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Metal acquisition and virulence in *Brucella*

R. Martin Roop II

Department of Microbiology and Immunology, Brody School of Medicine, East Carolina University, 600 Moye Boulevard, Greenville, North Carolina 27834, USA

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Abstract

Similar to other bacteria, *Brucella* strains require several biologically essential metals for their survival *in vitro* and *in vivo*. Acquiring sufficient levels of some of these metals, particularly iron, manganese and zinc, is especially challenging in the mammalian host, where sequestration of these micronutrients is a well-documented component of both the innate and acquired immune responses. This review describes the *Brucella* metal transporters that have been shown to play critical roles in the virulence of these bacteria in experimental and natural hosts.

Keywords: *Brucella*, iron, manganese, zinc, magnesium, nickel, bacterial iron acquisition.

Introduction

The *Brucella* spp. are Gram-negative bacteria that cause disease in a variety of mammalian hosts (Roop *et al.*, 2009). Although these bacterial strains are presently divided into 10 'nomenspecies' for diagnostic and epidemiological reasons, comparative genomic studies indicate that they are highly related at the genetic level (O'Callaghan and Whatmore, 2011). The brucellae are members of the α-proteobacteria (Moreno *et al.*, 1990), a phylogenetic group of bacteria that includes plant symbionts (*Sinorhizobium*, *Rhizobium*, and *Bradyrhizobium* spp.), plant pathogens (*Agrobacterium* spp.), and mammalian pathogens (*Brucella* and *Bartonella* spp.). It has become readily apparent that there are remarkable parallels between the interactions of these bacteria and their eukaryotic hosts (Batut *et al.*, 2004), and studies of their comparative biology have made significant contributions to our understanding of the pathogenesis of *Brucella* infections (Sola-Landa *et al.*, 1998; O'Callaghan *et al.*,1999; LeVier *et al.*, 2000; Sieira *et al.*, 2000).

Brucella melitensis, *Brucella suis*, and *Brucella abortus* strains cause abortion and infertility in goats, sheep, swine, and cattle, respectively, and are a great concern to the agricultural communities in areas of the world where these infections are not controlled by surveillance and eradication programs (Corbel, 1997). As they are easily transmitted to humans through direct contact with infected animals or their products, these strains also represent a serious public health threat in regions where they remain endemic in food animals. In fact, brucellosis is considered to be the world's leading zoonotic disease (Pappas *et al.*, 2006). *B. melitensis*, *B. suis*, and *B. abortus* strains also possess biological characteristics that have historically made them attractive as agents of biological warfare (Franz *et al.*, 1997), and currently make them a potential threat for use in bioterrorism (Valderas and Roop, 2006). Specifically, they are easy to aerosolize, they have a very low infectious dose, and the disease they produce is difficult to treat in humans (Ariza *et al.*, 2007) and impractical to treat in animals (Nicoletti *et al.*, 1989).

Brucella ovis and *Brucella canis* strains are also important veterinary pathogens. *B. ovis* causes epididymitis and infertility in rams and occasionally abortion in ewes (Blasco, 2003), and *B. canis* produces abortion and infertility in dogs (Wanke, 2004). *B. canis* infections associated with contact with infected dogs have been reported in humans (Lucero *et al.*, 2010), although these infections occur much less frequently and appear to be less severe than those caused by *B. melitensis*, *B. suis*, or *B. abortus*. Human disease caused by *B. ovis*, on the other hand, has not been documented.

Brucella pinnipedialis and *Brucella ceti* strains are naturally found in marine mammals (Dawson *et al.*, 2008). Reproductive tract pathology has been associated with

Corresponding author. E-mail: roopr@ecu.edu

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B. ceti infections in cetaceans (e.g. dolphins and porpoises), but whether or not *B. pinnipedialis* causes disease in pinnipeds (e.g. seals and sea lions) is presently unknown (Nymo *et al.*, 2011). Human infections with *B. ceti* strains have been reported (Sohn *et al.*, 2003), but the source of these infections is unclear. Other *Brucella* strains have been isolated from wild rodents [e.g. *Brucella neotomae* (Stoenner and Lackman, 1957) and *Brucella microti* (Scholz *et al.*, 2008)], and human clinical specimens [*Brucella inopinata* (Scholz *et al.*, 2010)], but neither the capacity of the *B. neotomae* or *B. microti* strains to produce human disease, nor the natural host for *B. inopinata* strains, is known.

