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Survival of the fittest: how *Brucella* strains adapt to their intracellular niche in the host

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Abstract

Brucella strains produce abortion and infertility in their natural hosts and a zoonotic disease in humans known as undulant fever. These bacteria do not produce classical virulence factors, and their capacity to successfully survive and replicate within a variety of host cells underlines their pathogenicity. Extensive replication of the brucellae in placental trophoblasts is associated with reproductive tract pathology in natural hosts and prolonged persistence in macrophages leads to the chronic infections that are a hallmark of brucellosis in both natural hosts and humans. This review describes how *Brucella* strains have efficiently adapted to their intracellular lifestyle in the host.

Introduction

The *Brucella* spp. are Gram-negative bacteria that cause economically important diseases in food animals worldwide [44]. *Brucella melitensis*, *B. abortus* and *B. suis* strains cause abortion and infertility in their natural hosts - goats and sheep, cattle and swine, respectively. Humans can also acquire a severe, debilitating febrile illness known as brucellosis, or "undulant fever", as the result of contact with infected animals or their products [133]. Naturally-occurring human brucellosis is strictly a zoonotic infection. In areas of the world where *Brucella* infections in food animals have been controlled by successful eradication programs, human infections are predominately an occupational hazard for animal handlers, veterinarians, slaughterhouse workers and others who work with potentially infected animals [130]. In contrast, human brucellosis remains a significant public health concern in areas of the world where *Brucella* infections are endemic in food animals. Indeed, brucellosis has been described as being the leading zoonosis worldwide [133].

Brucella ovis is a natural pathogen of sheep where it primarily causes epididymitis and infertility in rams and occasionally abortion in ewes [23]. *B. canis* infection leads to abortion and infertility in dogs [187]. Although *B. ovis* and *B. canis* are important veterinary pathogens, human infection with *B. canis* is rare [44], and human infection with *B. ovis* has not been reported.

Brucella pinnipedialis and *B. ceti* strains are being isolated from marine mammals with increasing frequency [48], but the role of these bacterial strains in disease in these hosts or how these strains are disseminated between marine mammals is presently unresolved [83].

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Marine mammal strains of *Brucella* have also been isolated from human disease [171] indicating that these strains are potential zoonotic pathogens.

Brucella strains are highly infectious via the aerosol route [69]. Human brucellosis is also debilitating, long-lasting and difficult to treat with antibiotics, and there are no safe and effective vaccines available to prevent human infection [196]. This combination of characteristics has led to the inclusion of *B. melitensis, B. suis* and *B. abortus* strains on lists of etiologic agents considered to pose risks for use as bioweapons [134]. Accordingly, the possession and handling of *Brucella* strains in both clinical and research laboratories are subject to strict regulations in many countries.

The brucellae are members of the α–proteobacteria [126]. Other members of this group of bacteria include those in the genera *Bartonella*, *Agrobacterium*, *Rhizobium*, *Sinorhizobium*, and *Mesorhizobium*. All of these bacteria inhabit eukaryotic cells, and comparative genomic studies indicate that they evolved from a common ancestor [25]. There are remarkable parallels between the mechanisms and gene products employed by these bacteria to establish successful interactions with their plant and animal hosts [16,110]. Recognition of these parallels has greatly improved our understanding of the host-pathogen interactions that take place during *Brucella* infections.

Brucella strains are intracellular pathogens *in vivo*

Brucella strains live in close association with their mammalian hosts. They are not found free-living in the environment. Although many texts refer to these bacteria as being facultative intracellular parasites, it has been proposed that they are more appropriately termed “facultatively extracellular intracellular parasites” [127]. This is based on the fact that although the *Brucella* spp. are relatively easy to cultivate on artificial media, they maintain predominately an intracellular existence within their mammalian hosts. Within these hosts, the brucellae occupy both professional and non-professional phagocytes, and their interactions with these host cells dictate the outcomes of infection [101,125,156].

Interactions of *Brucella* strains with professional phagocytes

Macrophages

It is well documented that the capacity of *Brucella* strains to survive and replicate for prolonged periods within host macrophages underlies their ability to produce chronic, and sometimes life-long, infections [101,156]. This intracellular niche provides a safe haven for the brucellae in terms of protecting these bacteria from antibodies and complement during dissemination in the host. Localization of persistently infected macrophages in organs of the reticuloendothelial system such as the spleen and liver also provides foci for the maintenance of chronic infection [60,128]. An interesting feature of the interactions of *Brucella* strains with macrophages is that experimental evidence indicates that these bacteria have the ability to prevent apoptosis of the macrophages within which they reside [79]. This property conceivably allows the brucellae to extend the longevity of their safe haven.

Dendritic cells

The link between persistent infection of macrophages and the virulence of *Brucella* strains has been recognized for decades. Recent work, however, suggests that another type of professional phagocyte may also play a key role in the pathobiology of *Brucella* infections. Specifically, in contrast to several other intracellular pathogens, the brucellae survive and replicate in human and murine dendritic cells [21,159]. Strikingly, the intracellular replication of virulent *Brucella* strains interferes with the maturation of these host cells [22,159]. Considering the importance of dendritic cells in the development of host immune

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responses [12], it is easy to see how the capacity of the brucellae to inhibit the maturation of these antigen-processing cells allows these bacteria to circumvent host immune responses. As is the case with macrophages, it is also possible that dendritic cells serve as safe havens to prevent exposure of the brucellae to components of the immune response and act as vehicles for the dissemination of these bacteria in the host.

Interactions of *Brucella* strains with non-professional phagocytes

Placental trophoblasts

During pregnancy in natural hosts, *Brucella* strains can infect and replicate within placental trophoblasts [123,160]. These host cells are epithelial in nature, and although they are considered to be non-professional phagocytes, some placental trophoblasts acquire the capacity to engulf and degrade erythrocytes from the maternal circulation; hence they are known as erythrophagocytic trophoblasts [86]. This activity provides an important source of iron for the developing fetus. Large numbers of brucellae can be isolated from the placenta of infected ruminants (e.g. 10^{13} CFU/g of tissue in fetal cotyledons) [2], and this extensive intracellular proliferation of the brucellae can disrupt the integrity of the placenta leading to abortion or the birth of weak and infected offspring, two of the hallmark clinical presentations associated with *Brucella* infections in their natural hosts [5,6,23,60,187]. It seems likely that the physical and hormonal characteristics of the placenta that facilitate immune suppression and prevent maternal rejection of the developing fetus play an important role in allowing the brucellae to replicate to high numbers in the gravid reproductive tract of their natural hosts. The deposition of heavily infected placental tissues into the environment is important for transmission of *Brucella* infections between natural hosts [5,6,23,46,187]. In contrast to the situation in natural hosts, abortion is not a predominant clinical presentation associated with human brucellosis, but it does occur and is an issue of medical concern in regions where this disease is endemic [196].

