCD ferrous iron transport system. The replicative niche for *Brucella* is in association with the host endoplasmic reticulum, where there is a considerable flux of heme as a result of erythrocyte phagocytosis and recycling, making heme a relevant iron source for *Brucella* at this stage of infection. Our data indicate that *Brucella* has the ability to use both ferrous iron and heme in the mouse model of infection indicating that these are relevant iron sources for these bacteria. We cannot say with certainty where in the macrophage the brucellae are encountering these iron sources, however the acidification of the BCV and the association of the replicative BCV with the endoplasmic reticulum represent circumstantial evidence that ferrous iron and heme are available at early, and late stages of BCV trafficking, respectively (see Figure 1.1) Determining that there are brucellae present when these iron source are predicted to be available will be important to address in the future in order to confirm our model. Regardless, it is clear *Brucella* strains are well suited to deal with the iron deprivation they experience as intracellular pathogens.

The *Brucella* Irr protein coordinates the expression of iron regulated genes

While *Brucella* strains have a growth requirement for iron, excessive levels of intracellular iron can produce hydroxyl radicals through Fenton chemistry that have the potential to damage biological molecules leading to cell death (Lloyd et al., 1998). Bacteria utilize iron-

responsive transcriptional regulators to ensure that the expression of genes coding for iron acquisition systems are only expressed when cellular iron levels are low enough to warrant iron import. Members of the alpha-proteobacteria rely on the activity of Irr (iron-responsive regulator) and RirA (rhizobial iron regulator) to regulate the expression of genes involved in iron metabolism and acquisition (Johnston et al., 2007).

Because Irr regulates the expression of genes coding for iron acquisitions systems in other alpha-proteobacteria, and is required for wild type virulence of *Brucella* in the mouse model of chronic infection (see Chapter 2) we hypothesized that Irr is a major iron-responsive transcriptional regulator in *Brucella*. Irr was previously shown to be required for the expression of the genes coding for siderophore biosynthesis in response to cellular iron deprivation in *Brucella* (Martinez et al., 2005; Martinez et al., 2006) providing further reason to speculate that Irr is an important iron-responsive regulator. Chapter 2 provides evidence that the *Brucella* Irr protein directly regulates the expression of *bhuA*, the gene coding for the outer membrane heme transporter, when cellular iron levels are low. The *Brucella ftrABCD* operon that encodes a ferrous iron transport system is also under the direct transcriptional control of Irr in response to cellular iron deprivation (see Chapter 4) (Elhassanny et al., 2013). The genes coding for these three iron acquisition systems are all required for wild-type virulence when tested in the animal model of infection and are all directly activated by Irr when cellular iron levels are low. The regulatory activity of Irr in activating these genes is required for *Brucella* to establish and maintain infections in animals where the bacteria experience host mediated iron deprivation. In addition to activating genes that encode products that are involved in iron acquisition, the *Brucella* Irr protein represses genes that encode products that are involved in iron storage (bacterioferritin), iron export (MbfA) or require iron for their function (heme synthesis and

cytochrome biosynthesis) (see Figure 5.1). By repressing these genes, the limited available bacterial cellular iron is incorporated into proteins that are required for *Brucella* survival. The repressive activity of the Irr protein when cellular iron levels are low may prevent the need for a *Brucella* to use a functional homolog to the RyhB sRNA. In *Escherichia coli*, RyhB inhibits the production of proteins that are not absolutely essential for survival when cellular iron levels are low. Some of the RyhB regulated gene orthologs are repressed when cellular iron levels are low in *Brucella* as well, including genes coding for NADH dehydrogenase components, aconitase hydratase components, succinate dehydrogenase and fumarate metabolism components, all of which require iron for their enzymatic activity. It is unclear if Irr directly regulates these genes in *Brucella*, but the activity of Irr when cellular iron levels are low suggests that it can.

While Irr is a member of the Fur superfamily of transcriptional regulators, it is unique to members of the alpha-proteobacteria. The closest Fur homologs in the alpha-proteobacteria have evolved to respond to cellular manganese levels rather than iron levels and have been renamed Mur (manganese responsive regulator) (Hohle & O'Brian, 2009; Platero et al., 2007; Diaz-Mireles, et al., 2004; Menscher et al., 2012). Manganese is an essential micronutrient for *Brucella* (Evenson & Gerhardt, 1955) and a *Brucella* derivative that is not able to transport manganese into the cell is attenuated in the mouse model of chronic infection (Anderson et al., 2009). While manganese can be toxic to bacterial cells if cellular levels exceed those needed by the cell (Papp-Wallace & Maguire, 2006), *Brucella* exhibits no growth defect when cellular manganese levels are high (Menscher et al., 2011). The potential toxicity of manganese is considerably less than that of other metals, such as iron, and efficient manganese transport has been linked to reactive oxygen species resistance in *Brucella*, suggesting that manganese can be protective at certain cellular concentrations (Anderson et al., 2009).

In *B. japonicum*, cellular manganese levels have an effect on Irr stability (Puri et al., 2010). The binding of manganese to Irr inhibits heme binding to Irr, stabilizing the protein. When cellular manganese levels are low, heme binds to Irr and the protein is inactivated and degraded resulting in low expression of genes coding for iron acquisition systems and consequently lower cellular iron levels. Manganese protects cells against oxidative stress, whereas iron promotes it through the generation of reactive oxygen species (Imlay, 2008). It has been postulated that *B. japonicum* responds to cellular manganese limitation by lowering cellular iron content through the destabilization of Irr to prevent iron generated oxidative stress.