The mammalian host as a metal-restricted environment

With a few notable exceptions, all living things require magnesium, manganese, iron, copper, zinc, cobalt, and nickel as micronutrients to support their cellular metabolism and physiology (Summers, 2009; Waldron and Robinson, 2009). These metals play important structural roles in proteins and other cellular components. Owing to their redox activity at physiological pH, iron and copper serve critical functions in proteins that are components of electron transport chains or other proteins that undergo oxidation–reduction reactions. Unfortunately, iron and copper also have the capacity to react with the reactive oxygen species H_2O_2 and O_2^- in a series of reactions known as Fenton chemistry. These reactions produce the highly toxic $OH^\bullet$ radical, which can cause extensive damage to cellular proteins, nucleic acids and lipids (Summers, 2009). Improper incorporation of metals in proteins can also lead to their inactivation (Waldron and Robinson, 2009). To avoid these latter two problems, organisms possess homeostasis systems that ensure that they only accumulate the levels of metals they need to meet their physiological requirements. These homeostasis systems consist of efflux systems; chaperones, transfer and storage proteins that hold these metals in unreactive or non-toxic forms; and transcriptional and translational regulators that tightly regulate expression of the genes encoding these metal import, export and storage systems.

In mammals, metal homeostasis systems not only protect the host from metal toxicity, but they also deprive invading microbes of the metals they need to establish a productive infection. Sequestration of iron, for instance, is a well-documented strategy employed by mammals to limit the replication of microbial pathogens (Nairz *et al.*, 2010). Iron that is not incorporated into host proteins is bound tightly by iron binding proteins such as transferrin and lactoferrin in the extracellular environment (Griffiths, 1999). This is predominantly an oxidizing environment, and, the vast majority of this iron is present as Fe^{3+} at physiological pH, and it has been estimated that the amount of 'free' Fe^{3+} in the blood and tissue fluids is $<10^{-18}$ M. During infection, the protein hepcidin also inhibits the ability of the iron exporter ferroportin to release iron obtained from nutritional sources and recycled from senescent or damaged erythrocytes from the spleen, liver and intestine into the bloodstream, which further restricts the availability of iron in the extracellular environment in the host. This so-called 'hypoferremic response' is considered to be an important component of innate immunity (Weinberg, 1995; Nemeth *et al.*, 2004; Weiss, 2005).

Brucella strains are primarily intracellular pathogens in their mammalian hosts. Multiple independent studies by numerous research groups have clearly shown that the capacity of these strains to survive and replicate efficiently in host macrophages is critical to their ability to produce chronic infections in a variety of natural and experimental hosts (reviewed in Roop *et al.*, 2009). In pregnant animals, extensive intracellular replication of the brucellae within placental trophoblasts is associated with abortion and reproductive tract pathology (Enright, 1990). Within the intracellular environment in the host, iron is present as a dynamic equilibrium between Fe^{2+} and Fe^{3+}, and the ratio of these two types of iron present within an intracellular compartment is dictated by the redox status and pH of that intracellular compartment as well as the activity of cellular ferric reductases and ferroxidases (Anderson and Vulpe, 2009). Three mechanisms have been identified by which mammals can deprive microbial pathogens such as the brucellae that live within phagosomal compartments in host macrophages of iron. All three of these strategies are considered to be important components of the host immune response to infection. The first involves the natural resistance associated macrophage protein (Nramp1) (Cellier *et al.*, 2007). This protein is incorporated into the phagosomal membranes of macrophages and pumps divalent cations such as Fe^{2+} and Mn^{2+} out of the phagosomal compartment. Macrophages activated by interferon γ (IFNγ) also have reduced levels of transferrin receptors on their surface, which reduces the overall flux of iron through these host cells (Byrd and Horwitz, 1989). Finally, although there is a generalized inhibition of iron release via ferroportin from host cells during the hypoferremic response, the ferroportin activity of infected macrophages actually increases, which results in an active efflux of iron from these cells (Nairz *et al.*, 2007).