Ruminant placental trophoblasts produce erythritol during the third trimester of pregnancy [160]. This sugar alcohol is a favored carbon and energy source for many *Brucella* strains [174], and it has been postulated that the presence of this compound contributes to the rapid and extensive replication of the brucellae in the ruminant reproductive tract [169]. This proposed link between erythritol utilization and virulence of *Brucella* strains in ruminants, however, has yet to be verified experimentally.

Epithelial cells

The brucellae gain entrance into the host at mucosal barriers, and thus the interactions of these bacteria with host epithelial cells at these locations represent an important point of initial contact between the pathogen and host. *Brucella* strains have been shown to invade a variety of epithelial cells in culture [64,166], but the efficiency with which these bacteria “invade” epithelial cells is low compared to bacterial pathogens that are considered to be truly “invasive” [128]. Consequently, the extent of the contribution that epithelial cell invasion makes to the initiation of *Brucella* infections unclear, and some investigators have proposed that M cells located at mucosal surfaces serve as the major site of entry of *Brucella* strains into the host [128]. It is important to note, however, that the human epithelial cell line HeLa and the African green monkey kidney fibroblastic cell line Vero have both been used widely and effectively as models for studying the interactions of *Brucella* strains with mammalian cells [52,128,139].

The brucellae proactively alter their intracellular trafficking in host cells

When unopsonized *B. melitensis*, *B. abortus* and *B. suis* strains are ingested by cultured macrophages and epithelial cells, the *Brucella*-containing vacuoles (BCVs) enter into an

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intracellular trafficking pathway that results in the development of specialized membrane-bound compartments [8,32,96,129,139,150] known as replicative phagosomes [96], replicative vacuoles [128] or brucellosomes [101] (Figure 1). Interactions between the O-chain of the smooth LPS of these *Brucella* strains and lipid rafts on the surface of macrophages have been shown to be important for mediating entry into host cells in a manner that leads to the development of the replicative phagosome [141]. During the initial stages of intracellular trafficking of the BCVs, these compartments undergo transient interactions with lysosomes [176] which results in their acidification [8,140]. These vacuoles then begin to interact extensively with the endoplasmic reticulum [32] and eventually their intracellular pH rises to a level that allows intracellular replication of the brucellae. During development of the replicative phagosome in epithelial cells, the BCVs acquire properties resembling autophagosomes [139], but this does not appear to be the case during the development of the BCVs in macrophages [32]. Studies employing the human monocytic cell line THP-1 and *B. abortus* strains opsonized with hyperimmune IgG have also shown that when the brucellae enter host macrophages in this manner, the resulting BCVs also undergo transient association with the lysosomal compartment and become acidified, but these BCVs do not interact extensively with the ER [20]. An obvious potential benefit of this altered intracellular trafficking is that limiting the fusion of the BCVs with lysosomes minimizes the exposure of these bacteria to the bactericidal proteins that reside in these intracellular compartments. A potential nutritional benefit to the brucellae of the fusion of the BCVs with the ER in host cells will be discussed in a later section.

The Type IV secretion system (T4SS) of *Brucella* strains encoded by the *virB* operon plays an essential role in the development of the replicative vacuole within which these bacteria reside in host cells [32,40,50,96] (Figure 1). The T4SS of *Legionella pneumophila* secretes "effector" proteins into host cells that alter the intracellular trafficking of this bacterium [167], and genetic and biochemical studies support a similar function for the *Brucella* T4SS. Specifically, the vacuoles containing *B. melitensis*, *B. abortus* and *B. suis virB* mutants fuse extensively with lysosomes but do not interact with the ER in cultured macrophages, dendritic cells and HeLa cells (32,40,50,159]. Recently, de Jong et al. (49) identified two putative effector proteins (designated VceA and VceC) secreted by the T4SS of *B. abortus* 2308. These investigators showed that VceC is also secreted by *B. suis* 1330. The biological functions of VceA and VceC have yet to be defined, but the importance of the *Brucella* T4SS for virulence is clearly evident from the attenuation that *virB* mutants exhibit compared to their parental strains in cultured macrophages [67,131,146], HeLa cells [67,97,131,168], human and murine dendritic cells [21,159] and experimentally infected mice [84,92,146,168] and goats [93,198]. It is also notable that two of the major environmental stresses encountered by the brucellae during their intracellular residence in host macrophages, exposure to acidic pH and nutritional deprivation (see below), serve as important stimuli for induction of expression of the *virB* operon in *B. suis* [24].

BvfA is a *Brucella* protein discovered in a genetic screen designed to identify substrates of the T4SS [106]. *B. suis bvfA* mutants are highly attenuated in cultured murine and human macrophages, HeLa cells and mice. Although the *bvfA* gene in *B. suis* 1330 exhibits a similar regulatory pattern in cultured macrophages as the *virB* genes, whether or not BvfA is a substrate for the T4SS has not been definitively resolved, and the function of this protein is unknown.

Like their phylogenetic relatives *Agrobacterium tumefaciens* and *Sinorhizobium meliloti*, *Brucella* strains produce periplasmic cyclic β-1,2-glucans (CβG) [87], and these glucose polymers are required for the successful interactions of all three of these bacteria with their eukaryotic hosts [26,57,144]. In the case of the brucellae, experimental evidence suggests that CβG disrupts the integrity of lipid rafts in the membrane of the BCVs during

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intracellular trafficking, preventing extensive interactions of these vacuoles with lysosomes [10] (Figure 1). It is unclear how these CβG molecules, which reside in the periplasm of *Brucella* strains, make their way to the membrane of the BCV. A plausible proposition that has been put forth is that CβG may be released from intact bacterial cells as components of outer membrane vesicles [10].

The capacity of the brucellae to both avoid and interfere with components of the host immune response contributes to their intracellular persistence

The *Brucella* LPS is a weak inducer of the host inflammatory response compared to LPS molecules from many other Gram-negative bacterial pathogens (Figure 2). Mice infected with virulent *B. abortus* 2308, for example, do not show signs of sepsis. This is in contrast to mice infected with a virulent strain of *Salmonella typhimurium* [14], which typically exhibit malaise, wasting and eventually death. Unlike mice infected with *Salmonella*, those infected with *B. abortus* do not exhibit an acute phase response, do not recruit neutrophils to the site of inoculation and do not strongly induce production of the proinflammatory cytokines IL-1β, IL-6 or TNF-α. *Brucella* cells are also relatively inefficient at activating complement. These experimental findings support previous work showing that the *Brucella* LPS has greatly reduced "endotoxin" activity compared to similar molecules from other Gram-negative pathogens [125,148]. They are also in agreement with the fact that although human brucellosis is a febrile illness, *Brucella* infections do not elicit the same sepsis response observed in patients with systemic infections caused by Gram-negative bacteria possessing a highly endotoxic LPS such as the enterics and the *Pseudomonas* spp. [14].