Applying this model to *Brucella* provides a plausible explanation as to why evolution has selected for Mur and Irr regulation in response to manganese and iron. The environment that *Brucella* is exposed to during life in the macrophage is high in oxidative stress. If cellular manganese and iron levels are low, *Brucella* Irr is active, but may be more susceptible to inactivation and degradation by interacting with heme when cellular iron levels begin to rise than if cellular manganese levels were higher, inhibiting heme from interacting with Irr and protecting the bacteria from iron related oxidative stress. This would permit maximal expression of genes coding for *Brucella* iron acquisition systems only when cellular manganese levels are at levels that are protective from oxidative stress and suggests that one of the functions of the manganese acquisition system MntH is in protecting Irr by transporting manganese into the cell.

Brucella **Irr activity is controlled by heme dependent inactivation and degradation**

There are two paradigms to explain how the activity of Irr is controlled in response to cellular iron levels in the alpha-proteobacteria (see Figure 1.5). In *B. japonicum*, as cellular iron levels rise, heme is delivered to and binds to Irr through the enzymatic activity of ferrochelatase,

leading to the inactivation and degradation of Irr (Qi & O'Brian, 2002). Alternatively, in *R. leguminosarum*, heme binding to Irr inactivates the protein by promoting its oligomerization (Singleton et al., 2010; White et al., 2011). The *Brucella* Irr protein is a conditionally stable protein that follows the *B. japonicum* model for controlling the activity of Irr. *Brucella* Irr is degraded in response to rising cellular iron conditions and this is dependent on the enzymatic activity of ferrochelatase. By presumably binding directly to ferrochelatase, Irr can interact with a newly synthesized heme molecule acting as a signaling molecule without the need for cellular heme levels to rise. There are benefits to Irr sensing cellular iron levels through the biosynthesis of heme. Iron that is not incorporated into cellular proteins can damage biological molecules through Fenton chemistry (Lloyd et al., 1998). *Brucella* are exposed to oxidative stress because of their intracellular lifestyle and allowing free cellular iron to build up to levels high enough to act as a signaling molecule could enhance that stress. As iron is transported into the cell it is immediately incorporated into cellular proteins, including heme. While it is not known if *Brucella* Irr directly binds to ferrochelatase, the addition of a ferrochelatase inhibitor drastically reduces iron-responsive degradation of Irr, suggesting that there is a physical relationship between ferrochelatase and Irr. If Irr and ferrochelatase do physically interact, then when heme is synthesized it will be in close proximity to Irr and can signal in response to rising cellular iron levels at low concentrations. By preventing the need for free cellular iron or heme levels to rise to high levels before acting as a signal, the risk for iron related oxidative stress is reduced for *Brucella*.

Degradation of *B. japonicum* Irr depends on the ability of the protein to bind to reduced and oxidized forms of heme (Yang et al., 2004), whereas *R. leguminosarum* Irr only has the ability to bind to oxidized heme (White et al., 2011). An attractive proposal to explain the

degradation of *B. japonicum* Irr and not *R. leguminosarum* Irr is that the binding of both forms of heme is needed to cause the degradation of Irr. Supporting this proposition is the fact that heme can react with oxygen to form reactive oxygen species that damage proteins (Aft & Mueller, 1984). The *Brucella* Irr protein lacks the HRM found in *B. japonicum*, but has a conserved HXH heme binding motif and the ability to bind to heme (Martinez et al., 2005). A *Brucella* Irr derivative that has a mutated HXH heme binding motif (Irr^{AAA}) is stable regardless of cellular iron levels. An interesting consideration that could provide insight into the mechanism behind Irr degradation is whether the *Brucella* Irr protein has the ability to bind to both the reduced and oxidized forms of heme. *R. leguminosarum* Irr has a second, low affinity heme binding site that is required for Irr oligomerization, but again, only binds to ferrous heme (White et al., 2011). While this motif is conserved in the *Brucella* Irr protein, it is not known if this motif is functional in the *Brucella* Irr protein and warrants further investigation to determine its ability to bind to heme in different redox states. An important phenotype that was observed in a *Brucella* derivative producing Irr^{AAA} was the inability of this protein to activate the expression of genes coding for the three characterized iron acquisition systems (siderophore, ferrous iron, and heme), while retaining an intermediate level of repressive activity (see Chapter 4). One possibility is that the stable, mutated version of Irr has a reduced DNA binding activity, either through improper protein folding or the inability to diamerize effectively, preventing it from binding to lower affinity promoters, but still retaining the ability to bind to higher affinity promoters. This suggests that the heme binding motif is more important for controlling the activity of Irr as an activator than as a repressor. Because of the host generated oxidative stress experienced by *Brucella* during an intracellular infection, controlling the expression of genes coding for iron acquisition at a more sensitive level, or at lower cellular iron levels than genes that are repressed

by Irr (iron export) could be important for preventing bacterial-generated, iron-associated oxidative stress from metabolism. An important question to answer in the future is whether or not the *Brucella* derivative producing Irr^{AAA} exhibits an attenuated phenotype in the mouse animal model of infection. We would predict that this derivative would behave similar to an *irr* mutant because both of these derivatives are unable to activate the expression of genes coding for iron acquisition systems, which have been shown to be important virulence determinants in *Brucella*.