Recent studies indicate that mammals also actively deprive invading microbes of zinc and manganese as a defense mechanism (Kehl-Fie and Skaar, 2010). The identities of the proteins responsible for the sequestration of zinc in host tissues is unclear, but calreticulin, a protein produced by neutrophils, has been shown to be important for depriving *Staphylococcus aureus* of manganese during the formation of abscesses in a mouse model (Corbin *et al.*, 2008). In addition, as mentioned above, it is well documented that the capacity of Nramp1 to remove Mn^{2+} from the phagosomal compartment plays an

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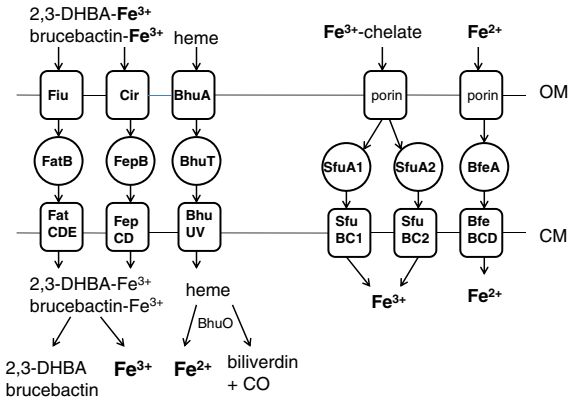


Fig. 1. Iron transporters in *Brucella*. Abbreviations: OM, outer membrane; CM, cytoplasmic membrane.

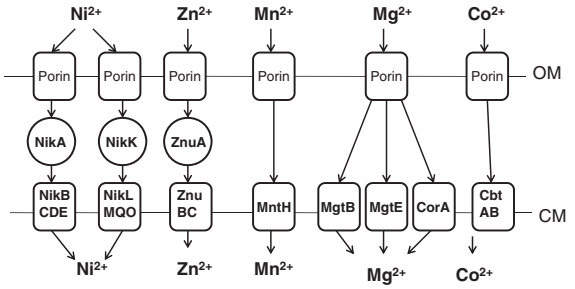


Fig. 2. Nickel, zinc, manganese, magnesium and cobalt transporters in *Brucella*. Abbreviations: OM, outer membrane; CM, cytoplasmic membrane. MgtC is not shown in this figure because its precise role in Mg^{2+} transport is not known (Günzel *et al.*, 2006; Alix and Blanc-Potard, 2007).

important role in the capacity of macrophages to limit intracellular replication by microbial pathogens (Zaharik *et al.*, 2004; Cellier *et al.*, 2007).

Brucella strains require iron, manganese, zinc, and magnesium transporters for wild-type virulence in natural and experimental hosts

Figures 1 and 2 show the iron, manganese, zinc, nickel, cobalt, and magnesium transport systems predicted to be present in *Brucella* strains based on surveys of the publicly available genome sequences, and Table 1 lists the genes in the *B. abortus* 2308 genome sequence that encode the individual components of these systems. For a more general and comprehensive review of the genes involved in metal acquisition and homeostasis in *Brucella* strains, the reader is directed to a recently published book chapter (Roop *et al.*, 2011).

Iron, manganese, and magnesium are required for the optimal growth of *Brucella* strains *in vitro* (ZoBell and Meyer, 1932; McCullough *et al.*, 1947; Sanders *et al.*, 1953; Waring *et al.*, 1953; Evenson and Gerhardt, 1955; Gerhardt, 1958), and phenotypic evaluations of defined mutants has shown that in addition to these three metals, efficient transport of zinc is also required for the virulence of these strains in experimentally infected animals (Fig. 3). The following sections will further describe the *Brucella* metal acquisition genes that have been experimentally linked to virulence.

Iron transport

Owing to its chemical versatility, iron serves as a co-factor for a wide range of proteins (Crichton, 2009). In fact, to the author's knowledge, bacteria in the genera *Lactobacillus* and *Borrelia* are the only organisms that have been documented to be able to live without this metal (Archibald, 1983; Posey and Gherardini, 2000). Presumably, a large and diverse group of *Brucella* proteins require iron for their activity. Some examples for which this requirement has been verified experimentally include catalase (Waring *et al.*, 1953), aldolase (Gary *et al.*, 1955), and CobG, an enzyme involved in cobalamin (vitamin B_{12}) biosynthesis (Schroeder *et al.*, 2009).