The endotoxin component of the *Brucella* LPS, the lipid A, has several biochemical features (e.g. diaminoglucose and long chain [C28] fatty acids) [88] that distinguish it from the "classic" lipid A found in the other Gram-negative bacteria that induces strong inflammatory responses in infected hosts [104]. Moreover, there is genetic evidence supporting the proposition that the *Brucella* lipid A plays a major role in the capacity of these bacteria to avoid the induction of a full-scale inflammatory response in the host (Figure 2). Specifically, a *Brucella bacA* mutant which has a lipid A that is deficient in its long chain (e.g. C28) fatty acid content [63] produces a stronger inflammatory response in experimentally infected mice than does its parental strain [136] and is attenuated. *Brucella bvrRS* mutants which also have lipid As with reduced long chain fatty acid content compared to their parent strains [117] are likewise highly attenuated in experimentally infected mice [172].

In addition to their ability to avoid induction of an optimal inflammatory response in the host, *Brucella* strains are also able to actively interfere with the host acquired immune response (Figure 2). The perosamine O-chain of the LPS of *Brucella* strains is poorly degraded by host macrophages [65] and forms complexes with components of the MHCII machinery of these phagocytes which interferes with their antigen processing capacity [66] (Figure 2).

Brucella strains also produce a protein designated TcpB [39,145] or Btp1 [159] that contains a Toll/interleukin-1 receptor (TIR) domain. TIR domain containing proteins serve as important components of the host cell signaling pathways that link the Toll-like receptors to NF-κB and are important for the induction of innate immunity [132]. When expressed in eukaryotic cells, the *Brucella* TcpB blocks TLR2- and TLR4-mediated induction of NF-κB expression [39,145,159, Sengupta et al, manuscript submitted] through its capacity to elicit the targeted degradation of the TLR signaling adapter MAL (also known as TIRAP) [Sengupta et al., manuscript submitted]. Although *Brucella tcpB* mutants are not attenuated in cultured murine macrophages, HeLa cells or immunocompetent mice (145,159, Sengupta et al., manuscript submitted], these strains do exhibit delayed virulence in the

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immunocompromised IRF-1$^{-/-}$ mouse model [145]. Studies employing a murine intestinal loop model indicate that TcpB plays a role in the capacity of *Brucella* strains to interfere with dendritic cell maturation and function [159] (Figure 2).

PrpA is another protein produced by *Brucella* strains that interferes with host immune response [173]. This protein is a proline racemase that acts as a T-cell independent B lymphocyte mitogen that stimulates the production of the anti-inflammatory cytokine IL-10 (Figure 2). *B. abortus prpA* mutants exhibit significant attenuation in experimentally infected mice at 12 weeks post infection and beyond. It has been proposed that PrpA induces a transient immune suppression that helps the brucellae maintain chronic infections.

Physiologic adaptation of the brucellae to their intracellular niche

Even though *Brucella* strains are able to actively alter the intracellular trafficking of the host cell vacuoles within which they reside and avoid the induction of a full scale inflammatory response, these bacteria still encounter formidable environmental stresses during their interactions with macrophages. These stresses include exposure to reactive oxygen (ROS) and nitrogen species (RNS), exposure to acidic pH, nutritional deprivation and at least transient exposure to the lytic peptides contained in lysosomes (Figure 3). Correspondingly, the brucellae are well equipped from both a physiologic and metabolic standpoint to withstand these environmental stresses [101,156]. This trait undoubtedly plays an important role in the success with which *Brucella* strains maintain prolonged residence in these host phagocytes. The intracellular “stresses” encountered by the brucellae within non-professional phagocytes such as epithelial cells are less severe than those encountered in professional phagocytes [128].

Resistance to oxidative damage

Brucella strains generate ROS such as O_2^- and H_2O_2 endogenously as a consequence of their aerobic respiratory-type metabolism [149]. Exogenous production of these ROS has also been shown to be important for the brucellacidal activity of macrophages [90]. Because O_2^- is a charged molecule, it does not readily cross bacterial membranes. Consequently, bacteria have compartmentalized defenses against this ROS. Periplasmic superoxide dismutases such as the Cu/Zn SOD, for instance, are important for protecting bacteria from O_2^- of exogenous origin [115]. Cytoplasmic SOD such as the Mn SOD (SodA) or Fe SOD (SodB), on the other hand, protect bacterial cells from endogenous O_2^- generated by aerobic metabolism. *Brucella* strains produce both SodC and SodA [175]. Studies have shown that SodC plays an important role in protecting *B. abortus* 2308 from the respiratory burst of host macrophages [74] and is required for maintenance of chronic infection in the mouse model [74,181]. Genetic analysis of *Brucella sodA* mutants indicates that SodA plays a major role in protecting these bacteria from the endogenous O_2^- that is generated by aerobic metabolism (Baumgartner and Martin, unpublished). The importance of SodA to *Brucella* strains during their residence in the host is presently under investigation.

Two major antioxidants with the capacity to degrade H_2O_2 have been described in *Brucella* strains, the periplasmic monofunctional catalase KatE [165] and the peroxiredoxin AhpC [155]. Genetic studies have shown that KatE detoxifies supraphysiologic levels of H_2O_2 [73,95,165] in these bacteria, while AhpC appears to be the major scavenger of the endogenous H_2O_2 that is generated by aerobic metabolism (Steele, manuscript in preparation). *B. abortus ahpC* and *katE* mutants exhibit wild-type virulence in experimentally infected mice [Steele, manuscript in preparation; 162] and a *B. melitensis katE* mutant produces abortion and fetal pathology in pregnant goats [73]. A *B. abortus ahpC katE* double mutant, in contrast, displays severe attenuation in both the C57BL6 and BALB/c mouse models (Steele, manuscript in preparation), and this attenuation is not

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diminished in C57BL6 knockout mice lacking a functional NADP oxidase or inducible nitric oxide synthase. These experimental findings indicate that, unlike SodC, neither AhpC and KatE plays a direct role in protecing *Brucella* strains from the oxidative or nitrosative bursts of host phagocytes. Rather, the combination of these two antioxidants appears to provides the brucellae with an efficient means of protecting themselves from potentially lethal levels of endogenous H_2O_2 that are generated as a consequence of their respiratory metabolism during residence in the host.

