# Abstract

## The *bhuTUV* and *bhuO* genes play vital roles in the ability of *Brucella abortus* to use heme as an iron source and are regulated in an iron-responsive manner by RirA and Irr

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*Brucella abortus* is a Gram negative intracellular pathogen that causes the zoonotic disease brucellosis. Antibiotic treatment for brucellosis in humans is prolonged and sometimes followed by relapses. Currently, the United States employs prevention of the illness in humans through cattle vaccinations, eliminating the bacterium in its natural host. Unfortunately, these vaccine strains cause the disease in humans, and *Brucella* research ultimately aims to identify new vaccine targets as well as alternative treatment options.

*Brucella abortus* resides in the phagosomal compartment of the host macrophage where essential nutrients such as iron are limited. Most bacteria need iron, and within the macrophage, heme is a likely source of iron due to the breakdown of red blood cells by the host macrophage. Heme transporters in Gram negative bacteria are highly conserved, and include components for outer membrane, periplasmic, and cytoplasmic membrane transport. BhuA has been previously characterized as the outer membrane heme transporter of *Brucella abortus* and here we report that BhuT, BhuU, and BhuV (BhuTUV) are the periplasmic and cytoplasmic heme transport components and that they are required in order for *Brucella abortus* to transport heme as an iron source. Utilization of heme as an iron source requires the breakdown of heme into ferrous iron,

carbon monoxide, and biliverdin by a heme oxygenase. BhuO has been identified as a heme oxygenase in *Brucella abortus*, and although there seems to be more than one heme oxygenase in *Brucella*, this study shows that BhuO is needed for the use of heme as an iron source under iron starvation conditions *in vitro*. Further, both *bhuTUV* and *bhuO* are regulated in an iron-responsive manner. The iron responsive regulator Irr directly represses *bhuO*, which shares an operon with *rirA*. Then the rhizobial iron regulator RirA in turn represses the *bhuTUV* operon. Together, these regulators help to maintain iron homeostasis within the bacterial cell, protecting it from damaging hydroxyl radicals produced by Fenton chemistry.

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## Dedication

This dissertation is dedicated to my husband Dan, who by all accounts earned this degree with me. He held the magic to obtaining the most obscure reference articles, the most visually appealing graphs, and had the wildest imagination when it came to scientific solutions. He rode the emotional turmoil of graduate school with me, and this work truly would not have been possible without his constant love and support.

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# Chapter 1: Literature Review

## *Brucella*

### The causative agent of brucellosis

*Brucella* spp. are Gram negative pathogens that belong to the phylogenetic branch of the alpha proteobacteria, which encompasses a large variety of organisms, including other pathogenic bacteria, such as *Bartonella quintana* as well as *Rhizobium leguminosarum*, a bacterium that has adapted a symbiosis with its plant hosts (Williams 2007). Despite their apparent differences, many of the bacteria within this grouping share similar t raits. For instance, several species within the alpha proteobacteria develop chronic, long term relationships with higher eukaryotic organisms (Batut 2004). In fact, the ability to evade the immune system and establish a chronic infection is the key factor in *Brucella* virulence (Barquero-Calvo 2007).

*Brucella* are found within a wide variety of animal reservoirs, with the individual strains named according to their natural hosts. *B. ceti* is found in porpoises and dolphins, *B. pinnipedialis* is found in seals, and *B. microti* is found in voles and red foxes (Pappas 2010). While there are many different species of *Brucella*, the three primary species that cause zoonotic infections in humans are *B. melitensis*, *B. abortus*, and *B. suis* strains which naturally infect goats, cattle, and swine, respectively (Solera 2010).

Humans are not a natural host for *Brucella* strains, and human brucellosis is strictly a zoonotic disease. Within the context of their animal hosts, most of these strains cause abortion and infertility, however, the symptoms are more flu-like in humans, characterized by a spiking and remitting fever and general malaise (Spera 2006). Due to the generalized nature of the

symptoms of brucellosis in humans, diagnosis is often slow, and in spite of heavy, sustained antibiotic treatment such as a combination of rifampin and doxycycline for six weeks, there is a 10-30% relapse rate (Skendros 2006). Since treatment of human brucellosis is not always effective, prevention is the best course of action.

Human consumption of unpasteurized milk from a diseased animal is a real problem in endemic areas of the world (Pappas 2010), and human brucellosis is a common occurrence; however, in the United States a successful vaccination program has led to the eradication of the disease in food animals. The vaccines used to control the disease in animals are RB51 and strain 19 in cattle, and in other parts of the world, Rev1 in sheep and goats (Ko 2003). Unfortunately, these vaccines cause the disease in humans, and to date there are no known human vaccines for the prevention of brucellosis (Olsen 2006). Therefore, the best prevention available is to keep our food animals brucellosis-free. Unfortunately, there is a large wildlife population that still carries *Brucella* species, and occasional interaction between livestock and these diseased animals can result in reintroduction of brucellosis to the unvaccinated mammals (Pappas 2010).

Cattle are the natural host of *Brucella abortus*, and within this host the brucellae establish either a chronic infection or, in a pregnant cow, an acute infection (Detilleux 1990). *Brucella* resides within the cells of its host, leading to evasion of the host immune response (Detilleux 1990). During an acute infection, the brucellae reside within the placental trophoblast epithelial cells, living on the unique erythritol carbon source that is only made during the third trimester in ruminants, which results in the third trimester abortion of the fetus (Acha 1980). Erythritol is the preferred carbon source for *Brucella*, and erythritol catabolism in *Brucella* is heavily iron-dependent, which greatly increases the cell's iron requirement as cofactors in this reaction

(Sperry 1975). The brucellae in this niche replicate in large numbers within the placental trophoblast, causing abortion. For the establishment of a more chronic infection in cattle, the brucellae take up residence within the bovine macrophages. Because erythritol is not found in humans, humans tend to develop a more chronic infection, and the ideal environment in this niche for the brucellae is within the macrophage (He 2006).

While most pathogenic bacteria rely on classical virulence factors such as toxins, pathogenicity islands and virulence plasmids, *Brucella* species utilize a more subtle approach to establishing and maintaining infection within their host. In fact, *Brucella* pathogenesis is mainly based on its ability to survive and multiply within the host macrophages. One coined term, "virulome" is used to describe the set of genes needed for survival of *Brucella* in the macrophages (Köhler 2002). The virulome genes are often associated with metabolism, and transposon mutagenesis of the *Brucella* chromosomes led to striking attenuation of the mutants in which amino acid biosynthesis genes were disrupted. This suggests that these corresponding metabolites are not available in the phagosomal compartment. Other attenuated mutants involved genes that are associated with cellular stress response, such as *hfq* and *rsh* (a *rel/spoT* homolog), and these mutations resulted in lowered resistance to stress induced by peroxides and acidic conditions. These results collectively lead to the conclusion that the *Brucella* environment is low in nutrients and oxygen (Köhler 2002). Therefore, it is important to take a closer look at the *Brucella* genes associated with nutrient uptake and metabolism in order to better understand the pathogenic nature of this bacterium.

**A stealthy intracellular pathogen**

Animal models of infection using mice and natural hosts (goats and cattle) have been developed (Kahl-McDonagh 2007) in order to gain a better understanding of what takes place during infection of a mammalian host with brucellae. Two popular mouse models are Balb/c mice and C57BL/6 mice. These mice strains provide different immunological responses to *Brucella* infection, with the C57BL/6 strain producing a more effective, cell-mediated response than Balb/c mice (Silva 2011). These differences have provided key insights into understanding the host immune response and have helped to reveal the relationship between cell-mediated Th1 host response and clearance of the brucellae organisms *in vivo*. The Th1 immunologic response of the C57BL/6 mice often results in clearance of the wild type brucellae after 2 months of infection, whereas the Th2 immunologic response of the Balb/c mouse during *Brucella* infection is ineffective and brucellae organisms may persist for over 6 months *in vivo*. It has been shown throughout the literature that the cell-mediated immune response (Th1), and not the humoral immune response (Th2), is required for an effective clearance of *Brucella* organisms that have chronically infected the host (de Jong 2010).

*Brucella* are extremely stealthy, and both avoid and suppress the host innate immune response (de Jong 2010). *Brucella* enter their host mostly through a mucosal route, via ingestion or inhalation, where they first encounter the host immune system. The O-chain component of the *Brucella* LPS interacts with the surface of the macrophage, allowing for unopsonized entry of the brucellae into these cells (Porte 2003). This intracellular niche protects the *Brucella* from complement and antibodies; however, even those brucellae that have not yet made it to their intracellular niche have ways to avoid the immune response. Most notably, the

lipopolysaccharide (LPS) of *Brucella* triggers an ineffective Th2 immunological response. The pathogen- associated molecular patterns (PAMPs) often recognized by the host toll – like receptors (TLRs) do not bind very well to the LPS of *Brucella*. For instance, the lipid A of *Brucella* contains a very long fatty acid residue that is not recognized well by TLR4, allowing the bacteria to avoid induction of a strong inflammatory response (Lapaque 2006). Also, the perosamine O-chain of the *Brucella* LPS is not broken down well by macrophages, and it forms complexes with MHC class II molecules, inhibiting their ability to present antigens for further host immune activation (Forestier 2000). Additionally, because of its aberrant O-antigen, *Brucella* LPS does not interact very well with C3 of the host's pro-inflammatory complement system, which also interferes with a robust response to infection (Hoffmann 1983).

In addition to having low TLR agonist and complement activity, *Brucella* produces proteins that interfere with the host immune signaling. TcpB contains a Toll/interleukin-1 (TIR) domain that blocks TLR2 and TLR4 – mediated induction of NF-kB expression by inducing the degradation of MAL, which reduces the ability of the host cell to produce proinflammatory cytokines such as TNF-α and IL-12 (Salcedo 2008). PrpA is a proline racemase produced by *Brucella* that stimulates the production of IL-10, an anti-inflammatory cytokine (Spera 2006). All of these initial evasions of the host innate immune response are vital to the establishment of infection.

Intracellular survival for the brucellae is not quite so stealthy, however, and within phagocytic cells about 90% of the bacteria are immediately killed by the oxidative burst of the macrophage (Jiang 1993). However, a small population of brucellae that enter the host survive, and it is with these successful pathogens that our interest lies.

Research to date has revealed a high level of interaction between the host and *Brucella* pathogen during the establishment and maintenance of chronic infection. A successful brucellae is able to resist being digested in the cellular phagosome, which temporarily fuses with the lysosome and acidifies, escape constitutive phagosomal fusion with the lysosome, and move to the safer compartment of the endoplasmic reticulum (ER), where it is able to replicate. *Brucella* strains enter the cell within a phagosomal compartment referred to as the *Brucella*-containing vacuole (BCV) (Celli 2005). The BCV interacts with early endosomal compartments, taking on several endosomal markers and causing acidification of the BCV (Bellaire 2005). The BCV quickly (10 minutes) loses its endosomal markers and takes on more lysosomal – like markers, such as Lamp-1, which remain detectable for up to 4 hours postinfection (Starr 2008). Simultaneously, the macrophage begins to express a natural resistance - associated macrophage protein -1 (Nramp1) within the membrane of the BCV. Nramp1 is a divalent cation transporter that creates an efflux of these precious nutrients from the phagosomal compartment, and is a vital protein for cellular resistance to *S. enterica* serovar Typhimurium, *Leishmania donovani*, or various species of *Mycobacterium* (Vidal 1995). This is a critical time for the brucellae, and they must survive in this extremely low- nutrient, acidic compartment. However, these harsh events in the BCV are necessary for *Brucella* survival, and it is this drop in pH and nutrients that signals *Brucella* to express genes to assemble the type IV secretion machinery (Porte 1999). The type IV secretion system releases effectors that are responsible for the BCV progression to the ER niche, and *Brucella* strains that lack a functional type IV secretion system are digested before they reach the ER (Celli 2003). At this point, the fusion between the BCV and the ER ensures the success of the brucellae, since this is a safe niche where it can replicate (Celli 2006).

# The bacterial necessity for iron

## The chemistry of iron

Iron, the most common metal on earth, is an essential metal for most living organisms. While iron can exist in an extensive range of oxidation states, the most common, biologically relevant forms are $Fe^{2+}$ (ferrous) and $Fe^{3+}$ (ferric). Ferric iron, which is insoluble at neutral pH, is predominantly found in the environment, and the ferrous form is typically found in environments low in either oxygen or pH (Rajasekaran, 2010). It is because of the ability of iron to alter its redox state that it is used as the catalytic site for many different proteins (Waldron, 2009). The reactivity of iron is a double – edged sword, however, since iron can also convert oxygen to reactive species (ROS). The Haber – Weiss reaction describes the interaction of ferrous iron with hydrogen peroxide. The product of this reaction is ferric iron, a hydroxyl radical, and a hydroxyl anion (Haber, 1932). Furthermore, the Fenton reaction involves ferric iron reacting with hydrogen peroxide to yield ferrous iron, a peroxide radical, and a proton (Fenton, 1894). The end result of these two reactions, commonly known as Fenton chemistry, is the production of very reactive, damaging hydroxyl radicals from the cyclic interaction of both forms of iron with hydrogen peroxide.

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

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

The hydroxyl radical is remarkably reactive and damaging to cellular components, and it can cause the potential for further damage by freeing iron from Fe-S centers of proteins and from the

iron storage protein ferritin (Arosio, 2009). Superoxide and ascorbate can also reduce ferric iron to its ferrous, reactive form (Kell 2010). Among other things, these hydroxyl radicals also react with DNA, causing damage that can lead to cell death (Loft 1996 and Lloyd, 1998).

Iron participates in a substantial number of cellular processes, including respiration, the tricarboxylic acid (TCA) cycle, oxygen transport, lipid metabolism, DNA synthesis, and gene regulation (Cairo 2006). Because iron is redox reactive, it is the metal commonly used in the active site of many important cellular respiration enzymes, such as cytochromes (Massey, 1963). In the TCA cycle, iron is needed for the aconitase enzyme, a critical enzyme in this metabolic pathway (Glusker, 1968). Iron also forms complexes with molecular oxygen in hemoglobin and myoglobin, both proteins are involved in oxygen transport within mammals (Perutz, 1960). In fact, within the host, heme is often the main source of iron. Heme consists of a tetrapyrolle ring surrounding an iron center, and is itself a very toxic molecule (Kumar 2005). Free heme damages lipids, proteins, and DNA through the generation of reactive oxygen species (Schmitt 1993). Heme and heme proteins have been implicated in a variety of toxic effects through the oxidation of lipids. Heme can aggregate in the cell membrane and promote oxidation, leading to the enhancement of permeability and membrane disruption, which may cause cell lysis.

**Bacterial adaptations to the chemistry of iron**

Bacterial iron metabolism encompasses three major processes: acquisition, storage, and release. The overall goal of these processes is to create and maintain iron homeostasis on a cellular level, since either too much or too little iron can wreak havoc on the survival of the organism. Bacteria acquire iron through specific transport systems (detailed later); any unused

iron must be either stored immediately or released out of the cell in order to protect the cell against the dangerous reactivity of iron.

Bacteria contain three types of iron storage proteins, the non-heme iron containing ferritin, the heme-containing bacterioferritin and a smaller protein, Dps (Andrews 1998). Ferritin contains 24 subunits that make up a protein "nanocage". Its center contains a ferroxidase activity that catalyzes the oxidation of ferrous iron to its ferric form, at least initially, as the iron core builds up. Likewise, bacterioferritin is very similar to ferritin and contains 24 heme molecules per holomer. Although research is still very active in this area, it is believed that a bacterioferritin associated ferroxidase (Bfd), which is genetically coded next to *bfr* in many bacteria, resides at the center of Bfr, performing a similar ferroxidase activity to that found in ferritin, within this protein aggregate by catalyzing the oxidation of ferrous iron (Hawkins, 1996). Dps forms smaller spherical cages made up of 12 subunits that can also function as a reservoir for iron (Andrews 1998).

Bacterial iron export is a fairly new area of research, with the recent identification of FieF in *E. coli* as a ferrous iron efflux pump fueled by the respiratory chain (Grass 2005), and a protective function against heme toxicity attributed to the product of *ght* in *Neisseria meningitidis*, which may be working as a heme exporter (Rasmussen 2005). Most recently, a TolC-dependent efflux system in *E. coli* has been shown to export porphyrins to avoid a toxic accumulation (Tatsumi 2008). Due to the toxic nature of both iron and heme, it is believed that more such protective export systems will be discovered in the near future.

Thus far emphasis has been placed on the importance of iron and heme storage and efflux in bacterial cells, however, as before mentioned, excessive iron in the cell interacts with

hydrogen peroxide, resulting in the production of toxic hydroxyl radicals. Therefore, iron uptake must be tightly regulated in order to maintain a delicate balance of iron within the cell. In the majority of Gram negative bacteria, the genes encoding iron uptake systems are regulated by the ferric iron uptake (Fur) regulator (Szafran 2008). Fur acts as a negative regulator of transcription by forming a dimeric complex with ferrous iron and binding specific sites within the promoter region of the target operon, blocking access by the transcriptional machinery. Therefore, when there is sufficient iron in the cell, Fur represses iron acquision genes. The equivalent iron – dependent regulator in some Gram positive bacteria like *Corynebacterium diphtheriae* is DtxR (Boyd 1990). DtxR was originally shown to regulate toxin production, but it also regulates the heme oxygenase genes in these organisms (Qian 2002). Interestingly, in the Gram negative alpha proteobacteria, Fur regulates manganese uptake genes, not iron metabolism genes, and is called Mur (manganese uptake regulator) (Rudolph 2006). The *Brucella abortus* Mur has recently been shown to regulate the manganese transporter *mntH* (Menscher 2012).

Instead of Fur, growing evidence implicates two regulators with a high degree of interplay in the alpha proteobacteria, RirA and Irr, in the iron – responsive regulation of iron metabolism genes. Originally found in *Rhizobium leguminosarum*, the *rhizobial* iron regulator (RirA) is a member of the Rrf2 family of regulators. Rrf2 itself regulates cytochrome biosynthesis in *Desulfovibrio* while IscR, another regulator of this family found in *E. coli* regulates the synthesis of iron-sulfur clusters (Johnston 2007). RirA can have both positive and negative effects on iron metabolism genes, and although it is not thought to directly bind iron like Fur does, it does contain an iron-sulfur center (Todd 2005). The iron-responsive operator (IRO) is the conserved sequence motif that RirA is proposed to bind, since mutation of this IRO box eliminates the iron responsive repression of the iron gene being studied (Yeoman 2004). To

date, RirA has been shown to regulate a variety of genes involved with iron uptake, energy metabolism, and heme biosynthesis (Rudolph 2006) (see Figure 1.2).