Siderophores

Siderophores are low molecular weight chelators that microbes release into their external environment to capture iron (Raymond and Dertz, 2004). *Brucella* strains produce two catechol siderophores when exposed to iron deprivation – 2,3-dihydroxybenzoic acid (2,3-DHBA) and the 2,3-DHBA-based molecule brucebactin (López-Goñi *et al.*, 1992; González-Carreró *et al.*, 2002). Owing to its instability in the laboratory, the precise structure of brucebactin is currently unknown. The biochemical features of the enzymes predicted to be encoded by the genes responsible for the production of these siderophores, however, indicate that brucebactin is likely to be a monocatechol consisting of 2,3-DHBA linked to a polyamine or an amino acid (Bellaire *et al.*, 2003a). Experimental evidence suggests that siderophore production plays a critical role in the virulence of *Brucella* strains in the gravid ruminant reproductive tract, but is not required for the persistence of these strains in host macrophages. A *B. abortus dhbC* mutant, which produces neither 2,3-DHBA nor brucebactin, for instance, does not cause abortion in pregnant goats (Bellaire *et al.*, 2000) or cattle (Bellaire *et al.*, 2003a) (Fig. 3). In contrast, this mutant and isogenic *B. abortus* mutants that produce 2,3-DHBA but cannot convert it to brucebactin display wild-type virulence in the mouse model of chronic infection (Bellaire *et al.*, 1999; González-Carreró *et al.*, 2002; Parent *et al.*, 2002).

One possible explanation that has been put forth for the apparent differential requirement for siderophore production by *B. abortus* in the ruminant reproductive

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Table 1. Designations of the genes in the *Brucella abortus* 2308 genome sequence predicted to encode the individual components of the metal transporters depicted in Figs. 1 and 2.

Gene product	Predicted function	Gene designation
DhbC	Biosynthesis of 2,3-DHBA	BAB2_0015
DhbE	Biosynthesis of 2,3-DHBA	BAB2_0014
DhbA	Biosynthesis of 2,3-DHBA	BAB2_0012
DhbB	Biosynthesis of 2,3-DHBA/ Conversion of 2,3-DHBA to brucebactin	BAB2_0013
EntD	Conversion of 2,3-DHBA to brucebactin	BAB2_0011
VibH	Conversion of 2,3-DHBA to brucebactin	BAB2_0016
Fiu	2,3-DHBA/brucebactin transport	BAB2_0233
FatB	2,3-DHBA/brucebactin transport	BAB2_0564
FatC	2,3-DHBA/brucebactin transport	BAB2_0562
FatD	2,3-DHBA/brucebactin transport	BAB2_0563
FatE	2,3-DHBA/brucebactin transport	BAB2_0561
Cir	2,3-DHBA/brucebactin transport	BAB1_1367
FepB	2,3-DHBA/brucebactin transport	BAB1_1366
FepC	2,3-DHBA/brucebactin transport	BAB1_1364
FepD	2,3-DHBA/brucebactin transport	BAB1_1365
BhuA	Heme transport	BAB2_1150
BhuT	Heme transport	BAB2_0483
BhuU	Heme transport	BAB2_0484
BhuV	Heme transport	BAB2_0485
BhuO	Heme degradation/Fe^{2+} release	BAB2_0677
SfuA1	Fe^{3+} transport	BAB2_0539
SfuB1	Fe^{3+} transport	BAB2_0538
SfuC1	Fe^{3+} transport	BAB2_0540
SfuA2	Fe^{3+} transport	BAB2_0519
SfuB2	Fe^{3+} transport	BAB2_0520
SfuC2	Fe^{3+} transport	BAB2_0521
BfeA	Fe^{2+} transport	BAB2_0840
BfeB	Fe^{2+} transport	BAB2_0839
BfeC	Fe^{2+} transport	BAB2_0838
BfeD	Fe^{2+} transport	BAB2_0837
MntH	Mn^{2+} transport	BAB1_1460
ZnuA	Zn^{2+} transport	BAB2_1079
ZnuB	Zn^{2+} transport	BAB2_1081
ZnuC	Zn^{2+} transport	BAB2_1080
NikA	Ni^{2+} transport	BAB2_0433/0434[a]
NikB	Ni^{2+} transport	BAB2_0435
NikC	Ni^{2+} transport	BAB2_0436
NikD	Ni^{2+} transport	BAB2_0437
NikE	Ni^{2+} transport	BAB2_0438
NikK	Ni^{2+} transport	BAB1_1384
NikL	Ni^{2+} transport	BAB1_1386
NikM	Ni^{2+} transport	BAB1_1385
NikO	Ni^{2+} transport	BAB1_1388
NikQ	Ni^{2+} transport	BAB1_1387
CbtA	Co^{2+} transport	BAB1_1329
CbtB	Co^{2+} transport	BAB1_1330
MgtB	Mg^{2+} transport	BAB2_0036
MgtE	Mg^{2+} transport	BAB2_0360
CorA	Mg^{2+} transport	BAB1_0583
MgtC	Mg^{2+} transport (?)[b]	BAB2_0039