In addition to their ability to directly detoxify ROS, the brucellae also appear to have developed mechanisms for indirectly avoiding oxidative damage. Cytochrome *bd* ubiquinol oxidases and the *cbb3*-type cytochrome *c* oxidases have high affinity for $O_{2,}$ and the O_2 "scavenging" capacity of these terminal cytochrome oxidases has been linked to the prevention of ROS toxicity in other bacteria [54,143]. Cytochrome *bd* ubiquinol oxidase and the *cbb3*-type cytochrome *c* oxidase are both required for wild-type virulence of *Brucella* strains in cell cultures and experimentally infected mice [59,91], and increased sensitivity to ROS has been experimentally linked to the attenuation of a *B. abortus abortus cydB* [59].

DNA is a target of ROS-mediated damage in all living cells, and experimental evidence indicates that DNA repair pathways such as base excision repair and recombination repair play an important role in protecting *Brucella* strains from ROS toxicity *in vitro* [85,158]. To date, however, *recA* mutants are the only *Brucella* strains with defects in DNA repair that have been shown to be attenuated in experimentally infected animals [182].

Resistance to nitrosative damage

Nitric oxide (NO) produced by the inducible nitric oxide synthase (iNOS) of murine macrophages has been shown to play a role in the capacity of these phagocytes to control the intracellular replication of the brucellae [78,90]. *Brucella* strains produce a nitric oxide reductase (Nor), and genetic studies suggest that in addition to its metabolic role in denitrification (see below), Nor may also play an important role in the detoxification of NO by *Brucella* strains during their replication in host macrophages [82,113]. Genetic studies have also uncovered a link between a D-alanyl-D-alanine carboxypeptidase (encoded by the *dacF* gene), expression of *norB*, and the resistance of *B. abortus* 544 to NO *in vitro* and in cultured macrophages [94], but the nature of this link is not readily apparent.

Peroxynitrite ($ONOO^-$) is produced as the product of the reaction of O_2^- with NO. This ROS-RNI hybrid has potent microbicidal activity, and it is considered to be an important component of the antibacterial arsenal of host macrophages [62]. In addition to their ability to detoxify H_2O_2 and organic peroxides, the AhpC proteins from *Salmonella*, *Mycobacterium* and *Helicobacter* have also been shown to have peroxynitrite reductase activity *in vitro* [28]. Genetic studies suggest that AhpC protects *Mycobacterium* strains from exposure to $ONOO^-$ *in vitro* and is required for their virulence in cultured macrophages [121] and guinea pigs [192]. A *B. abortus ahpC* mutant exhibits increased sensitivity to $ONOO^-$ generated by the compound SIN-1 in *in vitro* assays (K. Steele, unpublished), but whether or not AhpC protects this strain from exposure to $ONOO^-$ in host macrophages remains to be determined experimentally.

Resistance to acidic pH

It is well documented that the intracellular compartment within which *Brucella* strains reside in cultured macrophages and epithelial cells is acidified during the early stages of its development [53,140]. In fact, if this acidification is blocked by the addition of bafilomycin or neutralized by the addition of NH_4Cl during the early stages of development of the *Brucella* containing vacuole in these cells, the brucellae will not initiate intracellular

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replication in either cell type. As noted previously, this low pH apparently serves as an important environmental stimulus for the induction of the *virB* genes which encode the components of the Type IV secretion system [24].

Several gene products have been linked to acid resistance in *Brucella* strains. HdeA is a periplasmic chaperone that functions at low pH and plays an important role in acid resistance in *E. coli* [72] and *Shigella flexneri* [188]. A *B. abortus hdeA* mutant displays a decreased resistance to acidic pH (e.g. pH 4) compared to its parental strain [185], but this mutant is not attenuated in the mouse model. Asp24 is a putative “EF hands”-type Ca^{++}-binding protein (T. Ficht, personal communication) originally identified in a screen for gene products produced by *B. abortus* 2308 in response to exposure to acid pH [112]. Notably, *Brucella asp24* mutants are attenuated in mice [92] and goats [93] and have been proposed for use as vaccine candidates. Specifically how Asp24 protects *Brucella* strains from acid stress has not been described. Increased sensitivity to acid pH is a phenotype that has also been reported for *Brucella cydB* [59] and *hfq* mutants [152]. In the case of the latter strains, there is an as yet undefined regulatory link between the RNA chaperone Hfq (see below) and *hdeA* in *B. abortus* 2308 [185]. The basis for the acid sensitive phenotype of *B. abortus cydB* mutants is unclear.

Most *Brucella* strains produce a functional urease that protects these bacteria from extremely low pH (pH 2) under laboratory conditions when urea is present in the growth medium [13,163]. Results from experimental infections in mice suggest that urease may play an important role in protecting *Brucella* strains from the acidic conditions encountered during passage through the gastrointestinal tract after oral ingestion in the host. These same studies, however, indicate that urease does not play a role in protecting *Brucella* strains from the acidic conditions encountered within host cells [13,163].

Bacterial glutamate decarboxylases (GadA or GadB) and the associated γ–amino-butyric acid (GABA) exporters (GadC) represent important components of acid resistance because GadC is a proton symporter [31]. *Brucella* strains possess *gadC*, but the *gadB* genes appear to be pseudogenes, and mutational analysis indicates that neither GadB nor GadC contributes to the acid resistance of *B. abortus* 2308 *in vitro* or the virulence in this strain in mice [27].

Resistance to antimicrobial peptides

Brucella strains have an inherently higher level of resistance to many of the bactericidal cationic peptides found in mammalian hosts compared to other Gram-negative bacterial pathogens [120]. Experimental evidence indicates that this heightened resistance is linked to the acylation status of the lipid A moiety of the *Brucella* LPS [117]. Although this trait would be expected to be particularly beneficial to the brucellae in the extracellular environment in the host and during their interactions with neutrophils, this increased resistance to antimicrobial peptides may also provide these bacteria with protection from the lytic peptides contained in lysosomes during the transient interactions of the *Brucella*-containing vacuole with these organelles in host cells [176].