While RirA is more "Fur-like" in nature, its role in iron regulation appears to be minor in the alpha proteobacteria when compared with that of Irr. Whereas RirA senses cellular iron in the form of FeS clusters, Irr, an iron response regulator (Irr) first found in *Bradyrhizobium japonicum* (Hamza 1998), senses the metabolic intracellular concentrations of iron through direct interaction with heme molecules. Ferrochelatase is the last enzyme of the heme biosynthesis pathway, inserting an iron molecule into protoporphyrin IX to create heme. Irr binds to ferrochelatase in the absence of heme, however it is displaced in the presence of iron. The binding of the newly synthesized heme to Irr causes the protein to degrade, and therefore Irr is only present under iron limiting conditions (Qi 1999). Under iron limiting conditions, Irr represses the transcription of the heme biosynthesis genes, since heme biosynthesis requires iron, and at the same time increases the transcription of iron acquisition genes.

Bacteria compensate for iron starvation both by inducing iron transport systems and by decreasing the cell's iron demands, and in the alpha proteobacteria, Irr functions in both of those roles (Rudolf 2006) (see Figure 1.2). For example, under iron limited conditions, Irr transcriptionally represses the biosynthesis of heme, thus reducing the cell's iron needs (Martinez 2005). Under iron limitation, Irr also increases the expression of catechol siderophore and heme uptake genes, which allows the cell to bring in more iron (Martinez 2006, Anderson 2011). Irr only functions under low iron conditions, since the Irr protein is degraded in the presence of sufficient iron levels (Rudolf 2006). Irr binds to motifs known as iron control element (ICE) boxes within the promoter regions of several iron and heme responsive genes

(Rudolf 2006b), and Irr is implicated in the regulation of a growing number of iron transport genes of *Brucella*, including *bhuA*, the outer membrane heme transporter (Anderson 2011).

RirA primarily functions under sufficient iron conditions, whereas Irr is only present when cellular iron levels are low. The interplay of the RirA and Irr regulators allows for fine-tuning of cellular regulation of not only iron and heme, but also those genes inevitably affected by iron levels such as those genes implicated with the oxygen and carbon utilization and cellular stress (Johnston 2007). As a result, in high iron medium RirA will be more repressive due to the accumulation of FeS clusters and Irr will be less so, because the increased heme levels will lead to degradation of this regulator. However, under iron limited conditions, Irr would be the major player, allowing for transcription of genes both bringing in more iron and repressing the cell's iron needs.

Another heme – sensing regulator of iron metabolism genes is the two component system ChrSA, first discovered in *Corynebacterium* to positively regulate the heme oxygenase gene (Schmitt 1999) as well as an ABC transporter, HrtAB, that protects the cell against heme toxicity (Bibb 2010). In *Brucella abortus*, the *chrSA* genes are operonic and are predicted to encode a sensor kinase (ChrS) and a cognate response regulator (ChrA). Preliminary data suggests that both iron- and heme- responsive transcription of the genes encoding the outer membrane heme transporter are dependent on ChrA in *Brucella*, suggesting a similar role for ChrSA in this organism (Paulley 2007 dissertation). Ongoing experiments will tell us if ChrSA is truly a heme responsive regulator.

# Availability of iron within the host

## Iron trafficking in the host

Because iron is a necessary nutrient for most organisms, there are many different methods employed by host cells to take up iron. Systemically, within mammals, dietary iron is absorbed into the luminal side of duodenal enterocytes of the gut. Ferric iron must be reduced to ferrous iron in order to be transported across the cell membrane. Dcytb is a ferric reductase that often performs this function (Wyman 2008). The ferrous iron is then transported across the membrane by DMT1 (Cannone-Hergocts 2001). Moreover, heme constitutes a minor part of dietary iron, but it is transported very efficiently through the host cell membrane by HCP1 (Shayeghi 2005).

The diffusion of $Fe^{2+}$ across the basolateral membrane of the duodenal enterocyte and into the blood is assisted by iron-regulated transporter 1 (IREG1), a transmembrane iron transporter protein (McKie et al., 2000). Hephaestin, a membrane-bound protein, promotes oxidation of $Fe^{2+}$ to $Fe^{3+}$ (Vulpe et al., 1999). Once it is exported, this ferric iron is promptly bound to apotransferrin and circulated in the blood plasma. Overall, the level of duodenal iron absorption decreases as the total body iron levels increase, but an increase in red blood cell production or hypoxic conditions leads to an increase in iron uptake (Cairo, 2006).

Within mammals, all cells need to take in the iron absorbed from the gut in order to perform metabolic functions. The iron of the body is often found in the blood plasma, transported by a protein called transferrin. Transferrin (Tf) has two binding sites for iron (Baker 2003), and this diferric transferrin is taken into cells via Tf receptors expressed to varying

degrees on the surface of every cell (De Silva 1996). In times of higher metabolic need, Tf receptor gene expression can be increased to allow for higher iron uptake into the cell. Receptor – mediated endocytosis engulfs Tf, which releases its iron at the acidic pH of the endosome (Cheng 2004, Dautry-Varsat 1983, Dhungana 2004, Giannetti 2003, Klausner 1983). The iron is transferred to the cytosol by DMT1 and the apo-transferrin is returned to the cell surface for release into the blood plasma to bind and transport more iron.

Yet another great source of iron uptake in host cells is the phagocytosis of senescent red blood cells. Erythrophagocytosis involves the engulfment of a damaged red blood cell into the phagosomal compartment of a macrophage. Trafficking of the red blood cell follows phagolysosomal fusion resulting in acidification of the vacuole and breakdown of hemoglobin into heme molecules (Taketani 2005). Importantly, Nramp1, a divalent metal transporter expressed within phagolysosomal membranes, is required for the efficient recycling of heme in these macrophages. Without Nramp1, iron accumulates within the liver and spleen of mice (Soe-Lin 2009). Through an unknown mechanism the heme is trafficked to the endoplasmic reticulum where its iron center is liberated via a eukaryotic heme oxygenase (HO-1) and the iron gets recycled for use in the body. Heme oxygenases are required for this iron recycling, and provide a substantial amount of iron back to the body.

Heme oxygenase enzymes catalyze the oxidation of the tetrapyrolle ring of heme in order to break it open, releasing the iron and producing carbon monoxide (CO) and biliverdin in the process (Maines 1988). All three of these products of heme oxygenase activity have been implicated in host cellular protection during infection (Chung 2009). Specifically, initial pathogen – host interactions stimulate a host inflammatory response, where activated white

blood cells secrete cytokines, chemokines, antimicrobial molecules, and free radicals in order to aggressively fight the infection (Silva 2005). However, to avoid tissue injury from a continued inflammatory response, anti-inflammatory signals must be released to resolve the inflammation. Heme oxygenase-1 is thought to be responsible for this anti-inflammatory signaling, at least in part. For example, CO has been shown to reduce the inflammatory response through signaling through the p38 MAPK pathway (Otterbein 2000). Further, biliverdin and its downstream product bilirubin are both reducing agents, and are therefore considered antioxidants (Zhu 2011). They can reduce transcription of genes encoding iNOS and inflammatory cytokines, thus lowering the overall inflammatory response. Iron itself is a signal for the increased expression of ferritin, which protects the cell against oxidative damage (Balla 1992).

About 20% of the iron in the human body is stored iron, and this iron can be found mostly in the parenchymal liver cells and reticuloendothelial macrophages. These macrophages are particularly noteworthy to this discussion because they are found in the spleen, liver, and bone marrow and function to recycle senescent red blood cells. The iron released from heme acquired via erythrophagocytosis is first destined to be stored in ferritin, like all excess iron of the cell. Ferritin is a cytosolic molecule that can multimerize into a "nanocage" capable of binding and storing up to 4500 iron atoms (De Domenico, 2006). As the amount of iron in the cell increases, a larger percentage is deposited in hemosiderin, an insoluble, aggregated form of partially digested ferritin. The highest concentrations of hemosiderin in the body are found in the reticuloendothelial macrophages (Bothwell, 1979). Cells can remove iron from ferritin for heme synthesis and for export through ferroportin, and ferroportin expression levels decrease in response to hepcidin (discussed later) (Nemeth 2004). Together, it can be surmised that copious amounts of iron and heme are constantly trafficking through these macrophages. There are still

many areas of heme trafficking within the cell to be elucidated (Hamza 2006), and there appear to be other, as yet unknown, ways for macrophages to take in iron (Chen 2005).

**Host iron sources during infection**

A universal feature of host infection is the disturbance of normal iron homeostasis (Brock 1999). The host has adapted several mechanisms of iron deprivation in an attempt to starve pathogens of this necessary nutrient, the simplest being a decrease in DMT1 iron uptake in the intestinal lumen in response to hepcidin (Mena 2008). Hepcidin, a signal produced by the liver during inflammation, causes a systemic hypoferremia intended to resist microbial infection by decreasing the amount of extracellular iron available to the microbe (see Figure 1.1) (Roeser 1980). This hypoferremic response acts to posttranslationally regulate ferroportin expression. Hepcidin can bind directly to ferroportin, causing the iron exporter to be taken into the cell, which decreases the amount of cellular iron exported (Nemeth 2004). In the host serum, iron is tightly bound to proteins such as transferrin with a high affinity in order inhibit its availability, and during the hypoferremic response, inflammatory signals cause the upregulation of hepcidin, which increases the cell surface expression of Tf receptors (Ganz 2011). This response causes the temporary uptake of serum iron into cells, where it is stored as ferritin.

However, IFN-γ activation of the host macrophage by cells of the acquired immune response causes transferrin receptors on the surface of pathogen-infected cells to be downregulated, and also causes Nramp1 to be integrated into the phagosomal compartments (Wyllie 2002). It is postulated that Nramp1 can then function to efflux iron from the phagosomal compartment, creating an extremely low iron environment for the pathogen. Further,

this IFN-γ activation of infected cells serves to increase expression of ferroportin, causing intracellular iron to be transported out of the cell (Nairz 2010).

## Pathogenic bacteria acquire iron from the host

### Acquisition of ferric and ferrous iron from the host

Iron is an essential nutrient for most pathogenic microorganisms and plays a vital role in microbial pathogenesis. To survive within the iron-limited environment of the host, bacteria utilize iron-siderophore complexes, iron-binding proteins (transferrin, lactoferrin), free heme and heme bound to hemoproteins (hemoglobin, haptoglobin, hemopexin) as iron sources.

In aerobic environments where ferric iron is dominant, it is often necessary to chelate the iron using siderophores (Andrews 2003). Siderophores are molecules with a high affinity for ferric iron, and under iron-limitation, most bacteria produce and excrete siderophores into the host environment. Once bound to iron, these ferri-siderophore complexes are transported back into the cell using specific receptors that are energized by the TonB/ExbB/ExbD system. *B. abortus* produces two siderophores, 2, 3-DHBA and brucebactin (González-Carreró 2002). Brucebactin is constructed from 2, 3-DHBA because mutation of the genes involved in 2, 3-DHBA biosynthesis also results in loss of brucebactin production (González-Carreró 2002). The first gene in the 2, 3-DHBA operon encodes an isochorismate synthase, and mutating this gene (*dhbC*) results in a loss of all siderophore production. The role of siderophore production by *B. abortus* during infection of a pregnant cow is very significant, and a *dhbC* mutation results in loss of virulence within this acute model of infection (Bellaire 2003). However, 2,3-DHBA and

brucebactin are not required for the survival and replication of *B. abortus* 2308 in the mouse model of chronic *Brucella* infection suggesting that there must be an alternative source of iron within the macrophage of the host (Bellaire 1999).

Still, the brucellae within the BCV must be able to overcome the Nramp1 – associated nutrient deprivation of divalent cations such as iron (Moreno 2002) and although the iron requirements for *Brucella* are extremely low by bacterial standards, obtaining such small amounts of needed iron can be challenging (Waring 1953). In addition to ferric acquisition by siderophore, there are several identified high affinity ferric iron ABC transporters. For example, SfuABC makes up a high affinity ferric iron transport system in *Serratia marcescens* that transports $Fe^{3+}$ through the inner membrane of the cell (Angerer 1992). *Brucella* contains two operons with high homology to this *sfu* gene cluster, and these putative *Brucella* iron transporters have yet to be characterized to determine their role during infection (Roop 2011).

Host-associated bacteria such as *Brucella* and *E. coli* O157:H7 are often exposed to ferrous iron either within the acidified compartment of the host cell environment (*Brucella*) or in an anaerobic environment (*E. coli* K12), and therefore have adapted methods to take in these forms of iron (Halaas 2010). The most well-characterized ferrous iron transporter was first found in *E. coli* K12, and is known as the Feo system (Hantke 1987). Extracellular $Fe^{2+}$ is presumed to diffuse into the periplasm via undefined porins. It is then transported across the cytoplasmic membrane into the cytoplasm by FeoB through an apparent ATP/GTP-driven active transport process.

In an aerobic organism and because of the soluble nature of ferrous iron, a ferroxidase enzyme may convert $Fe^{2+}$ to the less reactive $Fe^{3+}$ form in the periplasm, and there is a conserved ferroxidase enzyme encoded on the *Brucella* genome near the putative ferrous transport genes.

While there are no *Brucella* homologs to *feoAB*, a more recently described *efeUOB* operon in *E. coli* 0157:H7 (Grosse 2006) bears homology to an operon in *Brucella* (recently named *bfe*). The ferrous transport system of *Brucella* is currently being investigated to confirm its function and evaluate its contribution to virulence, but initial studies of these genes in the mouse model of chronic infection suggests that they are highly correlated to *Brucella* virulence and that ferrous iron is a relevant source of iron during infection.

**Acquisition of heme iron from the host**

While both pathogenic and nonpathogenic bacteria have the ability to utilize heme as an iron source, in pathogenic bacteria this requirement for heme is often linked to virulence of the organism. In fact, in *Staphylococcus aureus*, heme is the preferred iron source over transferrin *in vitro* (Skaar 2004). Because extracellular heme in the host is found complexed with hemoproteins such as hemoglobin, extracellular pathogens in the host possess mechanisms for freeing the heme from these proteins, sometimes even directly from red blood cells. Hemolysins are exotoxins produced by bacteria like *S. aureus* that lyse red blood cells to release the hemoglobin (Kaplan 1963). Many bacterial outer membrane proteins can bind directly to hemoglobin before acting as proteases to remove the heme and transport it into the cell (Barton 2005). Still other bacteria produce hemophores, which are siderophore – like small molecules with a high affinity for heme (Cescau 2007). Like siderophores, hemophores require active transport and the genes encoding them are tightly regulated.

Intracellular pathogens within the host are faced with a different type of host iron availability. A major function of the host macrophage is the recycling of senescent red blood cells (RBCs) (Crichton 2002). These RBCs are phagocytosed and their components, such as

heme, are transported to the ER where the eukaryotic heme oxygenase (HO-1) degrades the heme in order to utilize its iron center (Taketani 2005). Many bacterial pathogens have adapted to live within host cells, including *Shigella, Salmonella, Mycobacterium,* and *Yersinia*, and have adapted to utilize iron sources specific to their final location within the cell. For instance, due to the interactions of the brucellae-containing vacuole with the ER, heme may represent a relevant iron source for the brucellae during chronic infection (Roop 2004).

Heme transport systems are present in both Gram positive and Gram negative bacteria. In Gram positive bacteria, the major challenge is overcoming the effects of the redox reactive heme molecule passing through the extensive peptidoglycan cell wall. As a result, there are several cell wall – anchored proteins that function to pass the heme molecule through the peptidoglycan layer. Heme transporters have been confirmed in *Streptococcus pyogenes* (Lei 2003), *Listeria monocytogenes* (Jin 2006), *Corynebacterium diphtheriae* (Allen 2011), and the most thoroughly studied, *Staphylococcus aureus* (Torres 2006). In *S. aureus*, IsdB and IsdH acquire heme from hemoglobin and other hemeproteins and pass it through to IsdA and IsdC. These proteins transfer the heme moiety to a transmembrane ABC transporter, IsdDEF, and IsdG and IsdI degrade the heme for its iron once it has entered the cell (Mazmania n 2003). The genes of these Gram positive bacterial heme uptake systems are, in all known cases, regulated by iron and heme either through ChrAS, DtxR, Fur, or some combination of these with other unknown regulators. While the heme transport system in *S. aureus* has been shown to contribute slightly to absess formation, mutation of components of the heme acquisition system in *L. monocytogenes* reduces virulence of the organism by over 50 fold (Torres 2006, Jin 2006). Together, these data support the hypothesis that the ability to derive iron from heme can contribute to survival of some bacteria *in vivo*.

Heme, a tetrapyrolle ring with iron as its center, is taken into Gram negative bacteria using a highly conserved method of transport. Bacterial pathogens including *Shigella dysenteriae* (Burkhard 2008), *Pseudomonas aeruginosa* (Ochsner 2000), *Escherichia coli* (Torres 1997), *Vibrio cholerae* (Mey 2001), *Yersinia pestis* (Thompson 1999), *Haemophilus influenzae* (Seale 2006), *Bradyrhizobium japonicum* (Nienaber 2001), and the *Bordetella* spp. (Murphy 2002) produce heme transporters and have the capacity to use heme as an iron source. In Gram negative bacteria, this transport involves the binding of a heme molecule to a TonB – dependent outer membrane spanning protein, where energy from the proton motive force shuttled from the inner membrane via a TonB, ExbB/D protein complex is used to transport the heme molecule into the periplasm. A periplasmic binding protein that is specific for heme binds to the molecule and the protein-heme complex then binds to an inner membrane permease, where ATP from an associated ATPase is hydrolyzed to provide energy to bring the heme molecule into the bacterial cell (see Tong 2009 for review). Once in the cytoplasm, the heme is either broken down for its iron center by a heme oxygenase or used directly as a cofactor.

A well-characterized heme transport system is that of *Shigella dysenteriae* (see Figure 1.3). The outer membrane transporter, ShuA, has conserved structural motifs consistent with TonB-dependent transport (a TonB box – Asp- Thr- Leu- Val- Val- Thr- Ala- Asn) (Köck 1987) as well as two critical histidine residues, His119 and His448, that are necessary for heme binding (Bracken 1999). ShuT is a monomeric protein having a molecular mass of 28.5 kDa following proteolytic processing of the periplasmic signaling peptide. ShuT binds one heme per monomer with high affinity (Eakanunkul 2005). The high-affinity PBPs are critical in maintaining the selectivity and specificity of transport. Not only do the PBPs determine specificity, but they also play an integral part in the transport process by complexing to the ATP-Binding Cassette (ABC)

proteins (U and V) to trigger release of their substrate. The release of substrate from the PBP to the ABC transporter is thought to be coupled to the conformational changes in the periplasmic protein (Eakanunkul 2005).

Translocation of heme into the cytoplasm is facilitated by a heme-specific ABC transporter, ShuUV. The heme ABC transporter consists of two membrane-spanning domains (MSDs) of ShuU, which form a translocation pathway, and two nucleotide-binding domains (NBDs) of ShuV. Interaction of the PBP with the transporter triggers the closing of the NBD interface, generating the open conformation of the PBP such that the affinity for the ligand is reduced. This conformational change in ShuUV opens the translocation channel and brings the NBDs together. Following ATP hydrolysis, the PBP is released, and the NBD dimer reopens, releasing the substrate and resetting the transporter (Burkhard 2008). In several bacteria, the release of the heme into the cytoplasm of the cell also requires binding of a shuttle protein ShuS, which transports the heme to a heme degrading enzyme.