[a]The region homologous to the *nikA* gene in other *Brucella* genome sequences is annotated as two adjacent pseudo-genes in the *B. abortus* 2308 genome sequence.
[b]The precise role of the MgtC in magnesium transport in bacteria is unknown.

tract is linked to the capacity of this bacterium to utilize erythritol as its preferred carbon and energy source (Anderson and Smith, 1965; Meyer, 1967). Ruminant placental trophoblasts produce copious amounts of this four carbon sugar alcohol during the latter stages of pregnancy (Enright, 1990), and it has been proposed that the capacity of the brucellae to efficiently utilize this carbon source is linked to their virulence in pregnant ruminants (Smith *et al.*, 1962). *In vitro* studies have shown that *B. abortus* 2308 displays a much greater need for iron when it is growing in the presence of erythritol than when it is growing with other readily utilizable carbon

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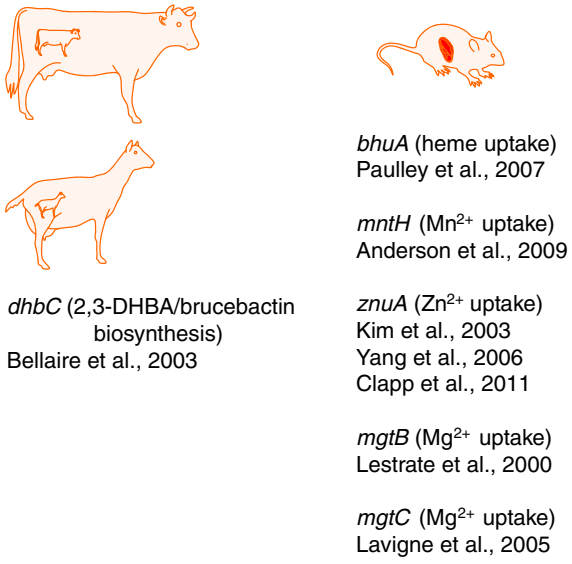


Fig. 3. *Brucella* genes experimentally linked to virulence in pregnant ruminants and mice. Virulence in pregnant ruminants is measured by bacterial colonization of the dam and fetus and fetal pathology (e.g. abortion or the birth of a weak kid or calf). Virulence in mice is measured by chronic colonization of the spleen.

and energy sources (Bellaire *et al.*, 2003b; Jain *et al.*, 2011). Accordingly, it has been proposed that siderophore production plays an important role in supplying this strain with the iron it needs to fuel rapid and extensive bacterial replication in placental trophoblasts that leads to abortion (Bellaire *et al.*, 2003b).

Not all *B. abortus* and *B. melitensis* strains produce catechol siderophores in response to iron deprivation *in vitro* (López-Goñi and Moriyón, 1995), and some of the siderophore biosynthesis genes in *Brucella* strains other than *B. abortus* 2308 are annotated as pseudo-genes in the genome sequences available in GenBank (Roop *et al.*, 2011). Thus, it will be important to better define the link between siderophore production and erythritol metabolism in *Brucella* strains and perform definitive experiments to determine whether or not this link is responsible for the extreme attenuation displayed by the *B. abortus dhbC* mutant in pregnant ruminants. Likewise, it will also be important to determine whether or not siderophore production is required for the virulence of other *Brucella* strains in a variety of pregnant and non-pregnant natural hosts.