The overlapping regulons of Irr and RirA in *Brucella*

Agrobacterium tumefaciens is a plant pathogen and a member of the alpha-proteobacteria. In this bacterium, the Irr and RirA proteins are both iron-responsive transcriptional regulators, but act opposite to one another (Hibbing & Fuqua, 2011). Irr is active under low-iron conditions where it inhibits the expression of genes coding for iron utilization proteins and activating those coding for iron acquisition systems, while RirA is active under high-iron conditions, where it represses the expression of genes coding for iron acquisition systems.

The work presented in this dissertation provides evidence that the activity of the *Brucella* Irr protein as a transcriptional regulator is similar to that of other alpha-proteobacteria, where it is active during growth in iron deplete conditions, repressing genes coding for iron utilization or storage proteins, and activates genes that encode iron acquisition systems. Data obtained from microarray analysis indicates that the *Brucella* RirA protein represses genes that encode iron acquisition systems, namely, the *dhbC* operon, coding for siderophore biosynthesis enzymes, the *fatA* operon, coding for siderophore transport proteins, *bhuA*, coding for a heme uptake

transporter, and *dhbR*, involved in activating siderophore biosynthesis (Figure 5.2A). In a *rirA* mutant, these genes are expressed at a significantly higher level than in the wild type strain during growth in high iron conditions. RirA exhibits wild type sensitivity to the iron activated antibiotic streptonigrin after growth in iron deplete conditions, suggesting that RirA has no role in the regulation of genes coding for iron acquisition systems when cellular iron levels are low (Figure 5.2B). These data indicate that RirA is responsible for repressing these genes during growth in iron replete conditions, similar to that observed in *A. tumefaciens*, while having no role in regulation during growth in iron deplete conditions.

The ability of Irr and RirA to regulate the expression of the same genes, but in response to opposite signals, allows *Brucella* to precisely control genes coding for iron acquisition systems and iron utilization activities during growth in conditions of both iron depletion and iron abundance (see Figure 5.3). The capacity of Irr to activate genes coding for iron uptake systems while inhibiting genes whose products are involved in iron utilizing or storage processes ensures that there is sufficient intracellular iron for bacterial growth. RirA activity ensures that iron toxicity does not occur by preventing the accumulation of dangerously high levels of iron by repressing iron uptake. Irr and RirA regulatory pathways are linked by the repression of *rirA* by Irr (see Chapter 3). The result of Irr and RirA regulatory activity on iron acquisition genes ensures that homeostatic iron levels are maintained within physiologically relevant ranges.

The process of natural selection ensures that organisms simple to complex are well adapted to their environments (Darwin, 1859). The transition from Fur to Mur and the emergence of Irr and RirA as iron-responsive transcriptional regulators in the alpha-proteobacteria has been analyzed by comparative genomics (Figure 5.4) (Rodionov et al., 2006). The last common ancestor of the alpha-proteobacteria likely used a Fur-like protein to control iron metabolism.

The Fur protein in the Rhizobiales and Rhodobacteraceae lineages of the alpha-proteobacteria evolved to become a manganese responsive regulator (Mur) and Irr emerged as the iron-responsive regulator. Nothing happens by accident in evolution, so the conversion of Fur to Mur and the emergence of Irr must have been beneficial to this ancestor. As the Rhizobiales lineage split from the Rhodobacterales lineage, *Bradyrhizobium japonicum* further split from Rhizobiales and now relies exclusively on Irr for iron-responsive gene regulation. The *B. japonicum* Irr regulon is large and consists of genes that are both activated and repressed by Irr. Another lineage split from the Rhizobiales and gained the *rirA* gene, whose product is a repressor of genes whose products are involved in iron acquisition. *Rhizobium leguminosarum* now relies heavily on the activity of RirA as its main iron-responsive regulator, whereas Irr has a limited role as a regulator. This suggests that in *R. leguminosarum*, repression of genes coding for iron acquisition systems is more important than activating these genes. *R. leguminosarum* is a plant symbiont, meaning both the bacteria and the host plant benefit from interacting with one another. In this niche, RirA may function to manage iron related oxidative stress from bacterial metabolism. Alternatively, the plant pathogen *Agrobacterium tumefaciens* and *Brucella*, an animal pathogen, rely on overlapping regulation by Irr and RirA. The hosts for *A. tumefaciens* and *Brucella* have a selective pressure to sequester iron away from these pathogens which could explain why Irr has retained the ability to activate genes that code for iron acquisition systems in these bacteria when bacterial iron levels are low. As pathogens, *A. tumefaciens* and *Brucella* must also deal with host mediated defenses that result in considerable oxidative stress, which is likely the selective pressure that resulted in these bacteria retaining RirA, which represses genes whose products are involved in iron acquisition when cellular iron levels are high, helping to protect against additional iron dependent oxidative stress. The alpha-proteobacteria are found in

a wide range of environments, from soil, to plants as symbionts and in mammals as pathogens, and the selection for Irr or Irr/RirA iron-responsive regulation over Fur suggests that these regulators afford some benefit to these bacteria in their diverse niches. The activity of Irr when cellular iron levels are low and the activity of RirA when cellular iron levels are high, allows for the precise transcriptional control of genes involved in iron acquisition and utilization over a wide range of cellular iron levels.