A second type of heme transport involves the use of hemophores, which function much like siderophores to bind with high affinity to heme molecules in the environment and then be transported back into the cell. Several Gram negative bacteria produce hemophores, including *Haemophilus influenzae, Serratia marcescens, Pseudomonas aeruginosa, Pseudomonas fluorescens, Yersinia pestis and Yersinia enterocolitica* (Wandersman 2004). Although the essential design for the ABC transporters is similar, more and more exceptions are being discovered. For example, the heme-binding protein HmuT of *Yersinia pestis* actually binds two heme molecules instead of the typical one (Mattle 2010). Also, in *Escherichia coli* K-12, which does not contain a genuine heme transport system, heme is transported by the Dpp peptide ABC transporter (Letoffé 2006). Another *E. coli* protein, NikA, binds heme in the periplasm and can

function in heme transport even though it also functions to bind nickel (Shepherd 2007). In *Sinorhizobium meliloti* and *Pseudomonas aeruginosa*, heme and the siderophore can share the proteins of an ABC transporter (Cuív 2008, Rossi 2001).

In most cases, both the heme and siderophore uptake systems are Fur regulated. Notably, *Shigella dysenteriae* heme transport regulation includes both Fur and a sRNA RhyB, and in the alpha proteobacteria, where Fur actually functions as a Mur, the heme transport machinery is regulated by both RirA and Irr. In many cases, the contribution of heme transport to virulence is unknown or insignificant, however heme uptake is required for virulence in *Haemophilus influenzae* (Morton 2007), enterohaemorrhagic *E. coli* (Torres 1997), and *Bordetella pertussis* (Brickman 2007).

Once inside the cell, the heme molecule must be broken down for iron utilization or storage. One or more heme oxygenase enzyme(s) is responsible for the degradation, and functions to release iron from its tetrapyrolle ring through a series of oxidative steps (Maines 1988). The reaction requires a total of three oxygen molecules and seven electrons for the cleavage of one heme molecule to release the iron atom (Montellano 2000). The HmuO heme oxygenase protein from *Corynebacterium ulcerans* was the first bacterial heme oxygenase to be identified, and has many similarities to the eukaryotic HO-1 heme oxygenase (Schmitt 1997). Other heme oxygenases were quick to be identified based on their homology to HmuO of *C. ulcerans*, including those of *Pseudomonas aeruginosa* and *Neisseria meningitidis* (Ratliff 2001 and Zhu 2000). More recently, novel heme oxygenases have been identified in pairs of two in both *Staphylococcus aureus* and *Bradyrhizobium japonicum* (Skaar 2004b and Puri 2006). In fact, a heme oxygenase from *Brucella*, BhuO, was identified based on its high amino acid homology to those from *B. japonicum*. Bacterial heme oxygenases are a hot topic in research

these days, and Wilkes *et al* have identified chemical heme oxygenase inhibitors with the intent to find better drug targets (Furci 2007).

It is worth noting, however, that although the main mechanism of heme uptake in these bacteria appears to be quite similar, there are often other genes of unknown function encoded within the same locus and shown to be required for utilization of heme as an iron source. Some are thought to function as heme shuttles, transporting the heme to the heme oxygenase within the cytoplasm, such as ShuS of *Shigella* (Burkhard 2008). Other proteins encoded within these heme uptake loci are still "hypothetical" or else denoted with letters from the end of the alphabet to follow the T, U, and V annotations of the cytoplasmic membrane transporter, and although often required for utilization of heme as an iron source such as HugWXZ, their exact function has yet to be elucidated (Henderson 2001).

*B. abortus* 2308 has been shown to utilize heme as an iron source *in vitro* (Paulley 2007), and in keeping with heme transport in other bacteria, *B. abortus* uses a specific outer membrane receptor (BhuA) as well as a cytoplasmic membrane – spanning heme transport complex. The *B. abortus* heme uptake proteins have high amino acid similarity with that of the well-characterized *Shigella dysenteriae* heme uptake system. A *B. abortus bhuA* mutant strain grows poorly in low iron media unless exogenous iron, such as $FeCl_3$, is added to the cells. This mutant strain cannot utilize heme as an iron source *in vitro* (Paulley 2007). Also, BhuA is required to maintain a chronic spleen infection in experimentally infected BALB/c mice, indicating a role for brucellae iron acquisition from heme *in vivo* (see Figure 1.4). While the regulation of *bhuA* is still being elucidated, recent work shows that transcription of *bhuA* requires the iron responsive regulator Irr (Anderson 2011).

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**Figure 1.1 Host iron sources during infection**. Bacterial infection of the host causes an increase in the production of hepcidin by the liver, which downregulates expression of DMT1, resulting in less intestinal lumen iron uptake. Hepcidin also starves extracellular bacteria for iron by increasing cellular transferrin (Tf) receptors and by causing ferroportin to be taken into the cell, both which result in decreased serum iron levels and increased intracellular ferritin iron storage. Alternately, intracellular bacteria stimulate IFN-γ production, which causes a decrease in Tf receptors on pathogen-infected cells as well as an increase in phagosomal expression of NRAMP1. IFN-γ also causes the increased expression of ferroportin on the cell surface, causing more iron to be pumped out of the cell. The result of IFN-γ is a decrease in overall cellular and phagosomal iron within infected cells.

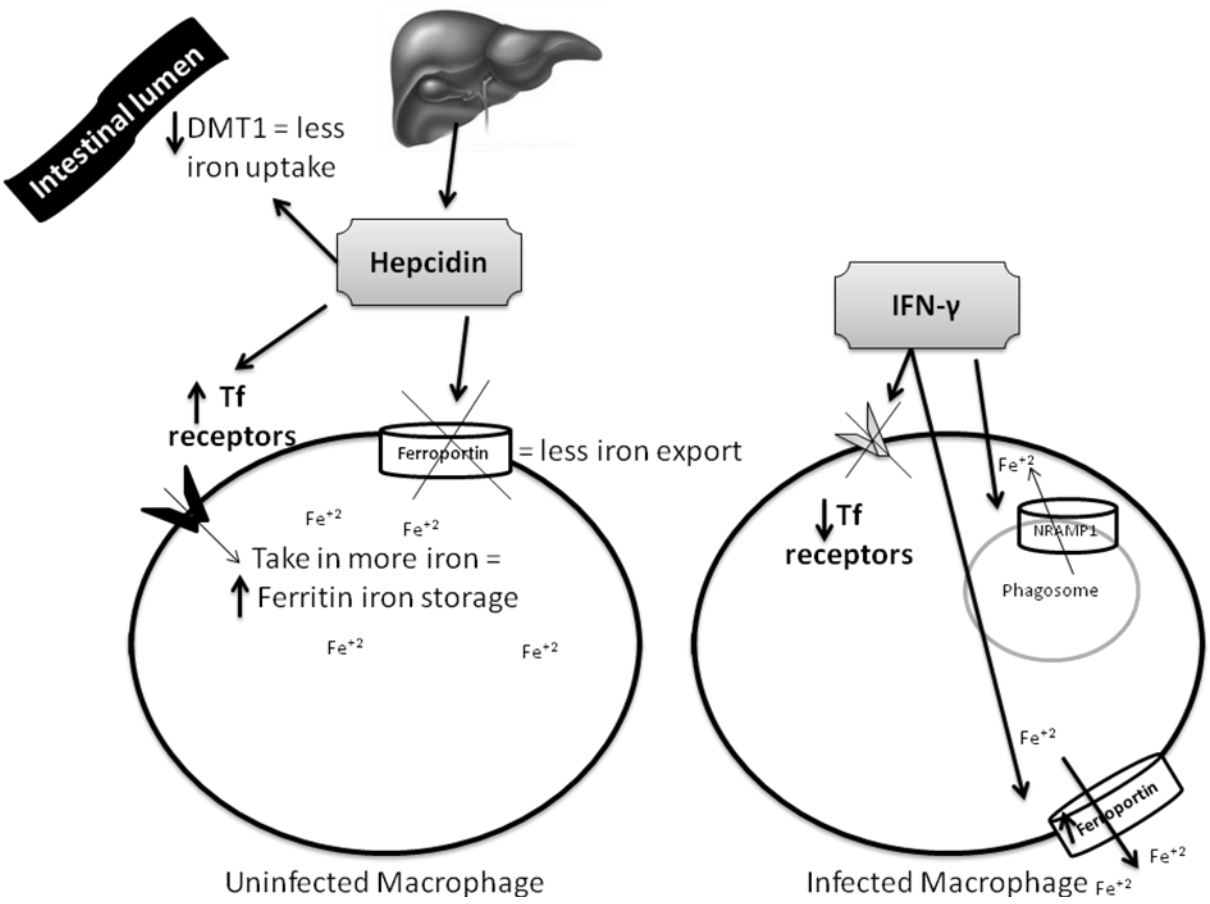
Intestinal lumen
DMT1 = less iron uptake
Hepcidin
Tf receptors
Ferroportin
= less iron export
Fe+2
Fe+2
Take in more iron =
Ferritin iron storage
Fe+2
Fe+2
Uninfected Macrophage
IFN-γ
Tf receptors
Fe+2
NRAMP1
Phagosome
Fe+2
Ferroportin
Fe+2
Infected Macrophage Fe+2

**Figure 1.1**

**Figure 1.2 RirA and Irr regulate several iron acquisition genes**. RirA senses iron through its FeS center, and under high iron cellular conditions it binds to its cognate IRO box motifs, repressing the expression of several iron acquisition genes. In contrast, Irr protein binds to its cognate ICE box motifs under low cellular iron conditions, and Irr is degraded under high iron conditions. Abbreviations of bacterial species: *Bradyrhizobium japonicum (B.j.), Rhizobium leguminosarum (R.l.), Brucella abortus (B.a.),* and *Sinorhizobium meliloti (S.m.).*

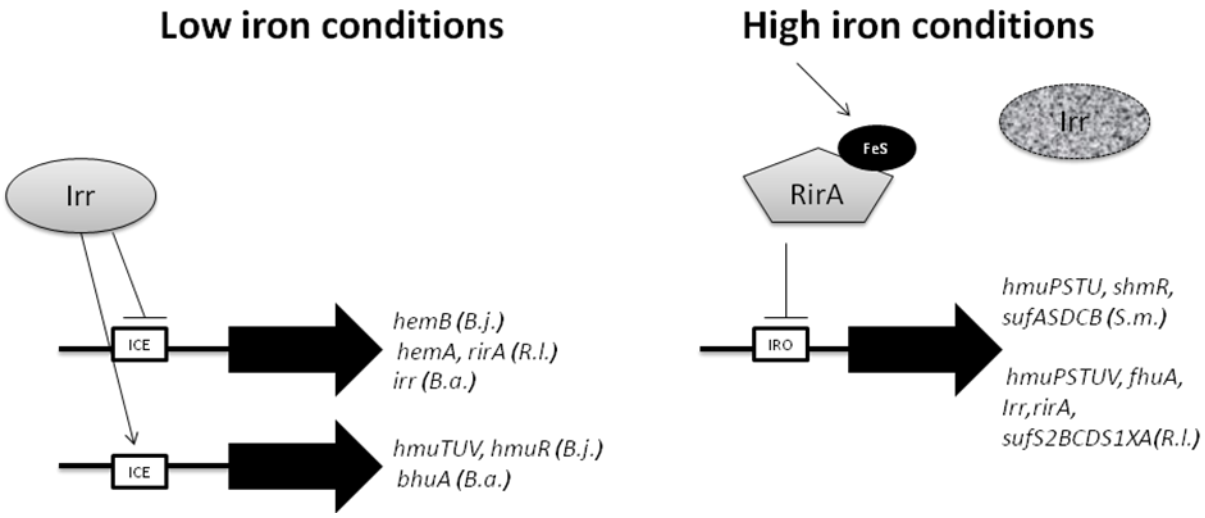
Low iron conditions
Irr
ICE
ICE
hemB (B.j.)
hemA, rirA (R.l.)
irr (B.a.)
hmuTUV, hmuR (B.j.)
bhuA (B.a.)
High iron conditions
Irr
FeS
RirA
IRO
hmuPSTU, shmR,
sufASDCB (S.m.)
hmuPSTUV, fhuA,
Irr,rirA,
sufS2BCDS1XA(R.l.)

**Figure 1.2**

**Figure 1.3. *Shigella* heme transport model**. Heme uptake in Gram negative bacteria is highly conserved and has been very well-characterized in *Shigella*. Transport of heme through the outer membrane is carried out by ShuA, a TonB/ExbB/ExbD – energized transporter. Heme is bound by ShuT in the periplasm and shuttled to ShuUV, a cytoplasmic permease energized by an ATPase. Once in the cytoplasm, the heme is shuttled to a heme oxygenase (HO) where it is broken down into iron, carbon monoxide, and biliverdin.

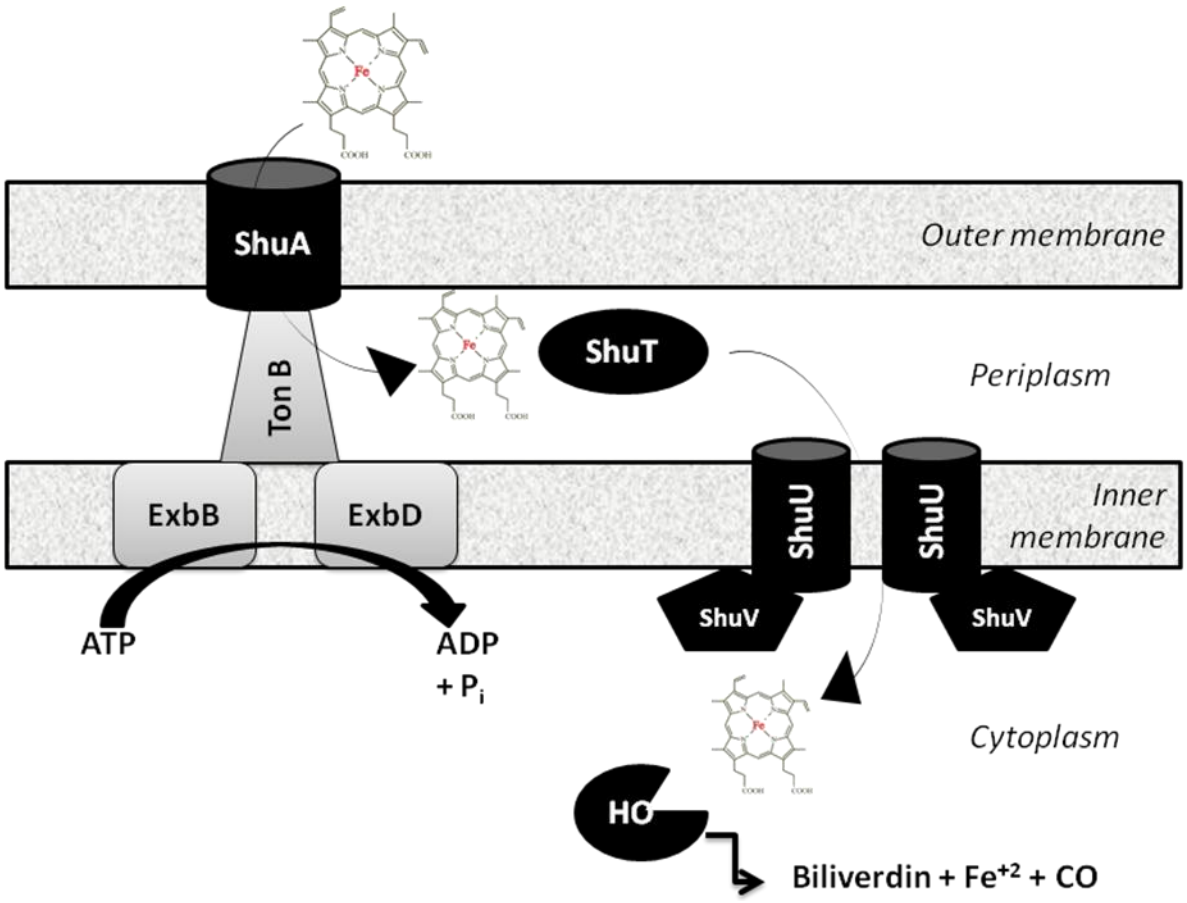
ShuA
Outer membrane
ShuT
Periplasm
Ton B
ExbB
ExbD
ShuU
ShuU
Inner membrane
ShuV
ShuV
ATP
ADP
+ $P_i$
Cytoplasm
HO
Biliverdin + $Fe^{+2}$ + CO

**Figure 1.3**

**Figure 1.4 BhuA contributes to the virulence of *B. abortus*.** Previous work with BhuA, the outer membrane heme transport protein of *B. abortus* 2308, has demonstrated a link between *B. abortus* heme utilization and its virulence within the mouse model. By the fourth week of *Brucella* infection within the Balb/c mouse, the *bhuA* mutant is significantly attenuated compared to *B. abortus* 2308 (Paulley 2007).

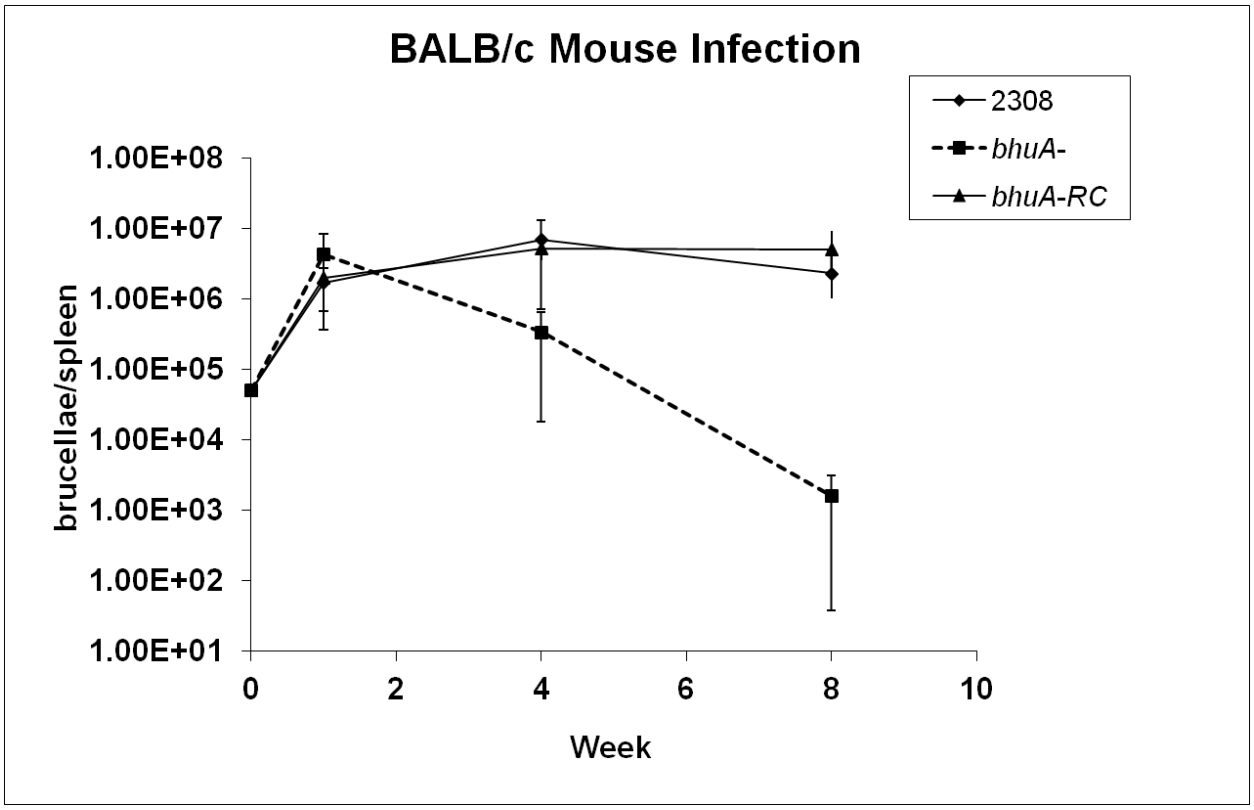
BALB/c Mouse Infection
2308
bhuA-
bhuA-RC
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
brucellae/spleen
0
2
4
6
8
10
Week

**Figure 1.4**

# Statement of the problem

*Brucella abortus* is a stealthy pathogen that lacks the classical virulence factors of other known pathogens. Instead, the pathogenesis of this organism depends solely on its ability to invade and replicate within the phagocytic cell of the host. The brucellae invade the macrophage into phagosomal compartments known as *Brucella* – containing vacuoles (BCV). The BCV acquires Nramp1, a transporter that pumps divalent cations such as iron out of this phagosome. Therefore, the ability to acquire iron in this niche is critical to *Brucella* survival.