Utilization of heme as an iron source

Degradation of senescent and damaged erythrocytes and the recycling of the iron released from these cells is one of the major functions of mammalian macrophages (Bratosin *et al.*, 1998). Ruminant placental trophoblasts also ingest maternal erythrocytes and degrade these cells to provide a source of iron to the developing fetus (Anderson *et al.*, 1986). During both processes, a considerable amount of heme is released into these host phagocytes. Both *B. abortus* 2308 and *B. melitensis* 16M can use heme as an iron source in *in vitro* assays (Bellaire, 2001; Danese, 2001; Paulley *et al.*, 2007). Heme transport is mediated by the TonB-dependent outer membrane protein, BhuA, and a periplasmic binding protein-dependent ABC-type transporter comprised of the proteins BhuT, U and V (Fig. 1), and the genes encoding these proteins appear to be well-conserved among *Brucella* strains (Roop *et al.*, 2011). *Brucella* strains also possess a heme oxygenase (Puri and O'Brian, 2008). Presumably, this enzyme, which we have given the designation BhuO (Roop *et al.*, 2011), allows the brucellae to use heme as an iron source by degrading the heme once it has been transported into the cytoplasm (Fig. 1). An isogenic *bhuA* mutant constructed from *B. abortus* 2308 displays significant attenuation in experimentally infected mice (Paulley *et al.*, 2007) (Fig. 3), suggesting that the capacity to transport heme represents a critical virulence determinant. Whether or not heme utilization plays an important role in the virulence of other *Brucella* strains, or in natural hosts, remains to be experimentally determined.

Due to its potential toxicity, the heme that is not incorporated into cellular proteins in mammalian cells is actively routed to the endoplasmic reticulum (ER) where it can be degraded by heme oxygenase (Taketani, 2005). Cell biology studies have shown that the membrane-bound vacuoles within which the brucellae replicate in host macrophages (known as the replicative *Brucella*-containing vacuoles or rBCVs) are derived through extensive interactions of phagosomes with the host cell ER (Celli *et al.*, 2003). The rBCVs initially interact with the ER exit sites, and eventually fuse with the ER (Celli *et al.*, 2005). Extensive interactions of rBCVs with the host cell ER have also been observed microscopically in experimentally infected HeLa and Vero cells (Detilleux *et al.*, 1990; Pizzaro-Cerdá *et al.*, 1998) and in placental trophoblasts from experimentally infected ruminants (Anderson *et al.*, 1986). Consequently, to gain a better understanding of the host–pathogen interactions in brucellosis, it will also be important to determine how the interactions of the rBCVs with the host cell ER influence the availability of heme as an iron source for *Brucella* strains during their intracellular residence in macrophages and placental trophoblasts.

Manganese transport

The ABC-type transporters exemplified by the SitABCD transporter of *Salmonella* and the proton-symporters of the MntH family are the most common types of manganese transporters that have been described in prokaryotes (Papp-Wallace and Maguire, 2006). Many bacteria possess both of these types of manganese transporters, but an analysis of the currently available

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Brucella genome sequences and phenotypic analysis of a *B. abortus mntH* mutant suggest that *Brucella* strains utilize MntH (Fig. 2) as their sole high affinity Mn^{2+} transporter (Anderson *et al.*, 2009). A *B. abortus mntH* mutant is extremely attenuated in the mouse model of chronic infection (Fig. 3). The basis for this attenuation is presently unknown. The *B. abortus mntH* mutant possesses reduced Mn superoxide dismutase activity compared to the parental strain, but an isogenic *sodA* mutant exhibits only modest attenuation in mice (Martin *et al.*, 2012), indicating that reduced SodA activity is not the basis for the severe attenuation exhibited by the *mntH* mutant. The *B. abortus mntH* mutant also exhibits aberrant expression of the genes encoding the Type IV secretion machinery (Anderson *et al.*, 2009), and although the relationship between Mn^{2+} transport and *virB* expression has not been investigated, one plausible explanation for this relationship is that orthologs of the (p)ppGpp synthetase/hydrolase known as Rsh (Dozot *et al.*, 2006), which is required for *virB* expression as well as induction of the stringent response in *Brucella*, are manganese-dependent enzymes (Papp-Wallace and Maguire, 2006).