Resistance to nutrient deprivation

Studies employing both defined mutants as well as those generated by transposon mutagenesis have demonstrated that *Brucella* strains experience a significant degree of nutritional deprivation during their intracellular replication in host cells. One of the major limiting nutrients in this environment is elemental O_2 that can be used as a terminal electron acceptor to fuel the respiratory metabolism of these bacteria. *Brucella* mutants that lack the *cbb3*-type cytochrome *c* oxidase (CcoNOQP), the cytochrome *bd* ubiquinol oxidase

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(CydCDAB) or components of the denitrification pathway (NarGHIJK/NirKV/NorBCDEFQ/NosDFLRXYZ) exhibit defective intracellular survival and replication in cultured macrophages [59,82,100,113] and HeLa cells [97] and are attenuated in experimentally infected mice [59,82,91]. The CcoNOQP and CydCDAB complexes allow these bacteria to respire efficiently at low O_2 concentrations, while the components of the denitrification pathway allow the bacteria to use NO_3^- as an alternative terminal electron acceptor instead of O_2. A recent comparative study of isogenic *B. suis ccoN* and *cydB* mutants in the mouse model [91] and an evaluation of *B. melitensis cydAB* mutants in pregnant goats [93] indicate that the *cbb3*-type cytochrome c oxidase may play a more important than the cytochrome *bd* ubiquinol oxidase in allowing *Brucella* strains to adjust to the environmental conditions encountered in the host, but this relationship merits further examination.

Brucella strains have a blocked Embden-Meyerhof pathway, and they rely on the pentose phosphate pathway and TCA cycle for the efficient use of carbohydrates as carbon and energy sources [61]. Several studies have shown that intact carbohydrate transport and metabolism pathways are essential for the successful intracellular replication of *Brucella* strains [67,84,97,100,103,108,194]. As noted in an earlier section, the capacity of *B. abortus* and *B. melitensis* strains to use erythritol as a preferred carbon source has been postulated to play an important role in their virulence in ruminant placental trophoblasts. Interestingly, experimental studies also suggest that some of the erythritol metabolism genes are required for the wild type virulence of *B. suis* 1330 in cultured human and murine macrophages and experimentally infected mice [29,100]. A derivative of *B. abortus* 2308 with Tn*5* inserted into the *eryB* gene, in contrast, exhibits wild-type virulence in the mouse model [164]. The role that the *eryB* and *eryC* genes play in the intracellular survival and replication of *B. suis* 1330 in murine and human macrophages is unclear [29] since erythritol is not considered to be a major constituent of murine and human tissues.

Multiple investigators have shown that intact purine biosynthesis pathways are essential for efficient replication of *Brucella* strains in cultured murine [1,194] and human [56,100] macrophages, HeLa cells [97] as well as the wild-type virulence of these strains in mice [1,45] and goats [38]. The biosynthesis of pyrimidines and several classes of amino acids also appear to be required for the successful adaptation of the brucellae to their intracellular niche [67,68,97,100,108,109].

In addition to the macronutrients listed above, a number of micronutrients that are essential for the intracellular replication of *Brucella* strains have also been identified. One class of micronutrient that appears to be particularly important for these strains is the divalent cations, which serve as co-factors for a wide array of cellular proteins. With the notable exceptions of the *Lactobacillus* spp. [190] and *Borrelia burgdorferi* [142], all of the other bacteria that have been examined require iron, and the brucellae are no exception. The activity of two *Brucella* iron acquisition systems has been described in the literature, and a survey of several publicly available *Brucella* genome sequences suggests that several more exist [157]. *B. abortus* 2308 produces two siderophores, 2,3-dihydroxybenzoic acid (2,3-DHBA) [114] and brucebactin [77], in response to iron limitation *in vitro*. Genetic evidence indicates that brucebactin is derived from 2,3-DHBA, but the structure of brucebactin has not been determined. Although the genes required for the biosynthesis and transport of 2,3-DHBA and brucebactin are strongly expressed in *B. abortus* 2308 during intracellular replication in cultured murine macrophages [103], neither siderophore is required for the virulence of this strain in cultured murine or human macrophages or in experimentally infected mice [17,77,135, Bellaire, unpublished]. In contrast, *B. abortus dhbC* mutants (which can produce neither 2,3-DHBA nor brucebactin) are highly attenuated in pregnant cattle [18]. *In vitro* studies have established a link between siderophore production and the

capacity of *B. abortus* 2308 to efficiently utilize erythritol as a carbon and energy source during growth under iron limiting conditions [19]. To what extent this link contributes to the attenuation of *B. abortus dhbC* mutants in ruminants, however, remains to be determined.

Brucella strains are also capable of transporting the intact heme molecule [4] and using it as an iron source [137]. A *B. abortus* mutant lacking the outer membrane heme transporter BhuA cannot maintain chronic spleen infection in experimentally infected mice [137], which suggests that heme represents a major iron source for the brucellae during their intracellular replication in host macrophages. The degradation of the hemoglobin contained in senescent erythrocytes by macrophages plays a central role in the recycling of iron in mammals [47]. Heme is a toxic compound, however, and unless it is directly used by these phagocytes, it is transported to their endoplasmic reticulum for degradation by heme oxygenase [179]. Thus, the possibility exists that one of the benefits to the brucellae of residence in an intracellular compartment that has extensive interaction with the endoplasmic reticulum is that it allows these bacteria access to a critical source of iron.

The efficient transport of Mg^{++}, Zn^{++} and Mn^{++} has also been shown to be critical for the success of *Brucella* strains as intracellular pathogens [7,98,105,195]. Although the brucellae possess a single high affinity Mn^{++} transporter, MntH, transport of this divalent cation appears to be very important for these bacteria in the mammalian host as *mntH* mutants exhibit extreme attenuation in mice that lack Nramp1 [7]. This mammalian divalent cation transporter plays a critical role in the metal withholding response in host macrophages that is an important component of the host innate immune response [33], and bacterial Mn^{++} transport mutants often do not exhibit attenuation in mice unless these animals possess a functional Nramp1 [197]. The precise basis for the importance of Mn^{++} as a micronutrient for *Brucella* strains is unknown, but experimental evidence suggests that MntH-mediated Mn transport plays an important role in facilitating optimal production of the Mn SOD in *B. abortus* 2308. Mn^{++} transport may also be important for wild-type activity of Rsh, the mediator of the stringent response in this bacterium (see below) [7].

Production of flagella contributes to virulence via an as yet undefined mechanism

Although *Brucella* strains are uniformly described as being non-motile, genome sequence data suggests that they have the genetic capacity to produce flagella. Moreover, under certain growth conditions *B. melitensis* 16M produces a polar organelle that resembles a flagellum [70]. Intriguingly, *B. melitensis flgI*, *fliF*, *fliC*, *flhA*, *motB* and *flgE* mutants which do not produce this flagellum are not attenuated in cultured bovine macrophages or HeLa cells, but do exhibit attenuation in experimentally infected mice [70]. A transcriptional regulator, FtcR, that appears to lie downstream of VjbR in the regulatory pathway of the flagellar biosynthesis genes in *B. melitensis* 16M, has been identified [107], and isogenic *ftcR* mutants constructed in this strain are also attenuated in mice. The basis for the attenuation of *Brucella* mutants that are defective in flagellar biosynthesis has not been resolved, but one possibility is that the polar organelle produced by the *Brucella* “flagellar” genes is a secretion apparatus rather than one linked to motility. The components of bacterial Type III secretion systems share many similarities with those involved in the transport and assembly of flagella [75].