Although iron is limited in the BCV, the macrophage recycles red blood cells, transporting heme to the eukaryotic heme oxygenase located in the endoplasmic reticulum (ER) of the cell. During its altered trafficking, the BCV fuses with the ER. The ER is the replicative niche for the brucellae, suggesting a sudden increase in the availability of nutrients. It is likely that the brucellae acquire their iron through the transport of heme in this environment. In fact, the ability to transport heme through the *Brucella* outer membrane heme transporter, BhuA (*Brucella* heme utilization), has been directly tied to the virulence of *B. abortus*. Because heme transport is necessary for the survival of *Brucella* in the mouse model of infection, it is important to identify and characterize the entire heme transport machinery. Typically, these components would be encoded together on the chromosome, but in *Brucella*'s genome there were no genes located near *bhuA* that contained the conserved motifs often associated with heme transport. NCBI BLAST searches using the well-characterized *Shigella* heme transporter amino acid sequences led to the identification of possible cytoplasmic membrane heme transport components in *Brucella abortus* (BAB2_0483-0485). The research described in this dissertation aims to evaluate the role of these proteins, BhuT, BhuU, and BhuV, (BhuTUV) in heme

transport by *B. abortus* 2308 with the intent to determine the contribution of these proteins to the ability of *B. abortus* to maintain infection in the mouse model.

Virulence of *Brucella* is dependent on the ability to obtain heme from its environment, but the brucellae must also be able to utilize this heme as an iron source. Freeing the iron from heme usually requires the presence of one or more heme oxygenases, and one has been identified in *Brucella* based on its high homology to HmuD in *Bradyrhizobium japonicum* as well as its ability to remove iron from heme in a reaction typical to heme oxygenases. The overall contribution of this heme oxygenase, renamed BhuO, to the ability of *Brucella abortus* 2308 to acquire iron from heme is also assessed in this dissertation.

Additionally, the separation of these heme uptake and utilization genes on the chromosome is unique to *Brucella* and raises the possibility of differential regulation of these genes. The environmental stimuli often associated with heme uptake gene regulation involve both heme- and iron- responsive mechanisms. In the other alpha proteobacteria, the RirA (rhizobial iron regulator) and Irr (iron responsive regulator) proteins work together to carry out this regulation. The research presented here aims to identify the roles of the iron – responsive regulators, RirA and Irr, in the expression of *bhuTUV* and *bhuO* under physiologically relevant conditions in *Brucella abortus* 2308.

The outer membrane heme transporter mutant, *bhuA*, establishes but cannot maintain infection within the host model, making it attractive as a vaccine candidate since live, attenuated vaccines are believed to be the most effective for prevention of brucellosis. Also, bacterial heme oxygenases are very different from mammalian heme oxygenases, and researchers are investigating them as potential drug targets. Improving our knowledge of the heme utilization

machinery in *Brucella* may one day be used by others to develop better vaccines and alternatives to the current intensive antibiotic regimen that is used to treat brucellosis in humans.

# Chapter 2: BhuTUV is required for heme utilization in *Brucella abortus* 2308

## Abstract

*Brucella abortus* is a Gram negative bacterium belonging to the alpha proteobacteria class. *B. abortus* resides in the phagosomal compartment of the host macrophage where essential nutrients such as iron are limited. Most bacteria need iron, and within this macrophage niche, heme is a likely source of iron due to the breakdown of red blood cells by the host macrophage. Heme transporters in Gram negative bacteria are highly conserved, and include components for outer membrane, periplasmic, and cytoplasmic membrane transport. While BhuA has been characterized as the outer membrane heme transporter of *B. abortus*, the periplasmic and cytoplasmic components have only recently been identified. NCBI BLAST searches using the well-characterized *Shigella* heme transporter amino acid sequences led to the identification of possible cytoplasmic membrane heme transport components in *Brucella abortus* (BAB2_0483-0485). Here we provide experimental evidence that these gene products, BhuT, BhuU, and BhuV (BhuTUV) are the missing components for heme transport and that they are required in order for *B. abortus* to use heme as an iron source. The *bhuTUV* locus is regulated in both an iron- and growth phase- dependent manner, and transcription of these genes is repressed by both Irr and RirA, the primary iron-responsive regulators in the alpha proteobacteria.

# Introduction

*Brucella abortus* is a Gram-negative bacterium that causes the zoonotic infection brucellosis in humans. Brucellosis is a flu-like illness that can be treated with a combination of doxycycline and rifampin for 6 weeks, however, relapses may occur and symptoms can continue for years (Ariza 2007). *B. abortus* is an intracellular pathogen, and primarily resides within the phagosomal compartments of host macrophages. An infected macrophage will rid itself of intracellular iron stores in order to deprive the pathogen of iron, and in the phagosomal compartment the brucellae must overcome this iron deprivation in order to survive (Cellier 2007, Byrd 1989, Nairz 2010). *In vitro*, *B. abortus* can utilize the iron sources 2,3-dihydroxybenzoic acid (siderophore), heme, and $FeCl_3$ (Bellaire 2003, Paulley 2007). A strain that lacks the ability to produce siderophore shows no growth defect in the mouse model of chronic infection, suggesting that iron uptake via siderophore is not the main source of iron within the macrophage. Since macrophages recycle aged red blood cells, a physiologically relevant source of iron for the brucellae within this niche is heme.

Many bacterial pathogens rely on heme for their iron, and the basic components of heme transport are highly conserved in Gram negative organisms. This iron transport includes TonB-dependent heme transport through the outer membrane and a periplasmic binding protein to shuttle heme to an ATP-dependent cytoplasmic membrane transporter (Siudeja 2005). Once the heme enters the cytoplasm of the cell, it is degraded for its iron component by one or more heme oxygenase(s) (Frankenberg-Dinkel 2004).

In *B. abortus*, the outer membrane heme transporter BhuA (*Brucella* heme uptake) has been characterized and is required for virulence in the mouse model of infection (Paulley 2007).

Because of this contribution of heme uptake to *B. abortus* virulence, it was imperative that we identify the rest of the heme transport machinery. The heme transport components of Gram negative bacteria typically share a genetic locus; however, in *B. abortus* there are no likely candidates for the genes that encode cytoplasmic heme transport components near *bhuA*. However, searches of the *Brucella* genome identified the locus BAB2_0483-0485 based on its high homology to the genes encoding cytoplasmic components of *Shigella* heme uptake machinery (*shuTUV*). Here we constructed an isogenic mutant in the locus encoding these gene products, BhuT, BhuU, and BhuV (BhuTUV) in order to determine its role in heme uptake.

Excess iron in the cell can react with oxygen, in a reaction commonly referred to as Fenton chemistry, creating toxic oxygen radicals that are very damaging to DNA and other cellular components. Due to the highly toxic nature of excess iron in the cell, iron uptake is regulated in an iron-responsive manner. In most bacteria, Fur is the iron-responsive regulator responsible for repressing the heme uptake genes in the presence of iron (Andrews 2003). However, most of the alpha proteobacteria lack Fur, and instead utilize two major regulators, Irr and RirA, in order to moderate iron uptake in the cell. For instance, in *Agrobacterium tumefaciens* RirA (rhizobial iron regulator) is an Fe-S protein that represses iron uptake genes such as those encoding siderophore and heme uptake under iron replete conditions (Ngok-Ngam 2009). Irr (iron responsive regulator) performs a similar function in the alphas, but can act as either a repressor or an activator, and is required for *bhuA* expression in *B. abortus* (Anderson 2011). Irr and RirA have been shown in the alphas to have interlinking roles that serve to control intracellular iron levels (Hibbing 2011). These interlinking roles are possible because Irr is only present in low iron conditions and RirA is only functional in high iron conditions (Hibbing 2011). This allows the cell to regulate iron under all conditions in a finite manner using both

regulators. Therefore, we sought to define the roles of these two regulators in controlling the expression of the heme uptake genes *bhuTUV* in *B. abortus* 2308.

# Materials and Methods

**Bacterial strains, media, and growth conditions.** *Brucella abortus* strains (Table 2.1) 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) and/or ampicillin (25 μg/ml) 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 (Sigma), or 45 μg/ml kanamycin (Sigma) when appropriate.

**Construction of a *B. abortus bhuTUV* mutant.**

Polymerase chain reaction (PCR) utilizing Taq polymerase (Invitrogen) with the oligonucleotide primers *bhuTUV*-1F and *bhuTUV*-1R (Table 2.2) was used to amplify a 4309 bp fragment encompassing the *bhuTUV* genes (BAB2_0483-0485) from *B. abortus* 2308 genomic DNA. This fragment was then cloned into pGEM®-T Easy (Promega). The resulting vector, pGEM*bhuTUV*, was digested with MscI to remove 705 bp from the 3' end of *bhuT* and most of *bhuU*. This fragment was ligated with a 1345 bp fragment containing the *aph3a* gene from pKS-Kn (Kovach, 1995). The resulting construct, pGEMΔ*bhuTUV*, was introduced into *B. abortus* strain 2308 by electroporation and transformants were selected on SBA supplemented with 45 μg/ml kanamycin. Putative *B. abortus bhuTUV* 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 prepared and oligonucleotides *bhuTUV*-1F and Kan R (Table 2.2) 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 705 bp in the middle of the operon (bhuTUV -2F and bhuTUV -2R). One confirmed *B. abortus bhuTUV* mutant was selected for further phenotypic evaluation and given the designation JFO2.

**Reconstruction of the *bhuTUV* locus in *B. abortus* JFO2.**

Reconstruction of the mutated *bhuTUV* genes in JFO2 was chosen as a strategy for verifying the link between genotype and the phenotype exhibited by this strain, rather than genetic complementation with a plasmid-borne *bhuTUV* operon. A 4309 bp fragment encompassing the *bhuTUV* operon from *B. abortus* strain 2308 genomic DNA was amplified using the primers *bhuTUV*-1F and *bhuTUV*-1R (Table 2.2) and cloned into pNPTS138Ap (Table 2.1), an ampicillin–resistant derivative of the *sacB*-containing ColE1-based pNPTS138 (Spratt, 1986). The resulting plasmid, designated pNPTS138*bhuTUV* was introduced into *B. abortus* JFO2 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 *bhuTUV* genes had been replaced by the parental *bhuTUV* genes. The genotype of the resulting *B. abortus* strain, designated JFO1RC was confirmed by PCR amplification and sequence analysis.

**Construction and genetic complementation of a *B. abortus rirA* mutant.**

A 757 bp region encompassing the *rirA* gene (BAB2_0678) was amplified from *B. abortus* 2308 genomic DNA by PCR (*rirA*F: 5'-CCGGCAGATCGCAGTAAATT-3', *rirA*R: 5'-CGCCGACTGTGTTACCTCAA-3') and cloned into pGEM®-T Easy (Promega) (Anderson 2006). The resulting plasmid (pEA19) was digested with SmaI/AscI releasing a 303 bp fragment internal to the *rirA* coding region. This linearized plasmid was then treated with the Klenow fragment of DNA polymerase I and ligated to a 987 bp SmaI/HincII fragment containing the chloramphenicol acetyltransferase (*cat*) gene from pBlueCM2 (Robertson 2000). This plasmid (pEA20) was used to construct a *rirA* mutant (designated BEA4) in *B. abortus* 2308 via gene replacement using previously described procedures (Elzer 1994). The genotype of the *B. abortus* BEA4 was confirmed by PCR analysis of BEA5 chromosomal DNA with *rirA*-, *cat*- and pGEM®-T Easy- specific primers.

A 775 bp fragment containing the *rirA* open reading frame was excised from pEA19 by EcoRI digestion and ligated into EcoRI-digested pMR10 (Gee 2005) to produce pEA21. The *rirA* containing EcoRI fragment was also inserted into pBBR1MCS4 (Kovach 1995) to produce pEA30. These plasmids were introduced independently into *B. abortus* BEA4 by electroporation (Elzer 1994).

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

*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 with the addition of 10 μM ethylenediamine-N,N'-bis(2-hydroxyphenylacetic acid) (EDDHA) 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 $FeCl_3$, 25 μM deferrated hemin (see below), or 25 μM deferrated hemoglobin (hemoglobin was deferrated by adding 20 μM desferoxamine, a siderophore that cannot be utilized by *Brucella*). 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% $CO_2$.

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

Free iron was removed from the hemin stock solutions used for the iron source utilization assays using the procedure described by Staggs and Perry (Staggs 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 phosphate-buffered saline (pH 7.2), and the optical density at 600 nm was adjusted to 0.15 (corresponding to $10^9$ CFU/ml). One hundred-microliter portions of these bacterial cell suspensions were then added to 500-ml flasks containing 100 ml low-iron minimal medium, and the flasks were incubated at 37°C with shaking at 165 rpm. Following 96 h of growth the optical densities at 600 nm of the bacterial cultures were adjusted to 0.15, and 100 μl portions of the bacterial cell suspensions were mixed with 4 ml 0.7% Noble agar (Difco). The mixtures were overlaid onto TSA plates containing 125 μM EDDHA. Seven-millimeter sterile filter paper (Whatman no. 3) disks were placed onto the plates, 30 μl of a 25 mM solution of hemin, or 20 μl of a 50 mM solution of $FeCl_3$ was added to the filter disks, and the plates were incubated for 96 h at 37°C with 5% $CO_2$. Following this incubation period, the diameter (in millimeters) of the zone of bacterial growth around each filter disk was measured and recorded.

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

Total cellular RNA was isolated from *B. abortus* 2308, BEA2 (2308 *irr*-) (Anderson 2011) and BEA4 (2308 *rirA*-) following growth in low-iron minimal medium and low-iron minimal medium supplemented with 50 µM $FeCl_3$ at 72 hours and 120 hours post inoculation using a previously described procedure (Caswell 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 (see Table 2.2). The differences in the levels of the *bhuT*- specific transcripts present under each condition 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.

**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

Efficient iron acquisition is critical for an invading microbe's survival and virulence. The erythrophagocytosis by the macrophage provides a considerable heme flux throughout the cell (Crichton 2002), making heme a biologically relevant source of iron for the brucellae (Roop 2011). *B. abortus* contains genes encoding several other iron acquisition pathways (Roop 2011), however, no other iron uptake system is able to obtain enough iron to compensate for the loss of the outer membrane heme transporter in *B. abortus* (Paulley 2007). Because heme uptake is linked to the pathogenesis of *Brucella abortus*, it was essential for us to identify the rest of the heme acquisition machinery. In most cases, the genes encoding heme uptake components are located together in the same chromosomal region; therefore the logical candidates for encoding the rest of the heme transporter are directly downstream from *bhuA* (BAB2_1148-1149) (Figure 2.1A). However, these genes, annotated as *nagC* and *tauC*, do not encode the conserved amino acid residues found to be required for cytoplasmic heme transport in *Shigella*, and real time PCR analysis of their transcripts (Figure 2.1C) showed a lack of iron-responsiveness that is usually observed with heme transporters. Further, a search of the *Brucella abortus* 2308 genome identified BAB2_0483-0485 as encoding much better candidates for cytoplasmic heme transport with high amino acid homology to ChuTUV of *Escherichia coli* as well as to the well-characterized ShuTUV transporter in *Shigella dysenteriae* (Torres 1997, Wyckoff 1998). In *Shigella*, the Tyr-94 of the ShuT periplasmic binding protein has been shown to be necessary for heme binding and correlates to the Tyr-89 of BhuT in *Brucella* (Eakanunkul 2005) (Figure 2.1D). ShuU contains two histidine residues required for binding of ShuT and subsequent release of the heme, and BhuU also contains these histidines (Burkhard 2008). BhuV includes the conserved features of an ATP – binding subunit. RT-PCR analysis using RNA from growth in

low iron conditions (Figure 2.1B) of this chromosomal region confirmed that *bhuT*, *bhuU,* and *bhuV* are all cotranscribed and since their open reading frames overlap each other by 4 base pairs, they are probably translationally coupled.

In order to determine whether or not *B. abortus* BhuTUV transports heme, an isogenic mutant was constructed (JFO2) and subjected to low iron conditions in liquid and on solid media. Growth in low iron miminal medium showed a limited growth defect for JFO2 in late stationary phase (data not shown). However, the addition of the chelator ethylenediamine-N,N'-bis(2-hydroxyphenylacetic acid) (EDDHA) to the low iron medium resulted in significant growth impairment of JFO2 compared to *B. abortus* (Figure 2.2A). This level of iron restriction is physiologically relevant because the brucellae require such low amounts of iron compared to most bacteria (Gerhardt 1950) as well as due to the severe iron restriction created by the host macrophage because of the active transport of iron out of the cell through ferroportin as well as the removal of divalent cations by Nramp1 from the phagosome where the brucellae resides (Cellier 2007, Byrd 1989, Nairz 2010). Importantly, growth of JFO2 can be restored with the addition of 50 µM $FeCl_3$ (Figure2. 2B), but not with the addition of 50 µM heme (Figure2. 2C) as well as by genetic reconstruction of the locus (JFO2RC). These data demonstrate that the *bhuTUV* mutant strain (JFO2) is unable to use heme as an iron source. To further confirm that JFO2 has a defect in its ability to use heme as an iron source, its growth was compared to both the parental *B. abortus* 2308 strain and HR1703 (2308 *bhuA*-) on chelated TSA plates with varying iron sources provided on a paper disc centered in the plates (Table 2.3). Under these conditions, both HR1703 and JFO2 are unable to grow when heme is provided as the sole source of iron, but all strains are able to grow using $FeCl_3$ as an iron source. These experimental

findings support the proposition that *bhuTUV* encodes the components that work in conjunction with BhuA for the transport of heme into *B. abortus* 2308.