Iron substrate-dependent transcriptional regulation in *Brucella*

Evolution has selected for bacteria with the ability to carefully regulate gene expression to ensure that energy is not wasted by synthesizing enzymes or transport systems when they are not needed. The idea of energy conservation folds over into how *Brucella* regulates genes coding for iron acquisition systems. Irr and RirA are global iron-responsive regulators that regulate large numbers of genes as a general response to low or high cellular iron levels ensuring that genes coding for iron acquisition systems are only expressed when cellular iron levels are low (Johnston et al., 2007). Synthesizing the components of iron transport systems is energetically costly, and there is evidence to propose a model that maximal expression of the genes coding for these systems requires a second, iron substrate specific signal (see Figure 5.5).

The AlcR type transcriptional regulator DhbR has been implicated in the direct activation of the genes coding for siderophore biosynthesis in *Brucella* (Anderson et al., 2008). In a *dhbR* mutant, expression of *dhbC* is about half as much as in the wild type strain during growth in iron deplete conditions. A *dhbC* mutant that produces no siderophore exhibits a similar phenotype as the *dhbR* mutant. In both the *dhbC* and *dhbR* mutants, there is still iron-responsive induction of siderophore production indicating that there is another regulatory system independent of DhbR.

The proposed model for the regulation of siderophore biosynthesis encoding genes involves Irr activating expression of these genes when cellular iron levels are low. Siderophore biosynthesis occurs, and when iron loaded siderophore is transported back into the cell, it interacts with DhbR which in turn activates the expression of siderophore biosynthesis genes to maximal levels (see Figure 5.4).

The gene coding for the outer membrane heme transporter BhuA is activated by Irr during growth in iron deplete conditions (see Chapter 3). The expression of the *bhuA* gene is also increased in the presence of extracellular heme (Paulley, 2007). In *C. diphtheriae*, the ChrSA two component regulatory system responds to the presence of extracellular heme and activates the expression of gene coding for the heme oxygenase, HmuO, that is needed to utilize heme as an iron source (Schmitt, 1999). *Brucella* has a homolog to the ChrSA system and a *chrA* mutant loses the ability to activate *bhuA* expression in the response to the addition of heme in the growth medium in *Brucella* (Paulley, 2007). We believe *bhuA* expression is first activated during growth in low iron conditions by Irr, and then in the presence of extracellular heme, ChrSA further activates the expression of *bhuA* (see Figure 5.4). The ability of ChrS to bind to the promoter of *bhuA* has not been determined, and will be important to confirm our model.

The genes coding for the *Brucella* ferrous iron transport system FtrABCD are directly regulated in response to low intracellular iron levels by Irr (see Chapter 4) (Elhassanny et al., 2013). Exposure to low pH growth medium also causes an induction of the expression of the *ftrABCD* operon (Elhassanny et al., 2013). In low pH conditions, the equilibrium between ferrous and ferric iron shifts heavily towards the ferrous form, making it a potential source of iron for *Brucella*. During an infection, the brucellae are exposed to low pH conditions while trafficking through the macrophage (Porte et al., 1999). It stands to reason that at this time during

infection, ferrous iron is a relevant iron source that *Brucella* could potentially transport and utilize. The data support the idea that *Brucella* has the ability to response to low iron conditions through Irr, and sense that the substrate for FtrABCD dependent transport is present through an unknown regulatory mechanism (see Figure 5.5). It has yet to be determined if low pH, or the presence of ferrous iron, which is only available in low pH conditions, is the signal to activate *ftrABCD* expression in an Irr independent manner.

During an infection, brucellae have the ability to utilize different forms of iron. Various forms of iron are available depending on the host cell type, and the stage of intracellular infection. *Brucella* has evolved a regulatory network to respond to and maintain homeostatic intracellular iron levels. This regulatory network extends to sensing and responding to what form of host iron is available for transport. The regulatory network allows for tight control of intracellular iron levels to ensure sufficient iron levels for growth, while preventing the buildup of toxic levels of iron, and to prevent the futile and energetically costly synthesis of transport systems until they are needed.

In summary, Irr is the main iron-responsive transcriptional regulator in *Brucella*, with RirA playing an important, but secondary role. Experimental evidence suggests that Irr activity when cellular iron levels are low allows *Brucella* to acquire sufficient iron for its physiological needs. The activity of RirA when cellular iron levels are high is thought to ensure that these bacteria do not experience iron related oxidative stress, although the role of RirA has not been as thoroughly examined as has been done for Irr. Together, these regulators act to maintain iron homeostasis in *Brucella* permitting both persistence and replication in the host, and transmission to other host organisms.

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Figure 5.1 The Irr regulon in *B. abortus* 2308. (A) Model depicting gene products that are produced at higher levels in iron deplete conditions than in iron replete conditions (green up arrows), or are produced at higher levels in iron replete conditions than in iron deplete conditions (red down arrows). Solid arrows indicate that there is evidence for Irr regulation of the genes coding for these proteins, and dashed arrows indicate that there is iron-responsive regulation, but the dependence of the regulation on Irr has not been determined.