Phosphatidylcholine is a major component of the OM of *Brucella* strains

Unlike many bacteria, *Brucella* strains and other α–proteobacteria have outer membranes enriched in phosphatidylcholine (PC), a phospholipid that is typically associated with eukaryotic cell membranes [170]. The presence of PC in the outer membrane appears to be

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required for the successful interactions of *Bradyrhizobium japonicum* [124] and *Agrobacterium tumefaciens* [191] with their plant hosts, and likewise *Brucella abortus* mutants lacking PC in their outer membranes are attenuated in the mouse model [41,42]. The precise role that PC plays in the virulence of *Brucella* strains is undefined, but studies suggest that this phospholipid may be important for maintaining the integrity and permeability characteristics of the outer membrane and in particular may be involved in resistance to complement and other antimicrobial peptides [42]. Because PC is a major component of eukaryotic membranes and the degradation of PC by eukaryotic cells produces two important eukaryotic cell-signaling molecules (diacylglycerol and phosphatidic acid); it has also been postulated that the presence of this phospholipid in the *Brucella* outer membranes plays a role in immune evasion via molecular mimicry [41,42]. Furthermore, studies with *Legionella pneumophila* [43] raise the possibility that the presence of PC in the outer membrane is important for the proper assembly or function of the components of the T4SS or flagella on the surface of *Brucella* strains.

The mystery behind the virulence of naturally occurring rough strains of *Brucella*

It is well-established that the LPS O-chain is a major virulence determinant of *B. abortus*, *B. melitensis* and *B. suis* strains, and O-chain deficient mutants derived from these strains (so-called "rough" mutants) are uniformly attenuated in experimental hosts [3,37,58,76,122,153,183,193]. *B. canis* and *B. ovis* strains, in contrast, naturally lack the LPS O-chain, yet they produce disease in their natural hosts. Compared to the naturally-occurring smooth strains, little work has been done on the interactions of *B. canis* and *B. ovis* strains with host cells. What can be derived from these studies is that, in general, naturally-occurring rough *Brucella* strains appear to be taken up into host cells with greater efficiency than smooth strains [52,64,151], but these strains do not replicate as well in host cells as their smooth counterparts [52,64,119]. This may be due to the fact that trafficking studies indicate that BCVs containing *B. canis* and *B. ovis* strains fuse more extensively with lysosomes in host cells than BCVs containing smooth *Brucella* strains [141,151]. Laboratory derived rough *B. abortus* mutants exhibit cytotoxicity in cultured macrophages [138], and it has been postulated that the spontaneous occurrence of these cytotoxic mutants may facilitate cell-to-cell spread of naturally-occurring smooth strains *in vivo*. It is notable in this regard, however, that *B. canis* and *B. ovis* strains are not cytotoxic for cultured macrophages [138]. It is clear that a lot more needs to be learned about the interactions of *B. canis* and *B. ovis* with host cells and how these interactions influence the progression of canine and ovine brucellosis.

Programmatic changes in gene expression required for intracellular survival by the brucellae

BvrRS

A genetic screen for *Brucella* mutants with decreased resistance to the antimicrobial cationic peptide polymyxin B led to the discovery of the two-component regulator BvrRS in *B. abortus* [172]. The outer membrane properties of *Brucella bvrRS* mutants are considerably altered compared to their parental strains [117], and these mutants are highly attenuated and exhibit altered intracellular trafficking patterns in cultured macrophages and HeLa cells [172]. These strains also exhibit significant attenuation in experimentally infected mice [172]. BvrRS controls the expression of the genes encoding the outer membrane proteins Omp3a and Omp3b [80,102] as well as yet undefined genes whose products play a role in proper acylation of the lipid A component of the LPS (Figure 2) [117]. As noted earlier, these latter genes appear to play a major role in the high level of resistance that *Brucella*

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strains display to killing by cationic antimicrobial peptides. Altered expression of the *omp3a* and *omp3b* genes, in contrast, does not appear to be linked to this phenotypic trait or the attenuation of *Brucella bvrRS* mutants in mice [118]. Proteomic analysis suggests that numerous other *Brucella* genes are also subject to either direct or indirect regulation by BvrRS [102], but the individual contributions of these genes to virulence remains to be determined.

From a phylogenetic standpoint it is notable the *Brucella* BvrRS system is homologous to the *Agrobacterium* ChvIG and *Sinorhizobium* ChvI/ExoS two component regulators. These regulators control expression of genes whose products make the modifications of the cell envelope for the wild-type interactions of *Agrobacterium* [34] and *Sinorhizobium* [36] strains with their plant hosts. The environmental stimuli recognized by the *Brucella* BvrS have not been reported, but studies indicate that ChvG senses acidic pH in *Agrobacterium tumefaciens* [111]. If the same is true for the *Brucella* BvrS, it is easy to envision the potential benefit of such a regulatory link for the brucellae in successful adaptation to their intracellular niche.

VjbR and BlxR

Cell-to-cell communication via the process known as “quorum sensing” has been shown to be important for the successful adaptation of many bacteria to changing environmental conditions [189]. This process is also critical for the virulence of many bacterial pathogens [30] and the successful establishment of symbiotic relationships [161]. *Brucella* strains produce an acyl-homoserine lactone (AHL)-type signaling molecule (C12-HSL) [180]. LuxR-type transcriptional regulators respond to AHL in bacterial quorum sensing systems [189], and two of these transcriptional regulators have been identified in *Brucella*. VjbR [51] controls expression of the *virB* operon, flagellar biosynthesis genes and genes encoding several outer membrane proteins [184], and *vjbR* mutants exhibit attenuation in cultured macrophages, HeLa cells and experimentally infected mice [9,51]. Another LuxR-type regulator, designated BlxR [147], has also been shown to play a role in the regulation of the *virB* and flagellar biosynthesis genes, but comparative studies in mice indicate that the loss of VjbR has a much more dramatic effect on the virulence of *B. melitensis* 16M than does loss of BlxR. As the authors of this study point out, these findings suggest although VjbR and BlxR are both LuxR homologs, they do not perform functionally redundant roles in *B. melitensis* 16M [147].