To our knowledge, this is the first formal report that *Brucella* can use hemoglobin as an iron source, and the physiologic relevance of this within the macrophage niche is as yet unknown. Also, because hemoglobin can be used as an iron source for *B. abortus* 2308 but not for the JFO2 heme uptake mutant (Figure 2.2D), there must be a protein responsible for removal of the heme from the hemoglobin. Perhaps, like *Shigella*, it is the outer membrane heme transport protein itself that is performing this function (Cobessi 2010).

Due to the generation of toxic hydroxyl radicals through Fenton chemistry, iron and heme transport genes are typically regulated in an iron – responsive manner (Andrews 2003). Real time PCR analysis of the *bhuT* transcript under low iron conditions at 72 hours post inoculation (log phase) and 120 hours post inoculation (stationary phase) demonstrated both an iron-responsive component as well as a growth phase-dependent component to the regulation of this operon (Figure 2.3). The two main iron responsive regulators found in alpha proteobacteria are Irr and RirA (Hibbing 2011). Irr was recently found to bind to the promoter region of *bhuA*, and is required for its iron - responsive expression (Anderson 2011). In order to assess the roles of Irr and RirA in the iron responsive regulation of *bhuTUV*, the *bhuT* transcript was examined in iron deplete and replete conditions in *B. abortus* 2308, BEA2 (2308 *irr*-), and BEA4 (2308 *rirA*-). In contrast to the positive effect of Irr on the expression of *bhuA*, Irr appears to repress *bhuT* expression during log phase growth; however it does not play an observable role later during stationary phase of growth. RirA appears to have an inverse relationship, having no observable effect on *bhuT* transcript during log phase growth, but clearly playing a repressive role on *bhuT* during stationary phase.

The gene that encodes BhuA, the outer membrane heme transporter, is highly upregulated in response to iron deprivation, but there is only a modest iron-responsive increase in expression of *bhuT* by comparison (Paulley 2007, Anderson 2011). While both regulators repress *bhuT* expression significantly in response to iron deprivation, there are no conserved binding motifs for these regulators such as the Irr iron control element (ICE) found in *Bradyrhizobium* (Rudolph 2006) or the RirA iron-responsive operator (IRO) found in *Rhizobium* (Yeoman 2004) for them in the promoter region of *bhuTUV* and the authors believe that this may be an indirect regulatory relationship involving a third regulator that may exhibit both iron- and growth phase-responsive characteristics. In *Bordetella avium*, heme utilization is regulated by a heme-responsive extracytoplasmic sigma function (ECF) called RhuI (Kirby 2001). RhuI belongs to a subfamily of $\sigma$-$^{70}$ type proteins that are iron regulated (usually by the global iron regulator Fur) and turn on various iron acquisition genes as well as genes encoding adaptive proteins necessary for pathogenesis of the organism. *Brucella* encodes the ECF homologs RpoE1 and RpoE2 that should be examined for their potential role as this third regulator. Alternatively, ChrAS is a heme-sensing two component regulator found in *C. diphtheria* to activate transcription of both a heme export system and a heme oxygenase in the presence of heme (Schmitt 1999; Bibb 2010). We have some preliminary evidence that ChrA is activating *bhuTUV* and also *bhuA*, which would provide a feedback mechanism for the increased expression of *bhuA*, possibly allowing for full expression of the heme uptake machinery.

These experimental findings conclusively show that BhuTUV is the cytoplasmic membrane heme transporter that works in conjunction with BhuA. The original *bhuA* mutant was constructed using a chloramphenicol resistance cassette. Recently the CDC instituted new guidelines for this select agent that no longer allows chloramphenicol resistance to be introduced

into the animal models, and therefore a new *bhuA* kanamycin resistant mutant is currently under construction with the intent to use it as a tool for comparison with the *bhuTUV* mutant in the mouse model of infection. Since BhuA was shown to be required for the maintenance of chronic infection in the Balb/c mouse model, it will be important to determine what role, if any, BhuTUV plays in the virulence of *Brucella*.

To our knowledge, the separation of heme transport genes appears to be unique to *Brucella,* and this genetic organization is conserved in all of the published *Brucella* genomes. The fact that *bhuA* and *bhuTUV* show different patterns of iron-responsive regulation certainly suggest that there is an evolutionary benefit to having these in separate loci. This separation probably allows for fine-tuned regulation of the genes responsible for heme transport. Perhaps the observation of growth phases is misleading, and actually counterintuitive to that which is physiologically relevant. For instance, there are probably low iron requirements for the brucellae as they first enter the macrophage, making more of a stationary phase phenotype that requires the presence of RirA. In other alpha proteobacteria, the absence of RirA has been linked to an increase in sensitivity to hydrogen peroxide (Ngok-Ngam 2009), and the presence of this regulator during a time when the brucellae would encounter the oxidative burst of the macrophage would make sense. Under these conditions, RirA would repress both *bhuTUV* and siderophore biosynthesis genes, since the uptake of iron during oxidative stress could be detrimental to the brucellae.

As the brucellae travel within the phagosome toward the endoplasmic reticulum (ER), it would become more iron-starved, allowing for the presence of Irr. Irr would repress *rirA* expression, as well as *bhuTUV*, since there would be little if any heme to transport. However, Irr would turn on *bhuA* expression, and as soon as the brucella-containing phagosome fused with the

ER, BhuA would begin to take heme into the periplasm of the cell. Through low levels of *bhuTUV* expression, cellular iron levels would increase and Irr levels would decrease, allowing for greater transcription of *bhuTUV*, and ultimately bringing more heme iron into the cell. This use of heme as an iron source would allow for the *Brucella* replication seen in the ER niche, which more closely resembles the log phase of growth seen *in vitro* (Köhler 2003). This is at best a hypothesis, and current work in our lab is aimed at understanding this regulatory loop.

The experimental findings presented here give a more complete picture of the genes involved with heme transport in *B. abortus*. While we can only postulate what other sources of iron these bacteria come into contact with during the course of infection, it is clear that no other iron source can take the place of heme. The ability of the brucellae to utilize heme as an iron source *in vivo* is paramount to the continuance of chronic infection in the mouse model, a model which may provide relevant insights into the chronic brucellosis disease acquired by humans. Therefore strains defective in their ability to use heme as an iron source may present viable options for vaccine development in the future. Also, the heme utilization components may make good targets for the development of antimicrobials, eliminating the need for the current intensive antibiotic regimen for brucellosis.

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**Table 2.1. Bacterial strains used in this study.**

TABLE 2.1. 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* $\lambda^-$ | Invitrogen |
| *Brucella abortus* | | |
| 2308 | Virulent challenge strain | Laboratory stock |
| JFO2 | 705 bp gene deletion of *bhuTUV* (BAB2_0483-0485) with an *ahpA3* kanamycin resistance gene insertion | This study |
| JFO2RC | JFO2 with *bhuTUV* (BAB2_0483-0485) reconstructed onto the chromosome using pNPTS138 | This study |
| BEA2 | *Δirr* | Anderson 2011, this study |
| BEA4 | *ΔrirA* | This study |
| **Plasmids** | | |
| pGEM-T Easy | ColE1-based cloning vector; ampicillin resistance | Promega |
| pKS + Kan | 794-bp *aphA3* gene from Tn*phoA* cloned into SalI-HindIII-digested pBluescript II KS+ | Kovach, 1995 |
| pNPTS138 | *sacB*-containing counterselection vector; ampicillin resistance | Spratt, 1986 |

**Table 2.1**

**Table 2.2. Primers used in this study.**

TABLE 2.2. Primers used for this study

| | |
|---|---|
| *REV* | 5’ –AATGCATCAAATGGCAGGCA -3’ |
| *bhuTUV*-1F | 5’-GAAGATCTTCTCGTGATCTTGTCCGC-3’ |
| *bhuTUV*-1R | 5’-CGGTTTTGCGAGGCATC-3’ |
| *bhuTUV*-2F | 5’- CCGTAAAACGCAAGTTTCTGG-3’ |
| *bhuTUV*-2R | 5’- ATGAGGGGATCGTCTGGAGC-3’ |
| *amp* - F | 5’-TATTTCGTTCATCCATA-3’ |
| *amp* - R | 5’-GTTTTCCAATGATGAGC-3’ |
| *Kan* R | 5’-CCACTCCAGCATGAGAT-3’ |
| 16S Fwd | 5’-TCTCACGACACGAGCTGACG-3’ |
| 16S Rev | 5’-CGCAGAACCTTACCAGCCCT-3’ |
| *bhuT* Fwd | 5’-TGGGTCTCGGTGACAGGGTT-3’ |
| *bhuT* Rev | 5’-TCACATTCCTGATGCCGAGC-3’ |

**Table 2.2**

**Figure 2.1. Genetic organization of the heme transport genes in *B. abortus* 2308**. (A) *Brucella* heme transport genes are located in separate areas of the chromosome. BAB2_1148 and BAB2_1149 are predicted to encode cytoplasmic transporters but do not encode conserved heme transport motifs. The black arrows indicate which genes are cotranscribed in an operon. (B) Reverse transcription PCR of *bhuTUV* and surrounding genes shows that the ORFs designated *hyp, bhuT, bhuU,* and *bhuV* are cotranscribed. Lanes are marked for ladder (L) and #1-6 are the intragenic regions denoted by the arrows in part (A). (C) Real time PCR of BAB2_1148, BAB2_1149, *bhuT* and *bhuA* transcripts in low iron medium compared to low iron medium containing 50 μM $FeCl_3$ which shows the iron-responsive nature of *bhuA* and *bhuT*, but lack of iron-responsiveness of BAB2_1148 and BAB2_1149. (D) Amino acid alignments of BhuT and ShuT as well as BhuU and ShuU.

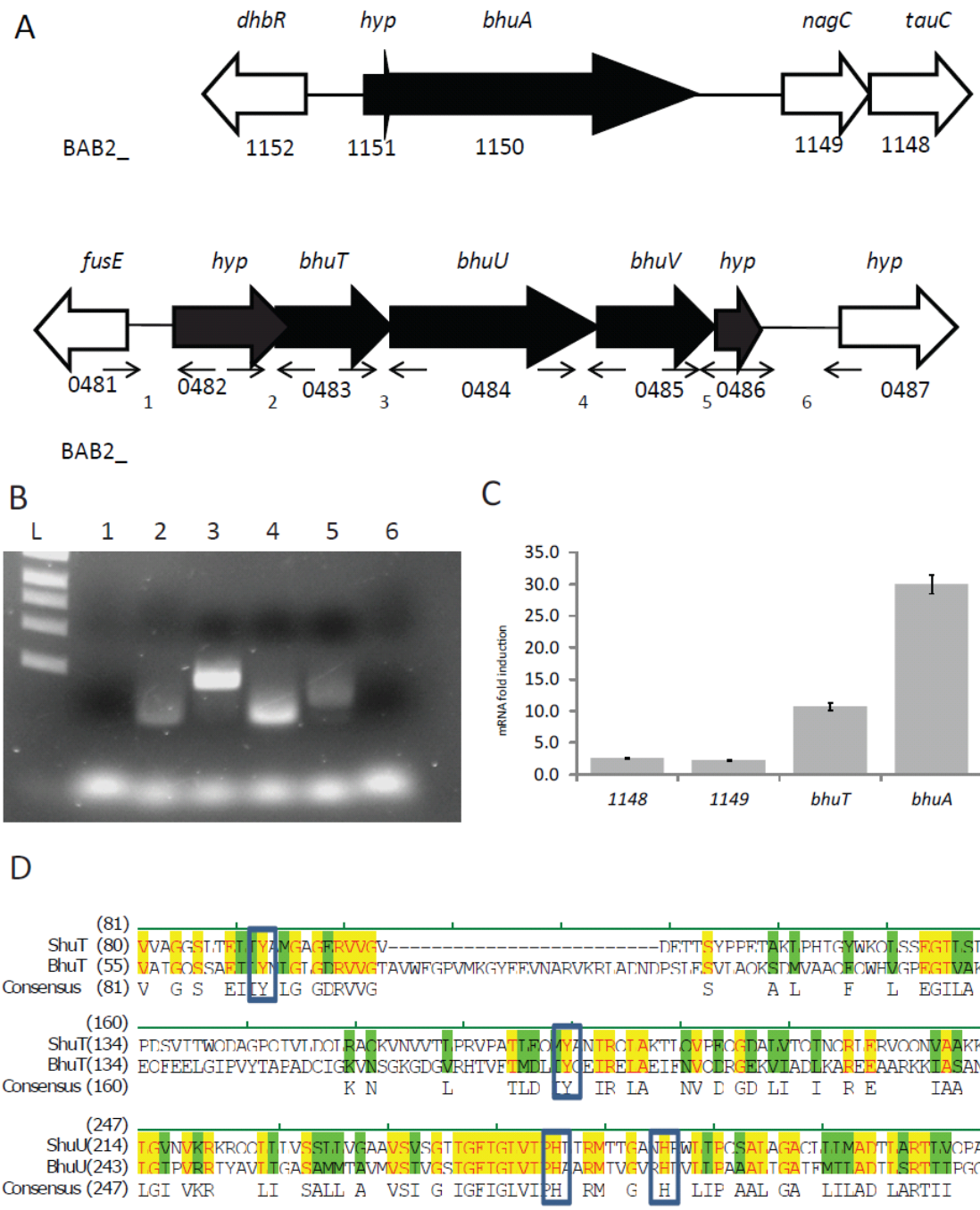
A
dhbR
hyp
bhuA
nagC
tauC
BAB2_
1152
1151
1150
1149
1148
fusE
hyp
bhuT
bhuU
bhuV
hyp
hyp
0481
0482
0483
0484
0485
0486
0487
1
2
3
4
5
6
BAB2_
B
L
1
2
3
4
5
6
C
mRNA fold induction
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
1148
1149
bhuT
bhuA
D
(81)
ShuT (80)
BhuT (55)
Consensus (81)
(160)
ShuT(134)
BhuT(134)
Consensus (160)
(247)
ShuU(214)
BhuU(243)
Consensus (247)

**Figure 2.1**

**Figure 2.2. The *bhuTUV* mutant exhibits a growth defect during iron deprivation that can be rescued by $FeCl_3$, but not heme or hemoglobin**. Growth of *B. abortus* 2308 (triangles), *B. abortus* JFO2 (2308 *bhuTUV)* (squares and dashed lines), and JFO2RC (JFO2 *bhuTUV*+) (circles) in (A) low iron minimal medium, (B) low iron minimal medium containing 50 µM $FeCl_3$, (C) low iron minimal medium containing 25 µM hemin, and (D) low iron minimal medium containing 25 µM hemoglobin. The data presented are from one experiment, but representative of multiple experiments (>3) from which similar trends were observed. ** = $P <$ 0.001 for comparisons of the data obtained for these strains in the Student's two-tailed t-test (Rosner, 2000).

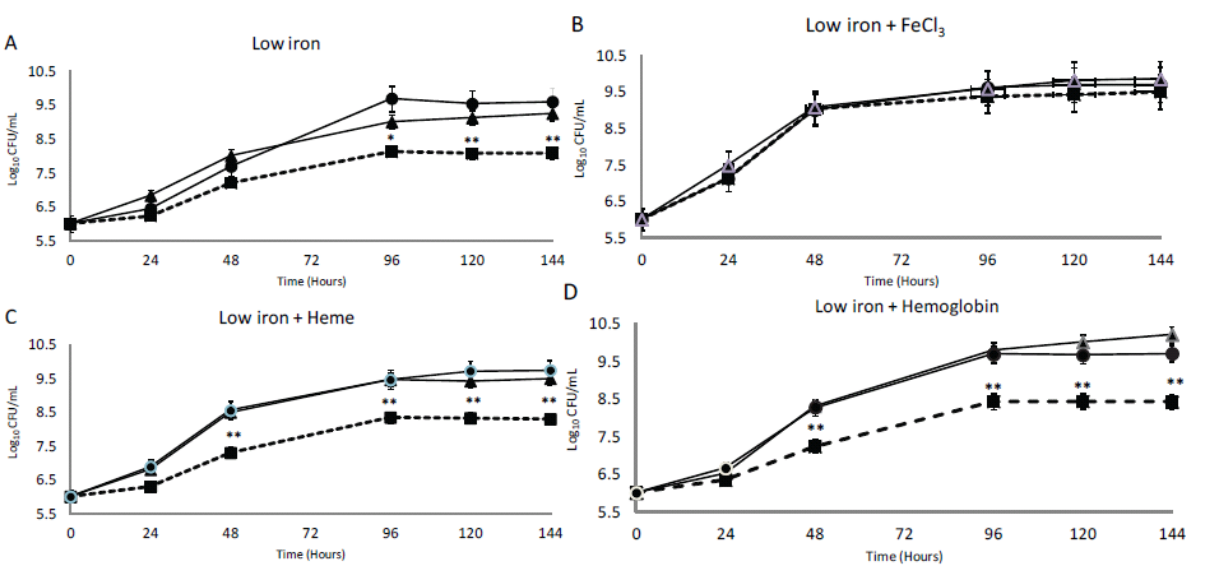
A
Low iron
B
Low iron + FeCl3
C
Low iron + Heme
D
Low iron + Hemoglobin
Log10 CFU/mL
Time (Hours)
10.5
9.5
8.5
7.5
6.5
5.5
0
24
48
72
96
120
144
*
**

**Figure 2.2**

**Table 2.3 Capacity of the *bhuTUV* mutant to use heme and $FeCl_3$ as iron sources in a solid medium-based assay.** Strains were spread onto chelated (125 µM EDDHA) tryptic soy agar plates following 72 h growth in low iron minimal medium. Either deferrated hemin or ferric chloride was added to paper discs and growth around the disks was measured in millimeters (mm). The data presented is representative of multiple experiments (>3) from which similar trends were observed.

| Strain | Heme (mm) | $FeCl_3$ (mm) |
|---|---|---|
| *B. abortus* 2308 | 25.5 | 44.3 |
| *bhuA* | 0 | 25 |
| *bhuTUV* | 0 | 45 |
| *bhuTUV* – RC | 26 | 44.7 |

**Table 2.3**

**Figure 2.3 *bhuT* transcription is iron-responsive in *B. abortus* 2308 and regulated by both Irr and RirA.** The fold induction presented on the Y- axis represents the difference between the levels of *bhuT* transcripts detected by real-time RT-PCR in RNA preparations from cultures after 72 h and 120 h of growth in low-iron minimal medium compared to low iron medium plus 50 µM $FeCl_3$ in *B. abortus* 2308 (black), BEA4 (*rirA*-) (grey), and BEA2 (*irr*-) (light grey). Both transcripts were normalized against the 16S ribosomal RNA housekeeping gene, whose expression is not altered by the conditions.