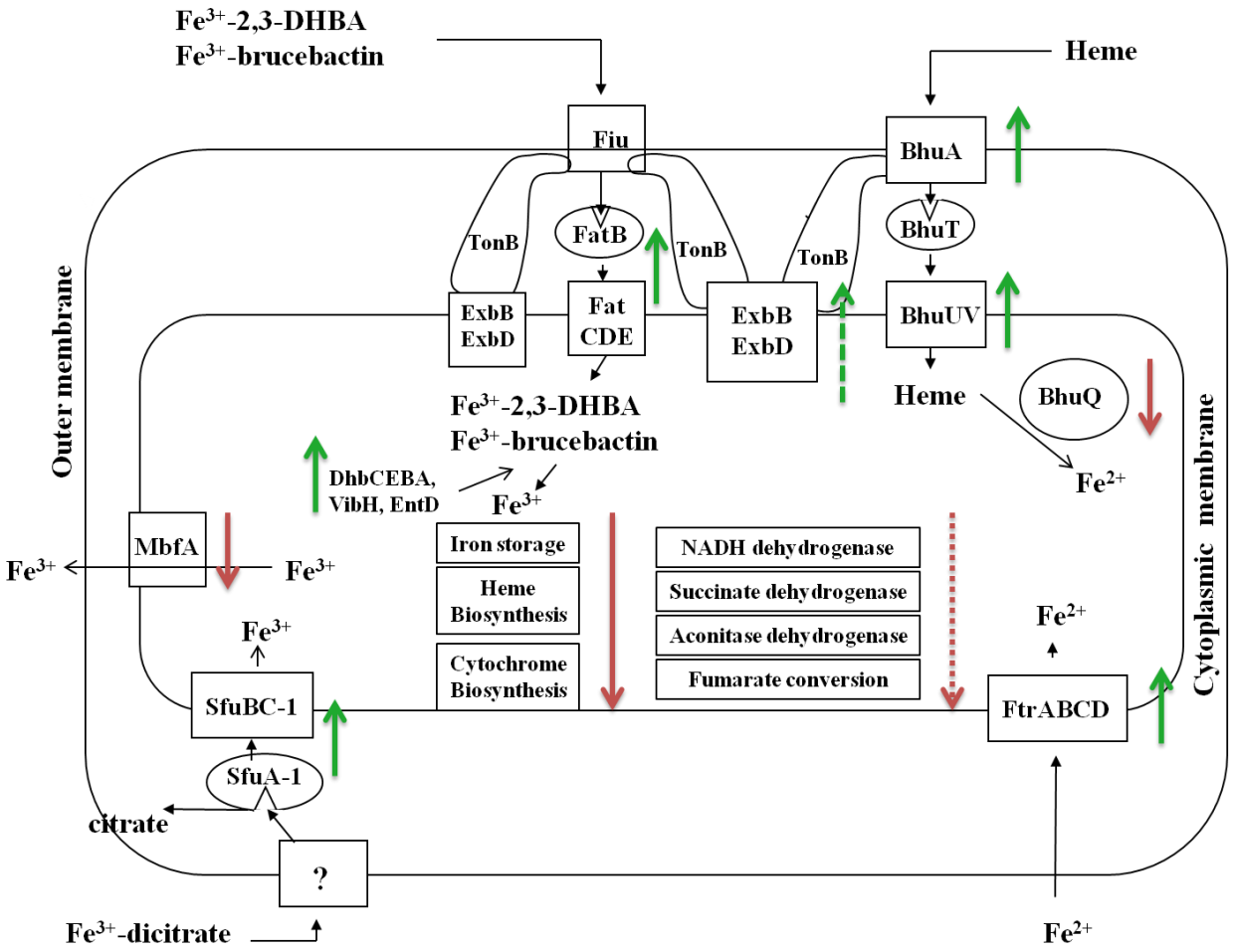
Fe3+-2,3-DHBA
Fe3+-brucebactin
Heme
Fiu
BhuA
TonB
FatB
TonB
TonB
BhuT
ExbB
ExbD
Fat
CDE
ExbB
ExbD
BhuUV
Outer membrane
Fe3+-2,3-DHBA
Fe3+-brucebactin
Heme
BhuQ
Fe2+
DhbCEBA,
VibH, EntD
Fe3+
MbfA
Fe3+
Fe3+
Iron storage
Heme
Biosynthesis
Cytochrome
Biosynthesis
NADH dehydrogenase
Succinate dehydrogenase
Aconitase dehydrogenase
Fumarate conversion
Fe2+
Fe3+
SfuBC-1
FtrABCD
Cytoplasmic membrane
SfuA-1
citrate
?
Fe3+-dicitrate
Fe2+

Figure 5.1

Figure 5.2 RirA represses genes coding for iron acquisition systems during growth in iron replete conditions in *B. abortus* 2308. (A) Microarray analysis of gene expression during growth in high iron conditions in the *rirA* mutant vs. wild type strains. Fold induction reflects transcript levels in the *rirA* mutant vs. wild type in high iron conditions. (B) Sensitivity of *B. abortus* 2308, and an isogenic *rirA* mutant (Δ*rirA*), to killing by streptonigrin, an antibiotic whose killing efficiency is dependent on cellular iron levels. The streptonigrin assay was performed after growth in low iron minimal medium. Strains were exposed to DMSO (white bars), or to streptonigrin (gray bars) and iron and were cultured followed by serial dilution to determine the number of viable brucellae present.