Rsh

Bacteria can undergo a global change in gene expression known as the stringent response when they are faced with severe nutrient deprivation [89]. In response to nutrient deprivation, bacteria produce the alarmone guanosine 3’,5’-bispyrophosphate (ppGpp) via the activity of the ppGpp synthetases RelA or SpoT. This alarmone, in turn, binds to RNA polymerase and changes the efficiency with which it recognizes promoter sequences, leading to reduced expression of genes encoding components of the translational machinery and increased expression of amino acid biosynthetic genes and other genes required for adjusting the cells metabolism to a maintenance mode [116]. *Brucella* strains produce a single ppGpp synthetase (designated Rsh for RelA/SpoT homolog), and studies have shown that *Brucella rsh* mutants quickly lose viability when subjected to nutrient deprivation *in vitro* and are attenuated in cultured macrophages, HeLa cells and experimentally infected mice [55,99]. These experimental findings suggest that the stringent response plays a key role in the successful adaptation of the brucellae to the nutritional deprivation they encounter during intracellular residence in the host. Notably, nutritional deprivation appears to be an important environmental stimulus for induction of the genes encoding the *virB* genes [24],

and the presence of Rsh is required optimal expression of these genes in *B. melitensis* 16M [55].

Hfq and sRNAs

Small regulatory RNAs (sRNAs) play an important role in regulating the expression of a wide variety of bacterial genes [177]. They perform this function predominantly by interacting with mRNAs and facilitating or interfering with the translation of these transcripts and/or accelerating or delaying their degradation by cellular RNases. Many of these sRNAs have limited complementarities with their mRNA targets and require the participation of the RNA chaperone Hfq for efficient interaction with these transcripts [186]. A *B. abortus hfq* mutant exhibits increased sensitivity to multiple environmental stresses compared to the parental 2308 strain and is attenuated in cultured murine and human macrophages and experimentally infected mice [20,152]. A *B. melitensis hfq* mutant is also attenuated in pregnant goats [154] and mice and non-human primates (M. J. Nikolich, personal communication). These experimental findings suggest that sRNAs play an important role in controlling the expression of genes required for successful adaptation of the brucellae to their intracellular niche in the host. Genetic and proteomic studies have linked Hfq to the wild-type expression of the *sodC* [71,74] and several other genes known to be required for the virulence in *B. abortus* 2308 [155, Gaines and Caswell, unpublished]. The nature of the regulatory links between Hfq and these genes and the identity of the sRNAs involved is presently under investigation.

NolR, MucR, and the LOV domain histidine kinase

The α–proteobacteria employ similar strategies to establish and maintain sustained interactions with their eukaryotic hosts, and as noted above for BvrRS, homologous regulatory systems appear to be responsible for proper expression of the bacterial genes required for these interactions. A couple of other examples of these shared regulatory networks have recently been discovered. NolR is a transcriptional regulator that controls the expression of nodulation genes in *Sinorhizobium meliloti* [35]. A targeted mutational analysis of genes predicted to encode transcriptional regulators in *B. melitensis* 16M has shown that a NolR homolog is required for wild-type expression of the *virB* genes and the virulence of this strain in cultured murine macrophages, HeLa cells and mice [81]. The transcriptional regulator MucR provides a regulatory link between exopolysaccharide synthesis and motility in *S. meliloti* [11], and insertion of the mariner transposon *Himar1* into a *mucR* homolog in *B. melitensis* 16M severely attenuates this strain in macrophages and mice [194].

A histidine kinase carrying an LOV (light, oxygen, or voltage) domain has recently been identified in *Brucella* strains [178]. Biochemical studies indicate that this protein is responsive to light, and genetic studies have shown that *B. abortus* strains lacking this protein or carrying a mutated version of the protein that is not light-responsive are attenuated in the murine J774 macrophage-like cell line. Because of their close association with the host, it is unclear when exposure to blue light would be a relevant environmental stimulus. One proposition that has been put forth is that this exposure may take place when the brucellae are expelled into the environment in the infected placenta, and this exposure may stimulate the expression of *Brucella* genes important for initiating infection in a newly infected host [178].

Summary

The capacity of *Brucella* strains to successfully survive and replicate in host cells is critical to their virulence. The brucellae employ several strategies to establish and maintain

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persistent intracellular residence in host cells. These bacteria are able to avoid a full blown inflammatory response during the initial stages of infecting the host. Once the brucellae enter into host cells they proactively influence the intracellular trafficking of the membrane bound compartments within which they reside so that these vacuoles avoid becoming "phagolysosomes". After the brucellae reach the vacuolar compartments within which they maintain their intracellular residence, they are well equipped from a physiologic standpoint to withstand the environmental stresses they encounter. The bacteria also appear to exploit some of the environmental stresses they encounter (e.g. acidic pH and nutrient deprivation) as stimuli for the induction of genes required for alteration of their intracellular trafficking. Finally, the intracellular brucellae alter the biological functions of the professional phagocytes within which they reside in such a manner that these cells lose their antigen-processing capacity and in the case of macrophages, become resistant to apoptosis.

Many of the cell components and strategies that the brucellae employ to successfully adapt to their intracellular lifestyle and produce chronic infections in the host appear to be same as those employed by other α–proteobacteria to establish and maintain prolonged infections of their plant and animal hosts [16]. Recent comparative studies with the closely related bacterium *Ochrobactrum anthropi* also provide insight into evolutionary pathways that the brucellae have followed to improve their adaptation to the specific challenges associated with their prolonged residence in mammalian cells [15]. Continued efforts to better understand how these remarkable intracellular pathogens have adapted to their intracellular niche will undoubtedly provide us with critical information that is needed for the rational design of better vaccines and chemotherapy for use against brucellosis in both natural hosts and in humans.