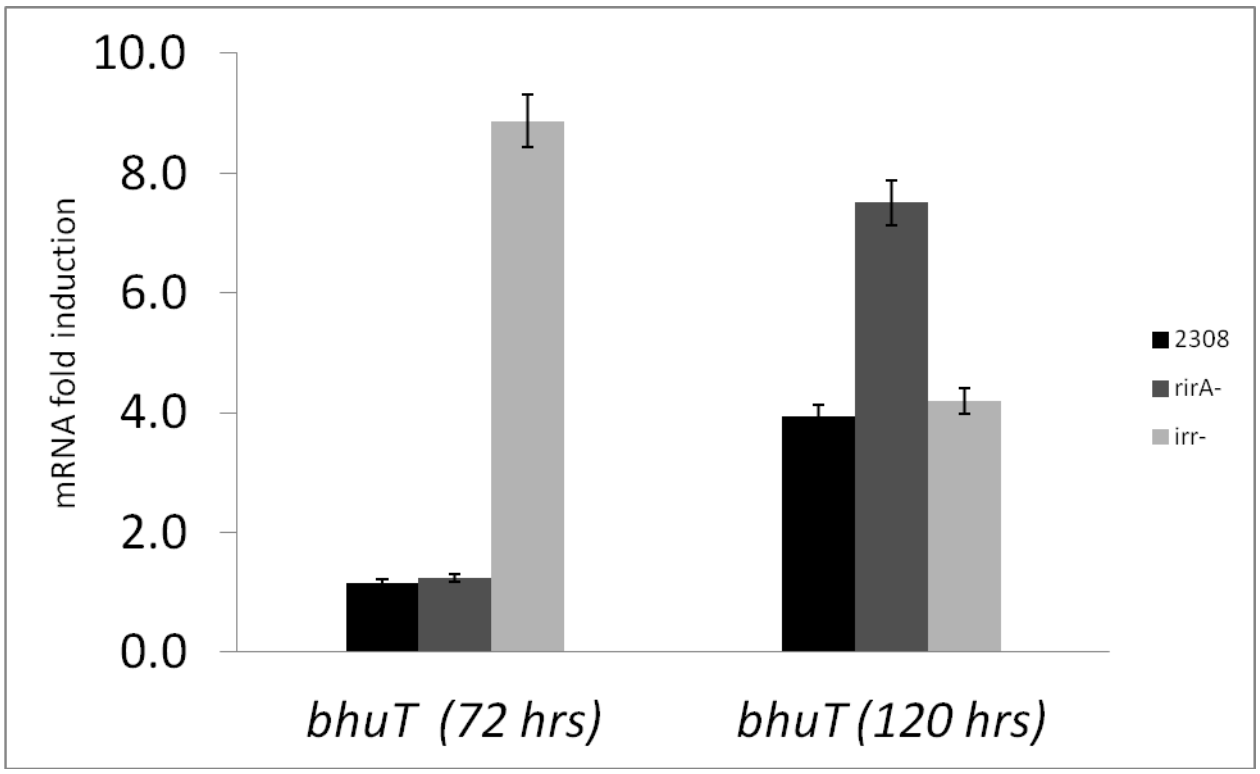
10.0
8.0
6.0
4.0
2.0
0.0
mRNA fold induction
2308
rirA-
irr-
bhuT (72 hrs)
bhuT (120 hrs)

**Figure 2.3**

# Chapter 3: The *bhuO* 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

## Abstract

The *Brucella* BhuO 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 *bhuO* mutant was constructed and its phenotype evaluated. Although the *B. abortus bhuO* 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 *bhuO* mutation into the *B. abortus dhbC* mutant BHB2 (which cannot produce siderophores) resulted in a severe growth defect in the *dhbC bhuO* 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 *bhuO* 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 BhuO to allow this strain to efficiently use heme as an iron source. The genetic organization of the *rirA-bhuO* operon also provides the basis for the proposition that BhuO 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 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 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 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 2000; Ratliff 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 2006). Based on its documented biochemical activity, we have given this protein the designation BhuO (*Brucella* heme utilization oxygenase). 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 1950) and low iron minimal medium (López-Goñi 1992) were prepared as previously described.

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

Polymerase chain reaction (PCR) utilizing Taq polymerase (Invitrogen) with the oligonucleotide primers *bhuO*-1F and *bhuO*-1R (Table 3.2) was used to amplify a 1605 bp fragment encompassing the *bhuO* 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 *bhuO*-2F and *bhuO*-2R (Table 3.2) was then employed to generate a blunt-ended linear fragment from which 70 bp internal to the *bhuO* coding region had been removed. This fragment was ligated with a 1345 bp fragment containing the *aph3a* gene from pKS-Kn (Kovach 1995). The resulting construct, pGEMΔ*bhuO*, was introduced into *B. abortus* strain 2308 by electroporation and transformants were selected on SBA supplemented with 45 μg/ml kanamycin. Putative *B. abortus bhuO*

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 *bhuO* 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 *bhuO (bhuO* F1 and *bhuO* R1). One confirmed *B. abortus bhuO* 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 *bhuO* mutation into *B. abortus* BHB2 (Bellaire 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 (López-Goñi 1992) or brucebactin (Gońzalez-Carrero 2002). The *B. abortus dhbC bhuO* double mutant constructed in this fashion was given the designation JFO1.

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

Because the *bhuO* gene is the terminal gene in an operon and lies downstream of a transcriptional regulator (Figure 3.4), reconstruction of the mutated *bhuO* 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 *bhuO* gene. A 920 bp fragment encompassing the *bhuO* gene from *B. abortus* strain 2308 genomic DNA was amplified by PCR using the primers *bhuO*-3F and *bhuO*-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 1986). The resulting plasmid, designated pNPTS138*bhuO* was introduced into *B. abortus* DCO1 and JFO1 by electroporation, and a previously described *sacB*-based counterselection strategy (Bellaire 2003) was used to select for derivatives of these mutants in which the mutated *bhuO* genes had been replaced by the parental *bhuO* 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 (López-Goñi 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 (Staggs 1991). 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 *bhuO* transcript levels using real-time RT-PCR.**

*B. abortus* 2308 and BEA2 (2308 *irr*) (Anderson 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 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 *bhuO* (see Table 3.2). The differences in the levels of the *bhuO*-, *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*, *bhuO*, 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 medium. Primers that span the intragenic regions between *ybaK* (BAB2_0679), *rirA* (BAB2_0678), *bhuO* (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*-*bhuO* operon.**

The *bhuO* 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 2011; Menscher 2012). Briefly, the oligonucleotide primers *rirA* F and *rirA* R (Table

3.2) were individually labeled with [$\gamma$-$^{32}$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 2012). Gels were dried under vacuum dryer 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* BhuO 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 2006) and the IsdI and IsdG proteins from *Staphylococcus aureus* (Skaar 2004). Moreover, BhuO contains the conserved Asn 7, Trp 67 and His 77 residues shown experimentally to be important for the heme oxygenase activity of IsdG (Wu 2005) (Figure 3.1), and BhuO has been shown to bind and degrade heme *in vitro* (Puri 2006). Despite the documuented heme oxygenase activity of BhuO, a *bhuO* mutant (DCO1) constructed from *B. abortus* 2308 exhibited a comparable growth pattern in low iron minimal medium (López-Goñi 1992) and an equivalent resistance to the iron specific chlelator ethylenediamine-N,N'-bis(2-hydroxyphenylacetic acid) (EDDHA) (Rogers 1973) 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 (Paulley 2007) (data not shown).

A distinctive characteristic of the *B. abortus bhuO* mutant that was noticed during its phenotypic characterization, however, was that this mutant consistently and reproducibly produced more siderophore during growth in low iron minimal medium than the parental 2308 strain (Figure 3.2A). This increase in siderophore production was accompanied by a corresponding increase in transcription of *dhbC* in the *bhuO* mutant in comparison to the parental strain (2308) following growth under iron limiting conditions (Figure 3.2B). This phenotype suggests that the *B. abortus bhuO* mutant is experiencing a greater degree of iron deprivation in the low iron minimal medium than 2308, and in turn increases its siderophore production to compensate. Enhanced siderophore production could explain why the *B. abortus bhuO* 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 BhuO and allow the *bhuO* 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 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 bhuO* mutant exhibits increased siderophore production in response to iron deprivation compared to the parental strain provides a potential experimental avenue for assessing the role of BhuO in iron metabolism. Specifically, if the loss of BhuO 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 bhuO* double mutant) would be expected to show an enhanced iron deprivation phenotype compared to *B. abortus* 2308 or the *bhuO* mutant when grown under iron limiting conditions. This is in fact the relationship that was observed. As shown in Figure 3.3A, the *dhbC bhuO* 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 bhuO* 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. To verify the link between the *bhuO* mutations in *B. abortus* DCO1 and JFO1 and the phenotypes exhibited by these strains, a *sacB*-based counterselection strategy (Bellaire 2003) was used to reconstruct the *bhuO* 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 BhuO 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 bhuO* 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 *bhuO* is the last gene in an operon transcribed as *rirA* (BAB2_0678) - *bhuO* (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 2002; Chao 2005; Ngok-Ngam 2009; Hibbing 2011). A predicted iron control element (ICE) motif is located in the (-10) region of the promoter of the *rirA-bhuO* operon (Figure 3.4), suggesting that the iron response regulator Irr regulates the expression of these genes in response to cellular iron levels (Martínez 2006). In fact, when the expression patterns of the *rirA* and *bhuO* 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 2011). Rodionov *et al* predicted a conserved ICE motif in the promoter region of *rirA* (underlined below) (Rodionov 2006). To investigate this possible direct interaction, DNase footprint analysis was used and indicated that Irr directly binds to the *rirA* promoter (Figure 3.6), protecting a 28 nucleotide sequence, 5'-CATATA<u>TTTTAAGAATGATTCTAAAG</u>TG-3'.

Genetic studies suggest that RirA functions as an iron-responsive repressor of iron acquisition genes in the α–proteobacteria (Todd 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 *bhuO* 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 *bhuO* and *rirA* in *B. abortus* 2308 is that heme oxygenases such as BhuO may have a secondary function in this strain. Specifically, it is conceivable that the release of iron from heme catalyzed by these enzymes allows RirA to recognize the utilization of heme 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 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 *bhuO* 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 BhuO 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 its toxicity in *Bartonella* (Minnick 2009). In *Brucella*, these proteins are known as the Omp25/31 family of proteins (Cloeckaert 1996). Heme export systems have also been proposed as a means by which bacteria protect themselves from heme toxicity (Anzaldi 2010). Although a heme exporter has not been identified in *Brucella* strains, genes that potentially encode orthologs of proteins linked to porphyrin (Tatsumi 2008) and heme (Rasmussen 2005) export in other bacteria can be found in the genome sequence of *B. abortus* 2308.

In order to fully understand the role of BhuO 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 2007). Brucellosis in humans is notoriously difficult to treat, requiring a combination of antibiotics for a prolonged period (Ariza 2007). Hence, the development of improved chemotherapeutic regimens for treating this disease would of great benefit to the medical community.

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**Table 3.1 Bacterial strains used in this study.**

TABLE 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 *bhuO* (BAB2_0677) with an *ahpA3* kanamycin resistance gene insertion | This study |
| DCO1RC | DCO1 with *bhuO* (BAB2_0677) reconstructed onto the chromosome using pNPTS138 | This study |
| BHB2 | Nonpolar, in frame deletion of *dhbC* | Bellaire 1999 |
| JFO1 | *ΔdhbC/ΔbhuO* | This study |
| JFO1RC | *ΔdhbC/ΔbhuO* with *bhuO* (BAB2_0677) reconstructed onto the chromosome using pNPTS138 | This study |
| BEA2 | *Δ*irr | Anderson 2011 |
| **Plasmids** | | |
| pGEM-T Easy | ColE1-based cloning vector; ampicillin resistance | Promega |
| pKS + Kan | 794-bp *aphA3* gene from Tn*phoA* cloned into SalI-HindIII-digested pBluescript II KS+ | Kovach 1995 |
| pNPTS138 | *sacB*-containing counterselection vector; ampicillin resistance | Spratt 1986 |

**Table 3.1**

**Table 3.2 Primers used in this study.**

| Primers used in this study | |
|---|---|
| *bhuO*-1F | 5’-GACATTTCGCTGAAGACATA -3’ |
| *bhuO*-1R | 5’-GAGCTTACTTTACCGTTGGC-3’ |
| *bhuO*-2F | 5’-TCTATGCATCCCACACAGTC-3’ |
| *bhuO*-2R | 5’-GAGCTGGGAGTCACGGTTCT-3’ |
| *amp* - F | 5’-TATTTCGTTCATCCATA-3’ |
| *amp* - R | 5’-GTTTTCCAATGATGAGC-3’ |
| *Kan* R | 5’-CCACTCCAGCATGAGAT-3’ |
| *bhuO*-3F | 5’-ACTGGATCCCGGAGCTGTTTCTATTC-3’ |
| *bhuO*-3F | 5’-ACTGTCGACTTCTAAATATGAGTTAA-3’ |
| 16S Fwd | 5’-TCTCACGACACGAGCTGACG-3’ |
| 16S Rev | 5’-CGCAGAACCTTACCAGCCCT-3’ |
| *dhbC* Fwd | 5’-GTGCCAAGCTTGGTCTGTACTTC-3’ |
| *dhbC* Rev | 5’-CGTGGATTGTTTACCGGC-3’ |
| *bhuO* Fwd | 5’-CGGTAGCGAAACCGATTTTGAGAC-3’ |
| *bhuO* 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/bhuO F | 5’-GGATAGCTGTGGCCTGAATG-3’ |
| rirA/bhuO R | 5’-GAGCTGGGAGTCACGGTTCT-3’ |
| bhuO/bfr F | 5’-GCTTCCGTATCGTCACTTTT-3’ |
| bhuO/bfr R | 5’-GAGCTTACTTTACCGTTGGC-3’ |
| *rirA* F | 5’-GCACCGCAGAAAATGGCCGA-3’ |
| *rirA* R | 5’-TGGAACATTCCCGAACCGGA-3’ |

**Table 3.2**

**Figure 3.1. The *B. abortus* BhuO protein shares amino acid homology with HmuD/Q and IsdG/I**. The *Brucella abortus* (Ba) BhuO 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 2004).

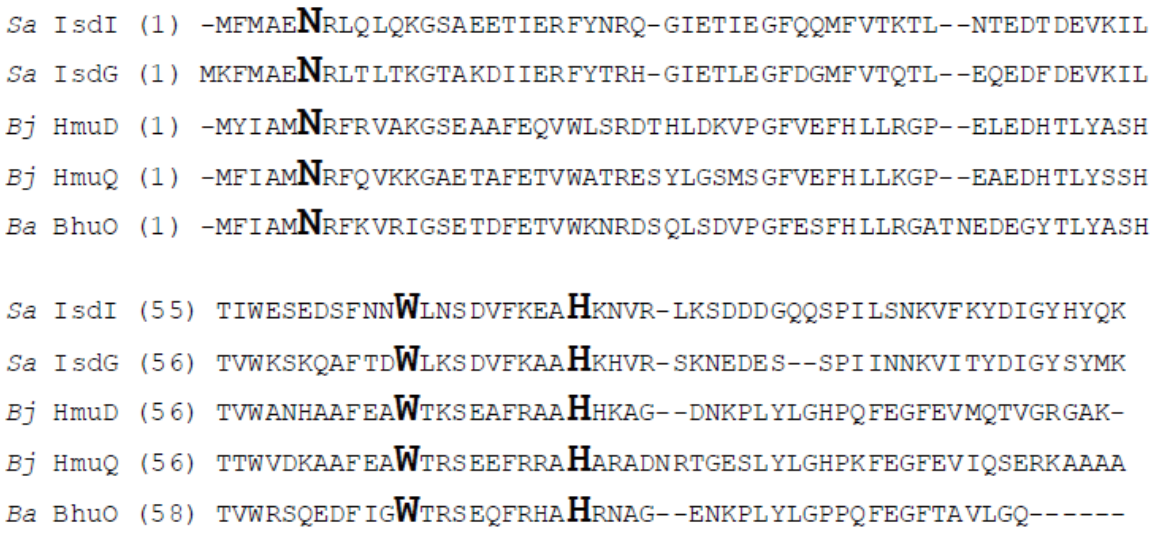
Sa IsdI (1) -MFMAENRLQLQKGSAEETIERFYNRQ-GIETIEGFQQMFVTKTL--NTEDTDEVKIL
Sa IsdG (1) MKFMAENRLTLTKGTAKDIIERFYTRH-GIETLEGFDGMFVTQTL--EQEDFDEVKIL
Bj HmuD (1) -MYIAMNRFRVAKGSEAAFEQVWLSRDTHLDKVPGFVEFHLLRGP--ELEDHTLYASH
Bj HmuQ (1) -MFIAMNRFQVKKGAETAFETVWATRESYLGSMSGFVEFHLLKGP--EAEDHTLYSSH
Ba BhuO (1) -MFIAMNRFKVRIGSETDFETVWKNRDSQLSDVPGFESFHLLRGATNEDEGYTLYASH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

**Figure 3.1**

**Figure 3.2. *B. abortus* DCO1 produces significantly more siderophore than *B. abortus* 2308 in response to iron deprivation.** (A) Siderophore production by *B. abortus* 2308, DCO1 (Δ*bhuO*) and DCO1RC (DCO1 *bhuO*$^{+}$) following 72 h growth in low iron minimal medium. The values on the Y axis represent the levels of catechol siderophore detected by the 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 *bhuO*). '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 $FeCl_3$.

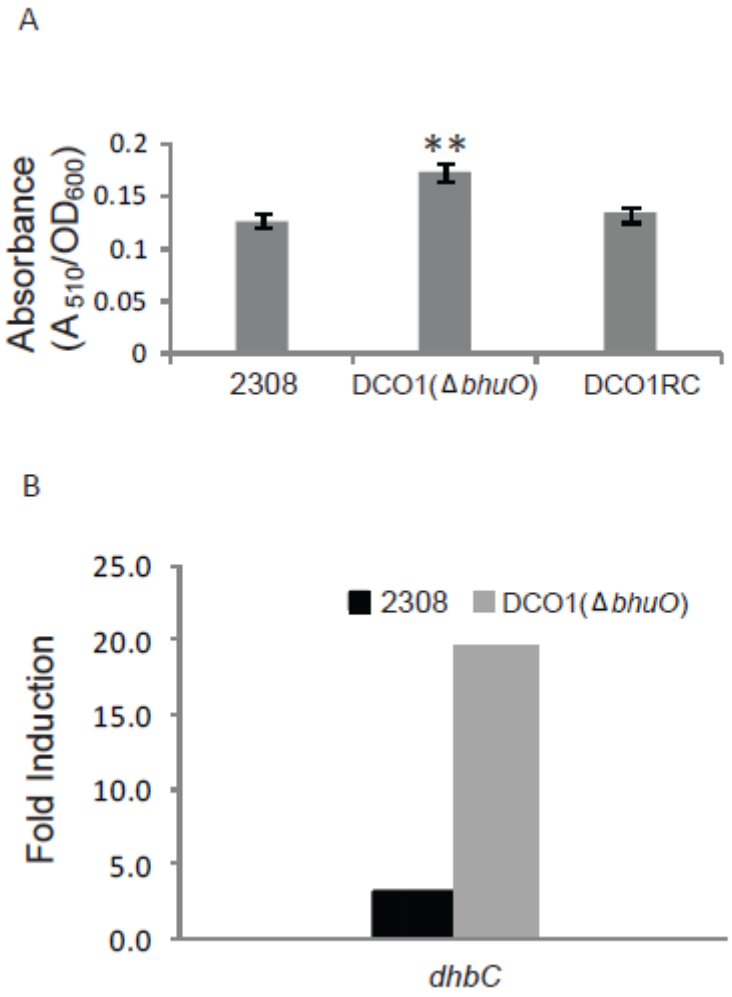
A
Absorbance ($A_{510}/OD_{600}$)
0.2
0.15
0.1
0.05
0
**
2308
DCO1(Δ*bhuO*)
DCO1RC
B
Fold Induction
25.0
20.0
15.0
10.0
5.0
0.0
2308
DCO1(Δ*bhuO*)
*dhbC*

**Figure 3.2**

**Figure 3.3. *B. abortus* JFO1 has a growth defect in the presence of heme as the sole iron source**. Growth of *B. abortus* DCO1 (2308 Δ*bhuO)* (squares), BHB2 (2308 Δ*dhbC)* (circles), JFO1 (2308 Δ*dhbC/*Δ*bhuO)* (triangles), and JFO1RC (JFO1 *bhuO*+) (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. 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).