A

Siderophore Biosynthesis

BAB2_0011	*vibH*	23.78
BAB2_0012	*dhbC*	24.83
BAB2_0013	*dhbE*	32.19
BAB2_0014	*dhbB*	42.84
BAB2_0015	*dhbA*	24.80
BAB2_0016	*entD*	25.70

Siderophore Transport

BAB2_0561	*fatA*	6.03
BAB2_0562	*fatD*	6.94
BAB2_0563	*fatC*	7.51
BAB2_0564	*fatB*	8.13

Heme Transport

BAB2_1150	*bhuA*	14.61

Siderophore Biosynthesis Regulation

BAB2_1152	*dhbR*	8.85

B

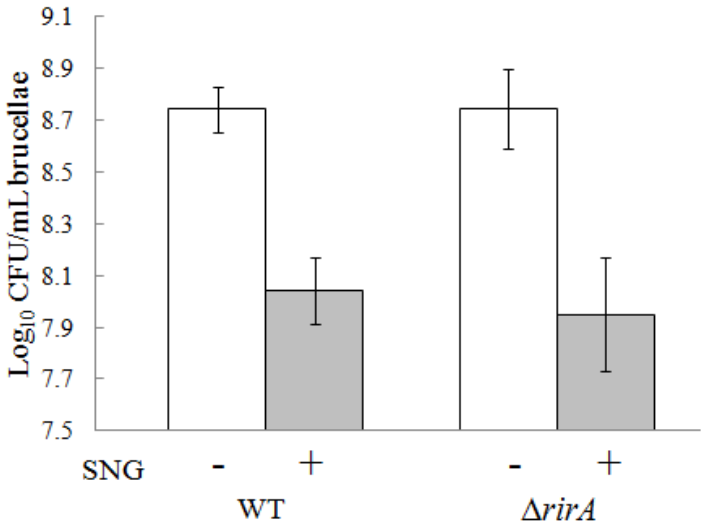

Figure 5.2

Figure 5.3 Proposed model depicting the overlapping regulons of Irr and RirA in *B. abortus* 2308. During growth in iron deplete conditions, Irr is active and promotes the expression of genes coding for iron acquisition systems. During growth in iron replete conditions, RirA represses the expression of genes coding for iron acquisition systems.

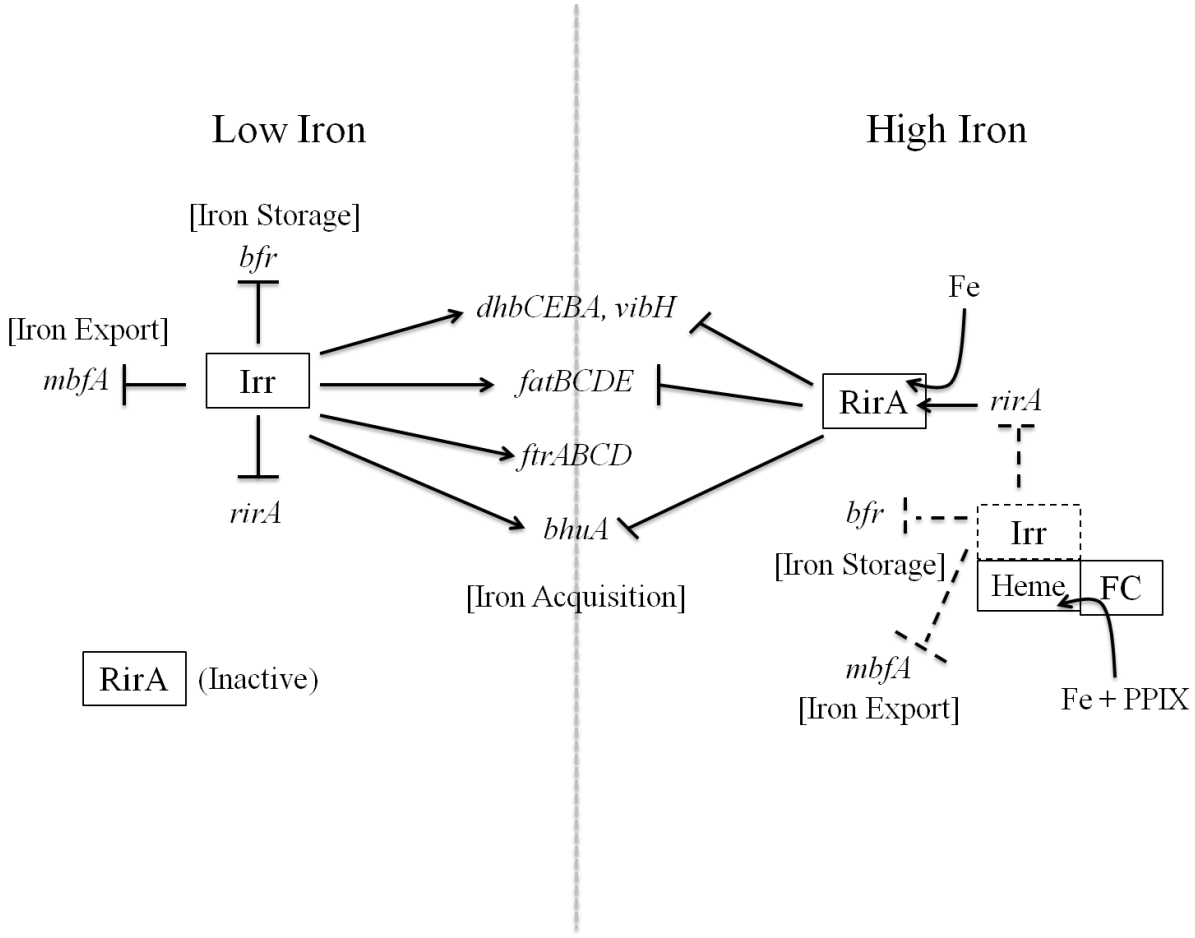
Low Iron
High Iron
[Iron Storage]
bfr
[Iron Export]
mbfA
Irr
rirA
dhbCEBA, vibH
fatBCDE
ftrABCD
bhuA
[Iron Acquisition]
Fe
RirA
rirA
bfr
Irr
[Iron Storage]
Heme
FC
mbfA
[Iron Export]
Fe + PPIX
RirA (Inactive)