Escherichia coli exhibits increased *mntH* expression in response to exposure to H_2O_2 (Anjem *et al.*, 2009), and recent genetic and biochemical studies indicate that by elevating the intracellular ratio of Mn^{2+}:Fe^{2+} this bacterium can substitute Mn^{2+} for Fe^{2+} in key metabolic enzymes such as ribulose-5-phosphate epimerase (Rpe), a major enzyme in the pentose-phosphate pathway (Sobota and Imlay, 2011). Unlike Fe^{2+}, Mn^{2+} does not participate in Fenton chemistry, hence this substitution protects Rpe from H_2O_2-mediated damage. Exposure of *B. abortus* 2308 to H_2O_2 *in vitro* also results in increased *mntH* expression (E. Menscher, unpublished results), and an isogenic *mntH* mutant displays an increased sensitivity to exposure to H_2O_2 in *in vitro* assays compared to the parental 2308 strain (Anderson *et al.*, 2009). Consequently, it will be important to determine whether *Brucella* strains have the same capacity to substitute Mn^{2+} for Fe^{2+} in metabolic enzymes as a mechanism for protecting these proteins from H_2O_2 mediated damage as has been demonstrated in *E. coli*.

Zinc transport

Zinc functions as a structural or enzymatic co-factor for a wide array of bacterial enzymes (Andreini *et al.*, 2006). The Cu/Zn superoxide dismutase SodC represents an important virulence determinant for *B. abortus* 2308 (Tatum *et al.*, 1992; Gee *et al.*, 2005), and the *Brucella* carbonic anhydrases I and II and histidinol dehydrogenase are zinc-dependent enzymes that have been proposed to be good targets for the development of antimicrobials (Lopez *et al.*, 2012). Two separate groups have independently shown that the *znuA* gene is essential for the wild-type virulence of *B. abortus* and *B. melitensis* strains in experimentally infected mice (Kim *et al.*, 2004, Yang *et al.*, 2006; Clapp *et al.*, 2011) (Fig. 3). This gene encodes the periplasmic metal-binding component of an ABC-type high affinity zinc transporter, with ZnuB and ZnuC being the cytoplasmic permease and ATPase components of this transporter, respectively, (Fig. 2).

Magnesium transport

Magnesium is present in bacterial cells at high (i.e., mM) concentrations. It plays an important role in maintaining the structural integrity of ribosomes and cell membranes, and serves as a structural and enzymatic co-factor for a variety of cellular proteins (Moomaw and Maguire, 2008). Erythritol kinase, the enzyme that catalyzes the first step in the catabolism of erythritol in *Brucella* strains, for instance, requires Mg^{2+} for its activity (Sperry and Robertson, 1975).

Homologs of two genes associated with magnesium transport in other bacteria have been genetically linked to virulence in *Brucella* strains. MgtB is a bacterial P-type ATPase (Fig. 2) and the activity of this protein as a magnesium transporter has been best described in *Salmonella* (Smith *et al.*, 1993). A *B. melitensis mgtB* mutant was isolated during a screen of signature-tagged transposon mutants derived from *B. melitensis* 16M for attenuation in experimentally infected mice (Lestrate *et al.*, 2000). Interestingly, this mutant did not exhibit a growth defect when cultured in magnesium limited medium. This suggests that similar to other bacteria, and as depicted in Fig. 2, the brucellae possess multiple transport systems for magnesium. Although the precise role of MgtC in magnesium transport has not been established (Günzel *et al.*, 2006; Alix and Blanc-Potard, 2007), a *B. suis mgtC* mutant does not grow well in a magnesium-restricted medium and displays significant attenuation in the murine macrophage-like J774 cell line (Lavigne *et al.*, 2005). More importantly, this attenuation can be partially alleviated by supplementation of the cell culture medium with $MgCl_2$.

Nickel transport

Urease is one of the few bacterial proteins that have been shown to require nickel as a co-factor (Li and Zamble, 2009). This enzyme is essential for the virulence of *B. abortus* 2308 and *B. suis* 1330 in mice when these strains are introduced via the oral route, but not when they are administered via the peritoneal route (Bandara *et al.*, 2007; Sangari *et al.*, 2007). *B. abortus* and *B. suis* urease mutants also exhibit wild-type virulence in mammalian cell cultures. The proposed explanation for these findings is that urease assists the brucellae in resisting the very low pH they encounter during passage