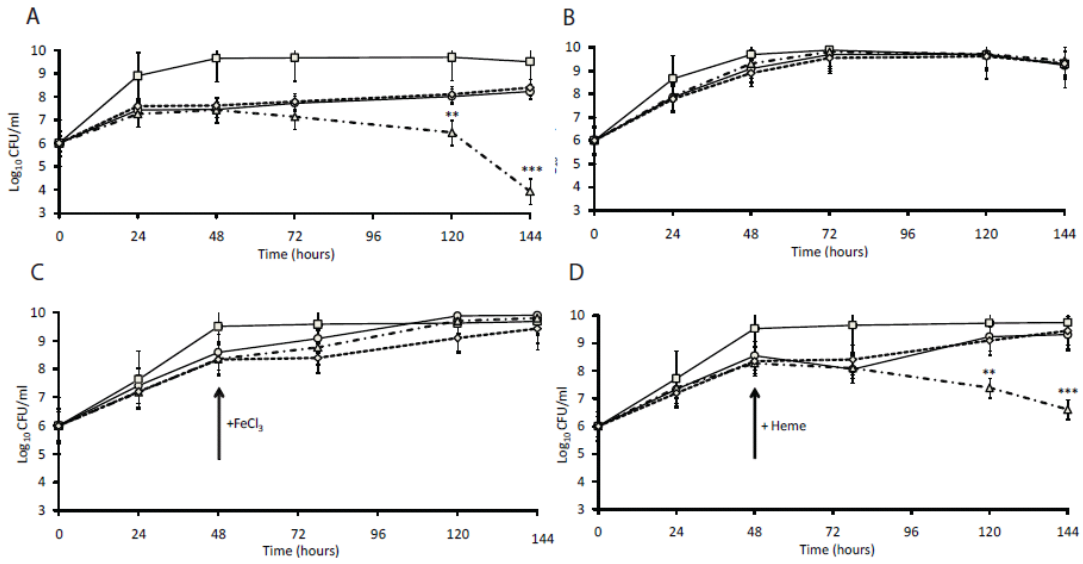


**Figure 3.3**

**Figure 3.4**. ***rirA* and *bhuO* are cotranscribed as an operon in *B. abortus* 2308.** The start site (+1) for the *rir-bhuO* transcript is located 134 base pairs upstream from 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 2004), 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.

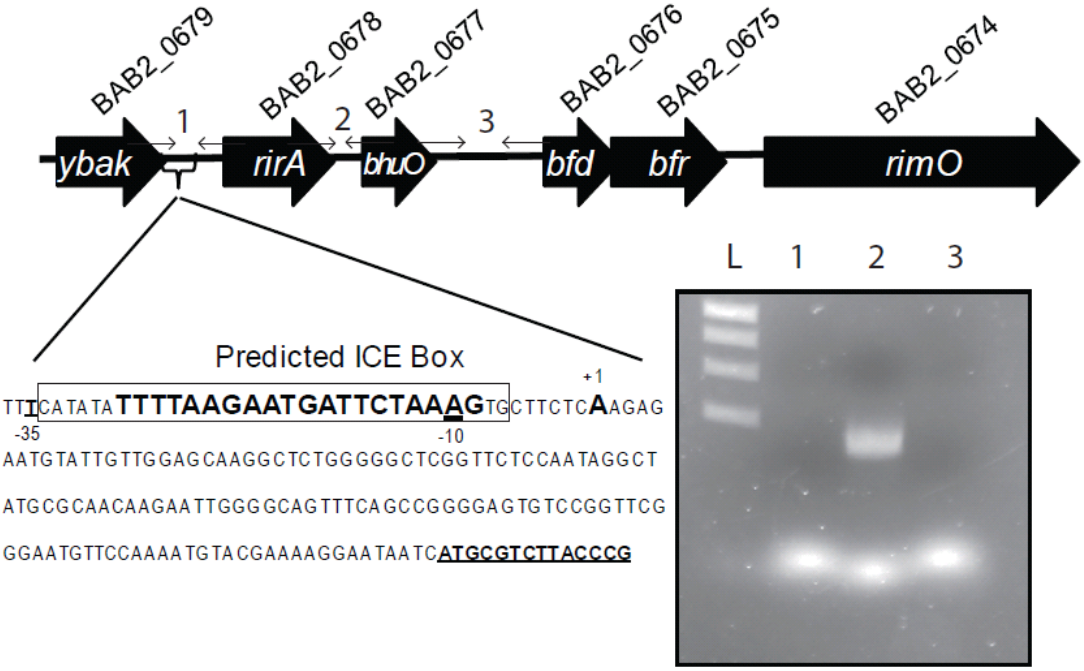
BAB2_0679
BAB2_0678
BAB2_0677
BAB2_0676
BAB2_0675
BAB2_0674
1
2
3
ybak
rirA
bhuO
bfd
bfr
rimO
L
1
2
3
Predicted ICE Box
+1
TTTCATATATTTTAAGAATGATTCTAAAGTGCTTCTCAAGAG
-35
-10
AATGTATTGTTGGAGCAAGGCTCTGGGGGCTCGGTTCTCCAATAGGCT
ATGCGCAACAAGAATTGGGGCAGTTTCAGCCGGGGAGTGTCCGGTTCG
GGAATGTTCCAAAATGTACGAAAAGGAATAATCATGCGTCTTACCCG

**Figure 3.4**

**Figure 3.5. *rirA* and *bhuO* transcription is increased in response to iron deprivation in *B. abortus* BEA2(Δ*irr*).** 'Fold induction' on the Y axis represents the difference between the levels of *rirA, bhuO* 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 2011).

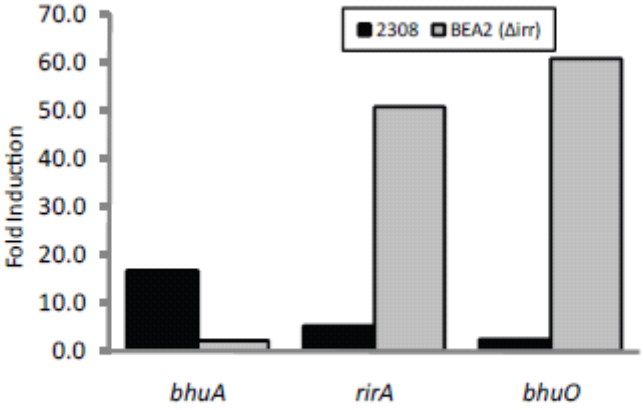
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Fold Induction
2308
BEA2 (Δirr)
bhuA
rirA
bhuO

**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.

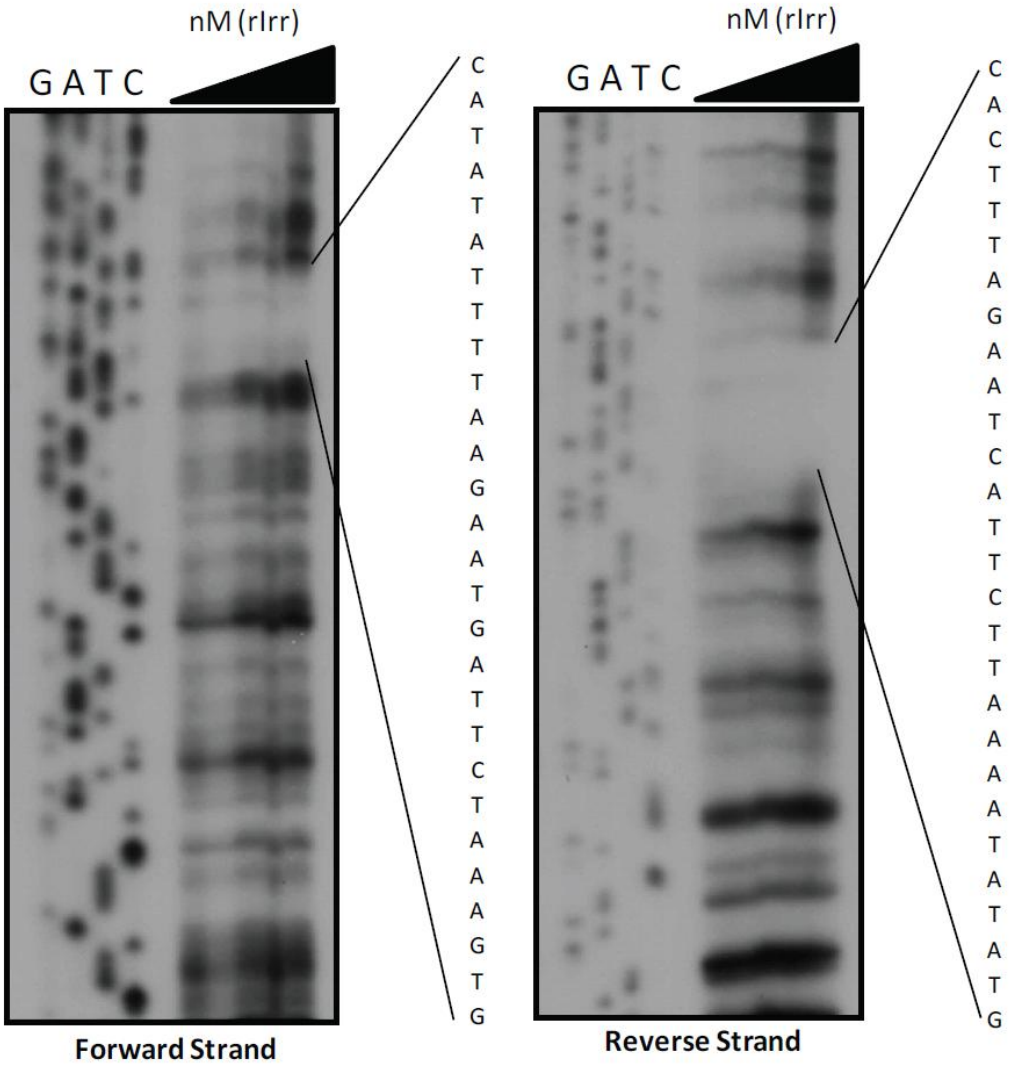
nM (rIrr)
G A T C
C A T A T A T T T T T A A G A A T G A T T C T A A A G T G
Forward Strand
nM (rIrr)
G A T C
C A C T T T T A G A A T C A T T C T T T A A A A T A T A T G
Reverse Strand

**Figure 3.6.**

**Figure 3.7. Proposed model for the role of BhuO in allowing the transcriptional regulator RirA to recognize heme as an iron source in *Brucella*.**

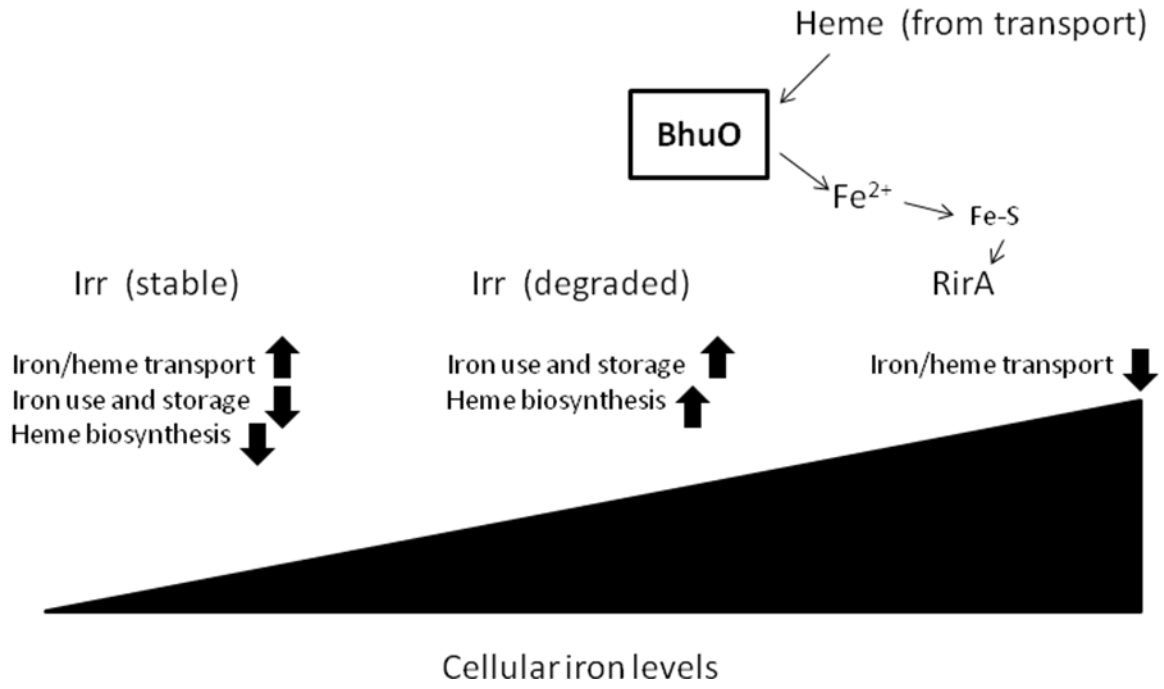
Heme (from transport)
BhuO
Fe2+
Fe-S
RirA
Irr (stable)
Irr (degraded)
Iron/heme transport
Iron use and storage
Heme biosynthesis
Iron use and storage
Heme biosynthesis
Iron/heme transport
Cellular iron levels

**Figure 3.7**

# Chapter 4: Summary and Future Directions

## The requirement for heme in *Brucella*

The virulence of *Brucella abortus* within the mouse model of infection is dependent on the ability of the brucellae to take in heme during their residence within the host macrophage. Many pathogens have heme transporters, but only a few have been shown to be important for virulence. To date, only *Bordetella pertussis*, enterohaemorrhagic *Escherichia coli*, *Listeria monocytogenes*, and the heme auxotroph *Haemophilus influenzae* contain heme transport systems that contribute significantly to virulence of the organism (Morton 2007, Torres 1997, Brickman 2007, Jin 2006). The data collected on BhuA and BhuTUV has demonstrated that without one or more of these components, the cell is unable to take in heme as an iron source, therefore this is the only heme transport system in *B. abortus* 2308. The original *bhuA* mutant was constructed using a chloramphenicol resistance cassette. Recently the CDC instituted new guidelines for this select agent that no longer allows chloramphenicol resistance to be introduced into the animal models, and therefore a new *bhuA* kanamycin resistant mutant is currently under construction with the intent to use it as a tool for comparison with the *bhuTUV* mutant in the mouse model of infection. While it seems likely that the absence of *bhuTUV* in the brucellae will produce a similar phenotype, experiments will need to be done to confirm this link between BhuTUV and virulence.

**Unknown heme transport components**

Encoded within the *bhuTUV* region, and clearly sharing the same operon (see Figure 2.1) are two hypothetical open reading frames (ORFs). The first hypothetical ORF is upstream, overlaps *bhuT* by 44 base pairs and encodes a protein that contains no known homology to any other protein. The second hypothetical ORF is located at the 3' end of *bhuV* and also lacks homology to any other known protein. The reason they are mentioned here is because it is not unusual for small hypothetical ORFs to actually encode proteins of unknown function within heme uptake operons, and it is possible that these two hypothetical ORFs do in fact serve a purpose for heme uptake. One component that is clearly absent in the *Brucella* heme uptake model (see Figure 4.1) is a shuttle to accept the heme once it is transported through BhuU. In *Shigella*, ShuS serves this function, chaperoning the heme to the heme oxygenase, and studies with reconstituted membranes have shown ShuS to be required for the heme to be released from the cytoplasmic permease (Burkhard 2008). The shuttle, HemS, from *Yersinia entercolitica* has been crystallized and the His-196 is required for functionality of the protein. This histidine is highly conserved across the known shuttles (Wilks 2011). While there has been no such protein found in *Brucella*, it is not unheard of for these proteins to only look similar in their native conformations, which cannot be searched for in a traditional BLAST search. This hypothetical protein is only 138 amino acids long, and therefore cannot have have a His – 196, however it does have two conserved H-X-X-H domains that are also known heme binding motifs (Matias 2002).

**The complexity of regulation of the *B. abortus* heme transport genes**

Of particular interest is the unique placement of the genes encoding *Brucella*'s heme transport machinery on different parts of chromosome II (see Figure 4.2). It is possible that this distribution of genes is the result of evolutionary inheritance or perhaps lateral gene transfer of the heme transport machinery in parts from other bacteria. While the brucellae do spend most of their time isolated within the host macrophage, they are considered facultative intracellular organisms because they can survive in the environment between infected animals. Evolutionarily, some argue that the second chromosome (which incidently is the one with the heme uptake genes) is auxiliary, and that it contains a region of homology with that of *Bartonella* species, suggesting a common ancestor for the "auxiliary replicon" (Batut 2004) .

Obviously there is an evolutionary benefit to having these in separate loci because *bhuA* and *bhuTUV* show different patterns of iron-responsive regulation. While we know that iron metabolism genes must be carefully regulated in order to maintain a safe homeostasis, perhaps in *Brucella* this seemingly complex web of regulation hints at the importance of the interactions of the brucellae with heme in the host. For instance, RirA has been shown to be protective against cellular oxidative stress, and an isogenic mutant *rirA* is more sensitive to hydrogen peroxide than *B. abortus* 2308 (unpublished laboratory observation). Is it possible then that RirA is meant not only to protect the brucellae against the oxidative burst of the macrophage as it first enters the cell but also to repress iron uptake genes at a time when the detrimental effects of the Fenton chemistry due to the combination of iron and oxygen would be most likely?