Figure 5.3

Figure 5.4 Model depicting the evolution of Irr and RirA in the alpha-proteobacteria. The common alpha-proteobacteria ancestor Fur protein shifted to Mur and Irr emerged as an iron-responsive transcriptional regulator (*1). The Rhizobiales lineage diverged into two separate lineages, one that now consists of *B. japonicum*, that relies solely on Irr for iron-responsive gene regulation, and another lineage that acquired RirA, possibly through genetic exchange from another bacterium (*2). This lineage diverged into two groups, one that consists of *R. leguminosarum*, that utilizes RirA as the main iron-responsive transcriptional regulator, and Irr as a minor regulator, and *A. tumefaciens* and *Brucella spp*. that utilize Irr as the main iron-responsive regulator with RirA playing a secondary, but significant role as a regulator. The thickness of outline around Irr and RirA indicates the relative contribution of each regulator in iron-responsive gene regulation by the bacteria listed above.

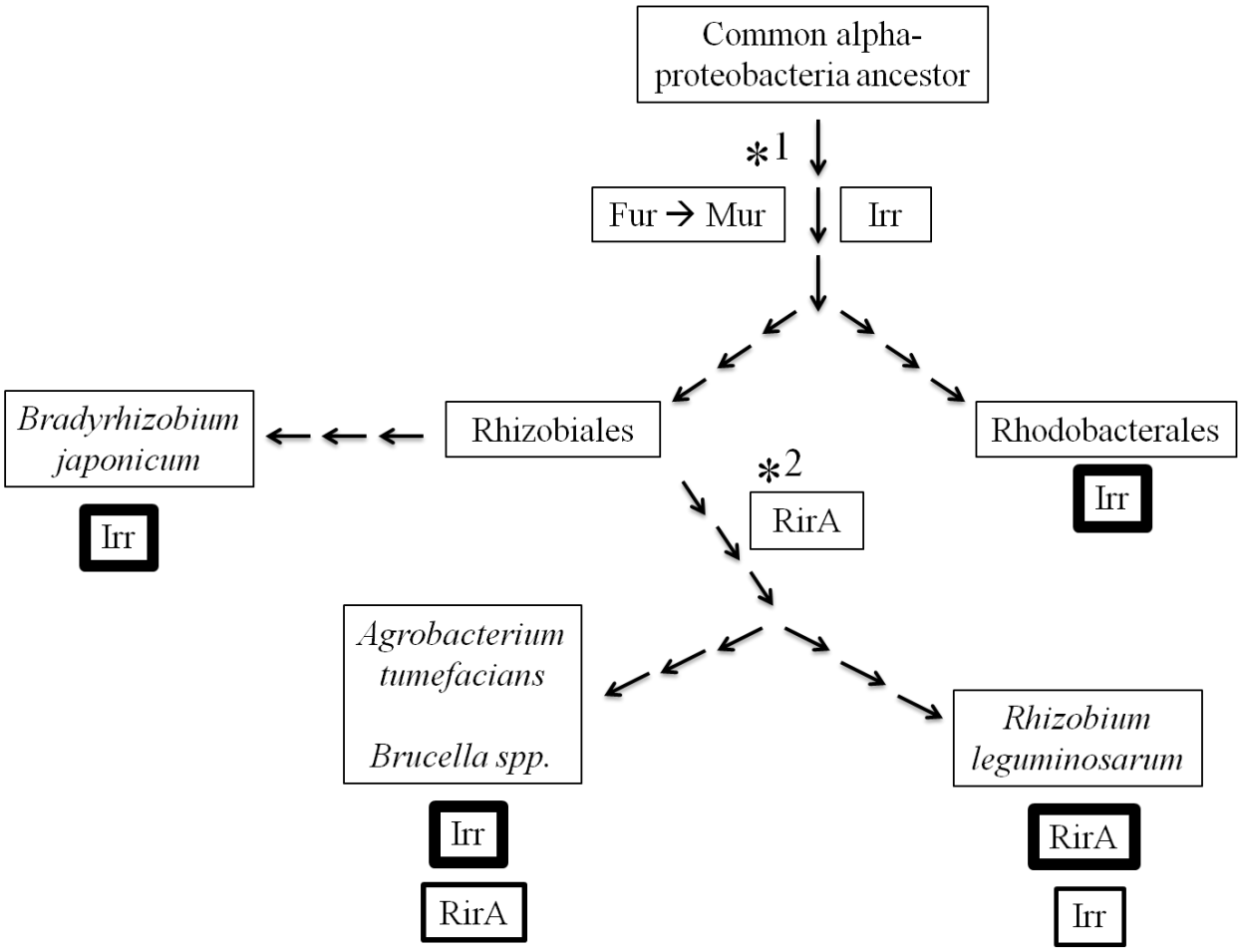
Common alpha-proteobacteria ancestor
*1
Fur → Mur
Irr
Rhizobiales
Bradyrhizobium japonicum
Irr
*2
RirA
Rhodobacterales
Irr
Agrobacterium tumefacians
Brucella spp.
Irr
RirA
Rhizobium leguminosarum
RirA
Irr