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through the stomach and gastrointestinal tract after ingestion, but is not required for intracellular survival in eukaryotic cells. Two nickel transporters, NikABCDE and NikKMLQO (Fig. 2) have been identified in *Brucella* (Jubier-Maurin *et al*., 2001; Sangari *et al*., 2010), but the role that these transporters play in virulence is unresolved. Although *nikA* expression is upregulated in *B. suis* 1330 during the intracellular replication of this strain in J774 cells, an isogenic *nikA* mutant derived from this strain displays wild-type virulence in the human monocytic cell line THP-1 (Jubier-Maurin *et al*., 2001). In order to gain a better understanding of the requirement for nickel transport by *Brucella* strains in the host, it will be important to assess the virulence properties of *Brucella* strains lacking either the NikABCDE or NikKMLQO transporter, or both, in cultured macrophages and in mice infected via both the intraperitoneal and oral routes. A comparison of the phenotypes displayed by these mutants with those exhibited by isogenic urease mutants in these experimental models will also be important for determining if *Brucella* strains require nickel for the proper function of enzymes other than urease.

Metalloregulators and metal storage/detoxification proteins p

As mentioned previously in this review, proteins that directly participate in metal homeostasis are essential for preventing toxicity due to the over-accumulation of these important micronutrients. Three transcriptional regulators that control the expression of *Brucella* metal acquisition genes have been characterized – Irr (Martínez *et al*., 2005, 2006), DhbR (Anderson *et al*., 2008) and Mur (Menscher *et al*., 2012). Irr is an iron-responsive transcriptional regulator that controls iron acquisition and iron metabolism genes; DhbR is an AraC-type transcriptional regulator that activates the transcription of the siderophore biosynthesis genes in *B. abortus* 2308 in response to Fe^{3+}-siderophore levels in the external environment; and Mur regulates the expression of the gene encoding the Mn^{2+} transporter MntH in response to cellular Mn^{2+} levels. *Brucella* strains also produce bacterioferritin (Bfr), a protein that stores and detoxifies intracellular iron (Denoel *et al*., 1995; Almirón and Ugalde, 2010). To date, only Irr and Bfr have been examined for their roles in virulence. A *B. abortus irr* mutant is attenuated in the mouse model (Anderson *et al*., 2011), but neither *B. abortus* nor *B. melitensis bfr* mutants exhibit attenuation in cultured human primary explant macrophages (Denoel *et al*., 1997), J774 or HeLa cells (Almirón and Ugalde, 2010), or experimentally infected mice (Denoel *et al*., 1997). The reader is pointed to a computational study described by Rodionov *et al*. (2006) and a recent book chapter (Roop *et al*., 2011) for a more comprehensive consideration of the *Brucella* genes involved in metal homeostasis.

Conclusion

It seems clear that *Brucella* strains are well equipped to deal with the metal deprivation they encounter in their mammalian hosts. However, the contributions of many of the metal transporters shown in Figs. 1 and 2 to virulence remain to be determined. Considering the conserved strategies the α-proteobacteria employ to establish and maintain chronic infections in their eukaryotic hosts (Batut *et al*., 2004), it will be particularly interesting to determine what role CbtAB-mediated Co^{2+} transport plays in the virulence of *Brucella* strains. Cobalt-containing enzymes play a critical role in the capacity of *Sinorhizobium meliloti*, a close phylogenetic relative of the brucellae, to maintain a symbiotic relationship with its eukaryotic plant host (Taga and Walker, 2010).

A final point that bears consideration is that the vast majority of the studies that have evaluated the contributions of *Brucella* metal acquisition to virulence have been performed in the mouse model of chronic infection, which is used as a measure of the ability of these strains to survive and replicate in host macrophages. But as the studies with *B. abortus* siderophore biosynthesis mutants well demonstrate (Bellaire *et al*., 1999, 2000, 2003a; González-Carreró *et al*., 2002; Parent *et al*., 2002), the results obtained with the mouse model may not always predict how a mutant will behave in the natural host, especially in pregnant ruminants. The sources of iron (e.g. Fe^{2+}, Fe^{3+}, and heme or heme-containing proteins) and other metals available and the metabolic requirements of the intracellular brucellae for these metals may differ depending upon whether or not these bacteria are residing in macrophages or placental trophoblasts, and pregnancy may have an impact on these differences. Consequently, it will be important in future studies to assess the importance of metal acquisition genes to virulence in a variety of pregnant and non-pregnant natural and experimental hosts.

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