The interplay of Irr and RirA hints at two different cellular conditions that the brucellae are exposed to *in vivo*, one that mimics log phase of growth and another that mimics stationary

growth phase, with an active iron-responsive regulator under each condition. The more static phase would involve entry into the cell in a BCV with trafficking to the ER, and the log phase would be seen in the replicative niche of the ER. In a previous study, *bhuA* was shown to be stationary phase – dependent in its expression, and in this report *bhuTUV* has also shown a similar regulatory link. However, while *bhuA* requires Irr for transcription, *bhuTUV* is transcribed in much higher levels without Irr. This effect of RirA and Irr on *bhuTUV* expression is probably indirect, but what is directly regulating these genes? One possible explanation is shown in Figure 4.4. Under high iron conditions in the cell, the presence of RirA would repress iron uptake genes (such as siderophore). Because of the availability of iron, heme biosynthesis genes would be expressed, causing the heme levels in the cell to increase. Encoded within the upstream hypothetical ORF in the *bhuTUV* locus is a conserved cobalamin riboswitch motif, with 233 base pairs of conserved mRNA structure (Vitreschak 2003). Cobalamin, better known as vitamin B12, shares the first steps of its biosynthetic pathway with that of heme, and in fact has a similar final structure to heme (see Figure 4.3). It is possible that under these conditions, heme binds directly to this riboswitch (Lester 2011) and that the altered mRNA conformation blocks transcription of the *bhuTUV* operon. However, under low iron (and low heme) physiological conditions, the Irr protein would be stable, both repressing heme biosynthesis in the absence of iron and activating expression of *bhuA*, the outer membrane heme transporter. In order to establish a testable model, it is necessary to invoke the presence of a third regulator, ChrAS. As mentioned in the Literature Review, ChrAS is a heme-sensing two component regulator found in *C. diphtheria* to activate transcription of both a heme export system and a heme oxygenase in the presence of heme (Schmitt 1999; Bibb 2010). In our model, under low iron conditions, BhuA would transport heme into the periplasm of the cell. The ChrS protein

embedded in the cytoplasmic membrane would sense the heme and phosphorylate ChrA. In turn, ChrA would activate expression of *bhuTUV*, allowing the heme to be transported from the periplasm into the cell (see Figure 4.4). We have some preliminary evidence that ChrA is activating *bhuTUV* and also *bhuA*, which would provide a feedback mechanism for the increased expression of *bhuA*, possibly allowing for full expression of the heme uptake machinery.

Alternatively, in Chapter 2, the possibility of an uncharacterized regulator is mentioned. This extracytoplasmic function sigma factor is an iron-responsive $\sigma^{70}$ protein regulator, and there are two such putative proteins in *Brucella* (RpoE1 and 2). The paradigm for iron-dependent sigma factors is FecI from *E. coli* which depends on the outer membrane ferric dicitrate transporter binding to ferric dicitrate to induce a signal to a plasma membrane protein (FecR) which then activates the cytoplasmic FecI to promote transcription of the ferric dicitrate operon (Kirby 2001). In fact, in *Bordetella* the FecIR homologs directly regulate expression of the *bhuRSTUV* genes that encode the inner membrane heme transporter components (King 2005). In *Brucella*, Irr activates the expression of *bhuA*, and perhaps BhuA binding to heme signals a *Brucella* FecR which then activates the RpoE to promote transcription of the *bhuTUV* locus.

### ***Brucella*** **must acquire iron from the heme**

There were two main goals to the research project described in this document: one was to identify the inner membrane heme transport components of *B. abortus* 2308 and the second was to determine the contribution of BhuO, a known heme oxygenase, to the iron requirements of the *Brucella*. Because the *bhuO* mutant strain was still able to grow around heme on an agar plate when heme was the sole source of iron and there was no significant phenotype for the *bhuO*

mutant strain compared to *B. abortus* 2308 *in vivo*, there is a second heme oxygenase in *B. abortus* 2308. This is not suprising since the closest homologs of BhuO, IsdI/G and HmuD/Q are both present in pairs (Skaar 2004, Puri 2006). What is suprising is that these other pairs of heme oxygenases share high amino acid sequence homology, whereas multiple NCBI BLAST searches using BhuO for comparison were unable to uncover a second *Brucella* heme oxygenase. In fact, all known heme oxygenases were used for comparisons in these NCBI BLAST searches, and still there is no likely candidate for this second heme oxygenase based on conserved amino acid residues. Searches of the annotated *B. abortus* 2308 genome yielded a gene whose product was a monooxygenase, a common annotation for heme oxygenases. This monooxygenase does not contain the conserved asparagine residue near its proximal end, but does have a tryptophan and histidine near the distal end of the protein, which in IsdG are known functional sites. Future work should target this gene, BAB1_1911, for mutagenesis in a *bhuO* mutant background to determine if the ability to use heme as an iron source has been completely removed. Alternatively, a transposon mutagenesis library made in the *bhuO* mutant may reveal candidates for the other heme oxygenase(s) by identifying any mutants with a heme utilization defect.

**Unanswered questions about bacterial heme oxygenases**

It has been shown in mammals that the products of heme degradation, biliverdin and bilirubin, perform downstream functions to protect the cell by reducing the inflammatory response (Chung 2009). While it has been observed for some time in our lab that there is a color change consistent with what has been previously described as the presence of porphyrins in the low iron medium containing growing *B. abortus* strains (Martinez 2005), no data to date has

been collected regarding what happens to the products of BhuO. If biliverdin in some form is being pumped out of the cell, what could the physiological significance of this be, given that the brucellae naturally reside within the mammalian cells? The mammalian HO-1 has been implicated in the production of anti-inflammatory signaling (see literature review), perhaps the *Brucella* heme oxygenase products are also released into the mammalian cell and act to "calm" the immune response, allowing for the maintenance of a chronic infection.

Additionally, a question yet to be addressed in any bacterium but often asked: Is exogenously acquired heme incorporated directly into bacterial proteins or is it always broken down for iron first? This question would be easiest initially to answer using known heme auxotrophs such as *Haemophilus influenzae*, *Porphyromonas gingivalis*, and even a close relative to *Brucella*, *Bartonella quintana* (Hanson 1992, Bramanti 1991, Battisti 2007). While it makes sense to conserve bacterial energy whenever possible and simply use the heme that is transported, studies have yet to confirm this.

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**Figure 4.1 Heme transport in *Brucella abortus* 2308.** In keeping with heme transport in other bacteria, brucellae utilize a heme–specific membrane transport complex in order to bind heme and bring it into the cell for use as an iron source. Once the heme has entered the cytoplasm of the cell, a heme oxygenase (BhuO) breaks it down into iron, biliverdin, and carbon monoxide.

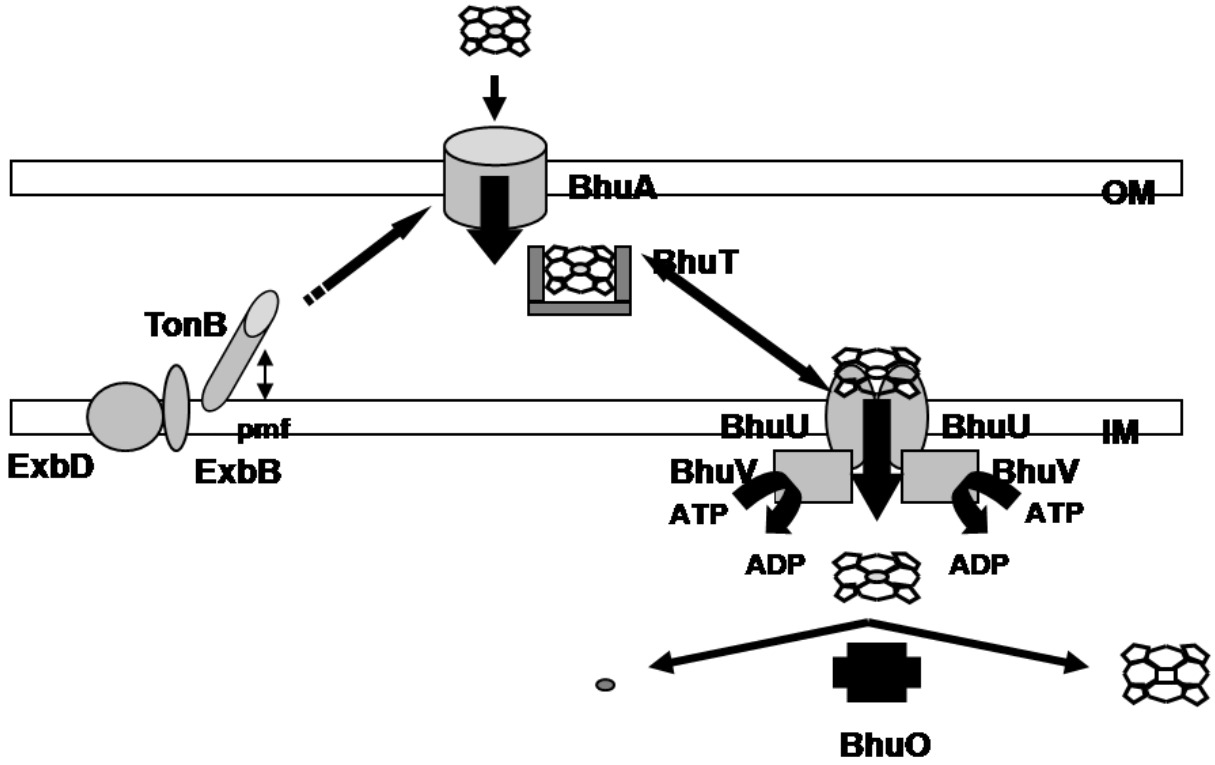
BhuA
OM
BhuT
TonB
pmf
ExbD
ExbB
BhuU
BhuU
IM
BhuV
BhuV
ATP
ATP
ADP
ADP
BhuO

**Figure 4.1**

**Figure 4.2 Genetic organization of the *B. abortus* genes associated with heme uptake and utilization.** The outer membrane heme transporter is encoded on one part of chromosome II, located near a siderophore uptake regulator, the genes encoding the periplasmic and cytoplasmic components of heme transport are located elsewhere on chromosome II and the heme oxygenase is encoded in an operon with *rirA*, also separately from the rest and directly upstream from the genes encoding the iron storage protein bacterioferritin. This unique separation of genes responsible for heme uptake is conserved across the *Brucella* genomes. The black arrows represent all of the genes in each operon.

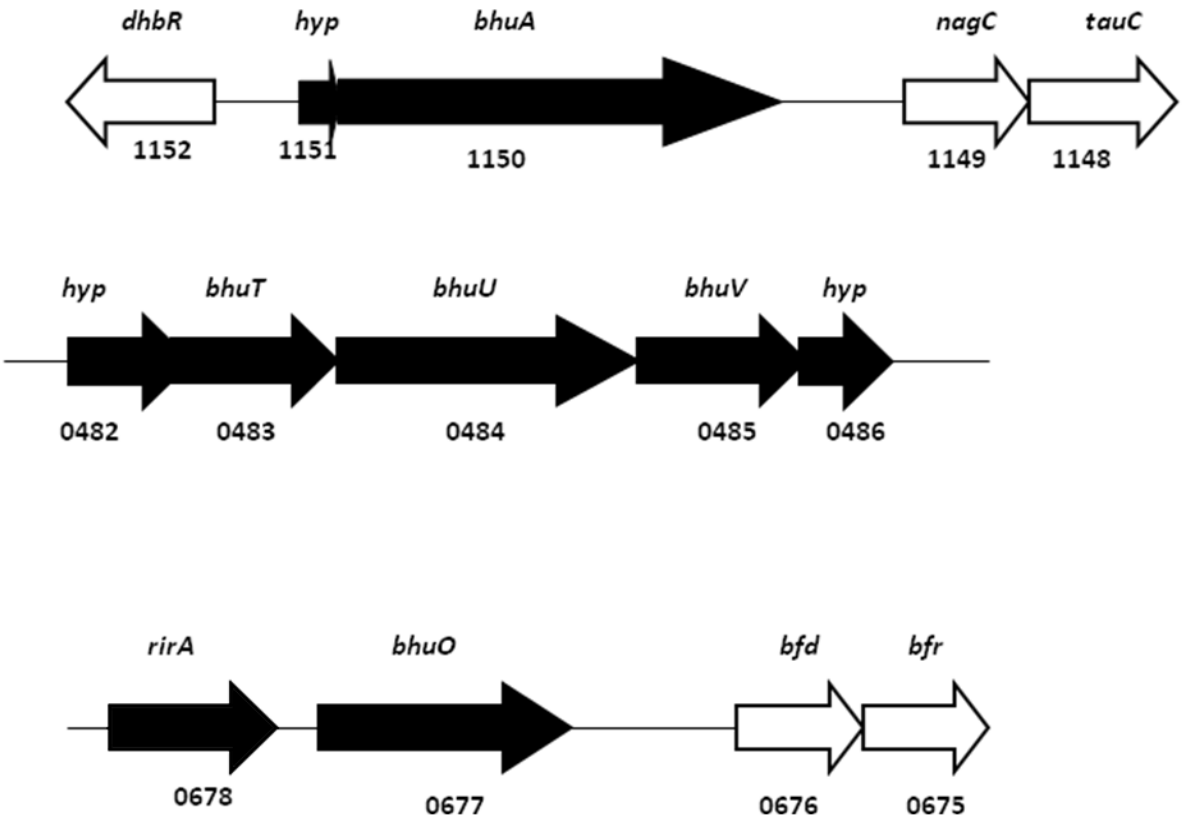
dhbR
hyp
bhuA
nagC
tauC
1152
1151
1150
1149
1148
hyp
bhuT
bhuU
bhuV
hyp
0482
0483
0484
0485
0486
rirA
bhuO
bfd
bfr
0678
0677
0676
0675

**Figure 4.2**

**Figure 4.3 The similar stuctures of cobalamin (B12) and heme.** Cobalamin and heme share the same initial biosynthetic steps, and the overall structures look similar.

**Cobalamin** **Heme**

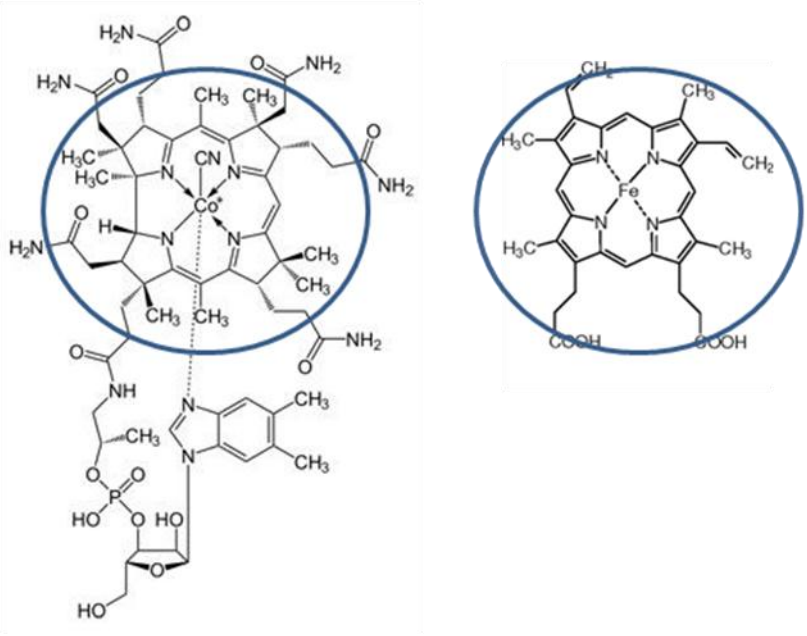

**Figure 4.3**

**Figure 4.4 Model of possible regulation of heme uptake genes in *B. abortus* 2308.**

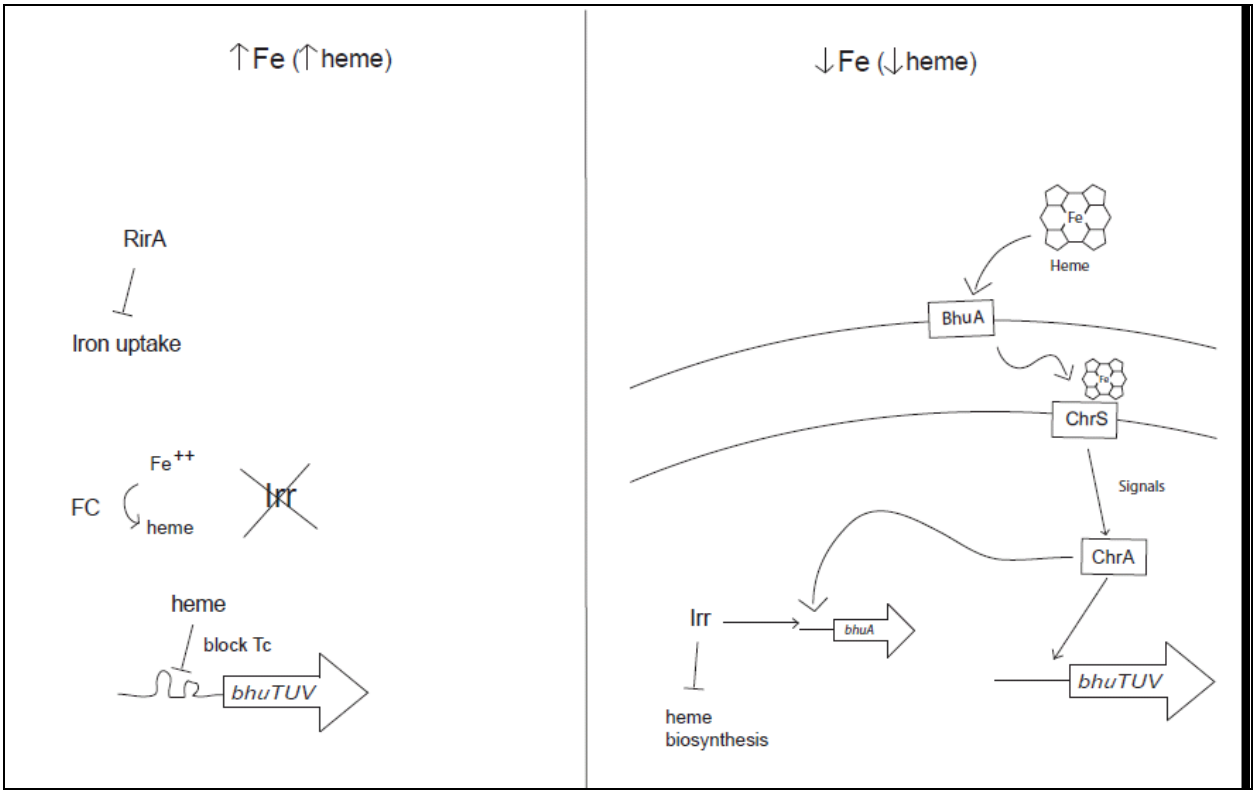
↑Fe (↑heme)
RirA
Iron uptake
Fe++
FC
heme
Irr
heme
block Tc
bhuTUV
↓Fe (↓heme)
Fe
Heme
BhuA
ChrS
Signals
ChrA
Irr
bhuA
heme
biosynthesis
bhuTUV

**Figure 4.4**