Figure 5.4

Figure 5.5 Proposed model depicting substrate-dependent regulation of genes coding for iron uptake systems in *B. abortus* 2308. When cellular iron levels are low, Irr activates the expression of genes coding for iron uptake systems as part of the global iron starvation response. Maximal expression of these genes occurs in the presence of the substrate specific for each transport system. ChrS responds to heme in the periplasm and signals to ChrA which then further activates *bhuA* expression. SenS, an unknown sensor kinase responds to extracellular low pH conditions or Fe^{2+} in the periplasm and signals to SenA, an unknown regulator, which then further activates *ftrABCD* expression. When iron bound siderophore returns into the cell it binds to DhbR which then further activates *vibH*, *dhbCEBA*, and *entD* expression.

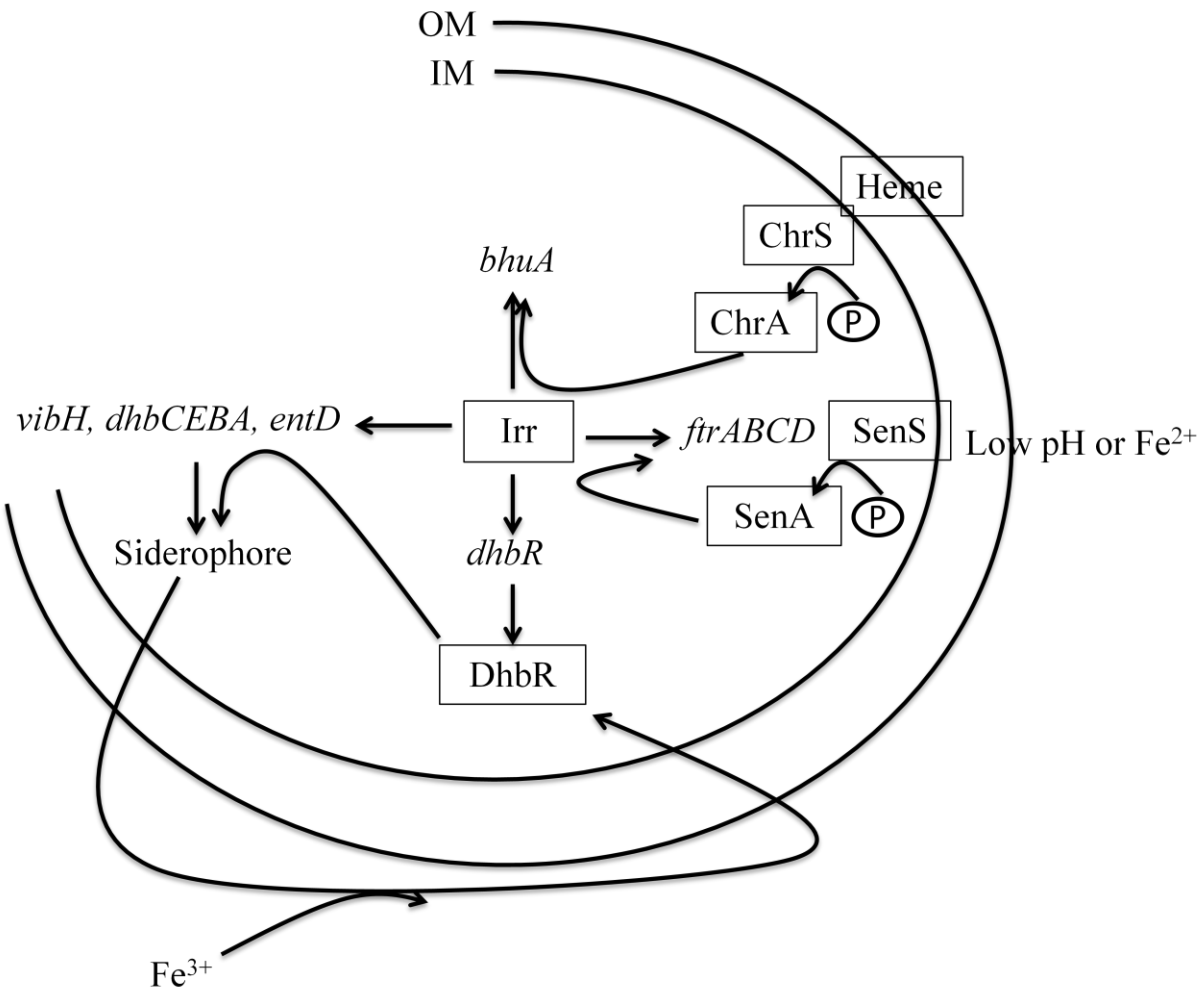
OM
IM
Heme
ChrS
bhuA
ChrA
P
vibH, dhbCEBA, entD
Irr
ftrABCD
SenS
Low pH or Fe2+
SenA
P
Siderophore
dhbR
DhbR
Fe3+

Figure 5.5