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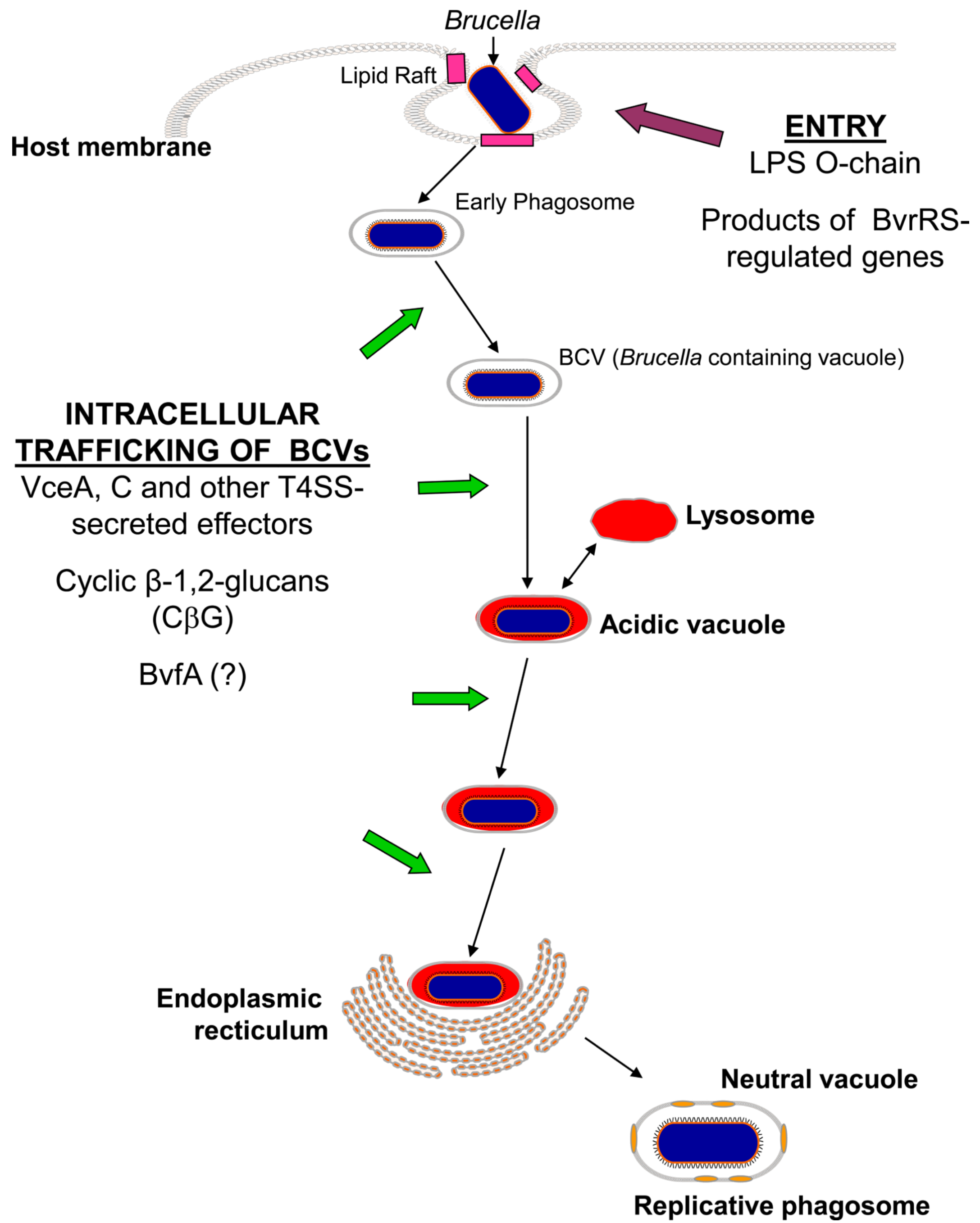

Figure 1.

Gene products that influence their intracellular trafficking of *Brucella* strains in host cells.

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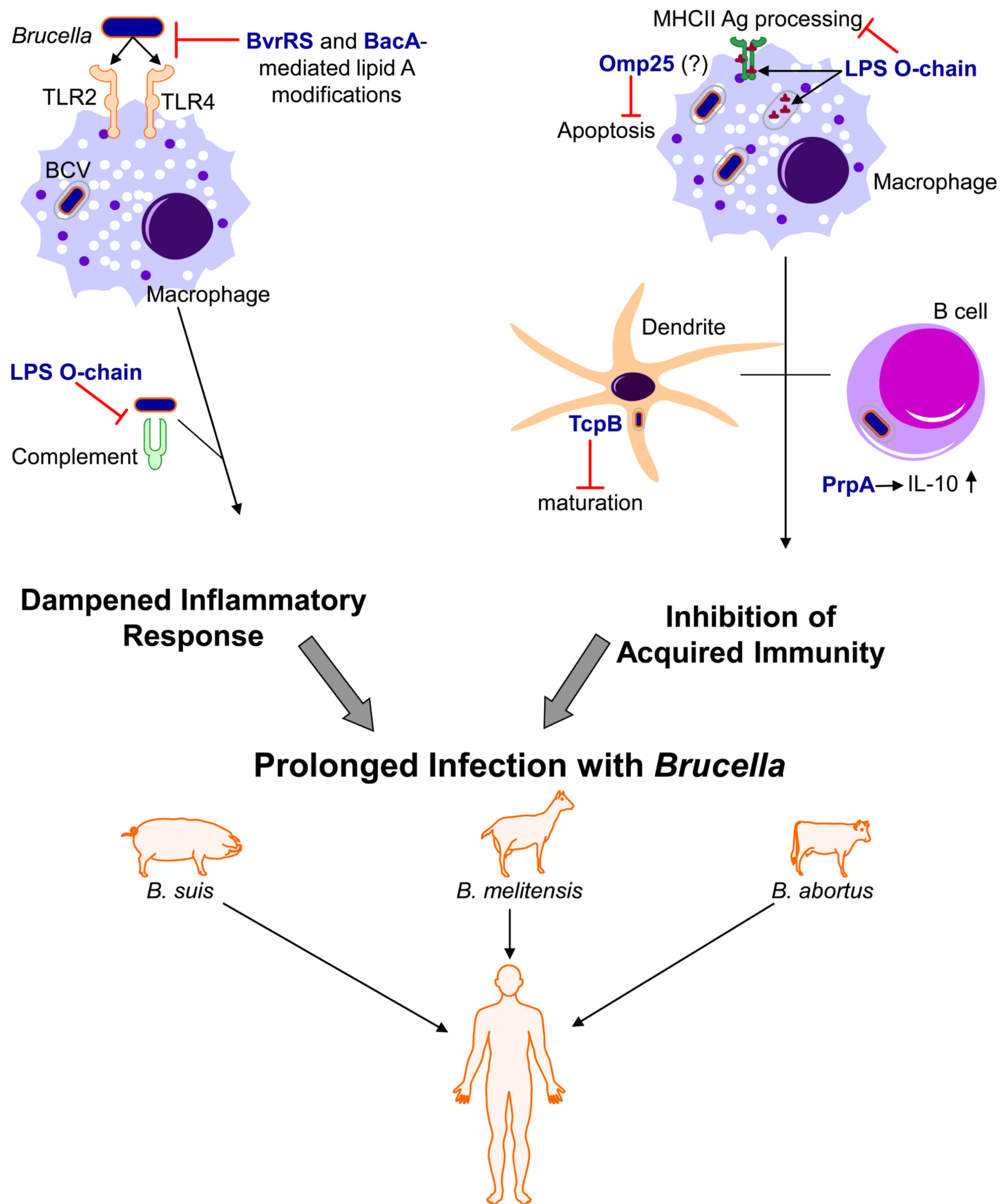

Figure 2.

Subversion of host immune defenses allows *Brucella* strains to establish and maintain chronic infections in their hosts. *Brucella* gene products linked to immune evasion are shown in blue font.

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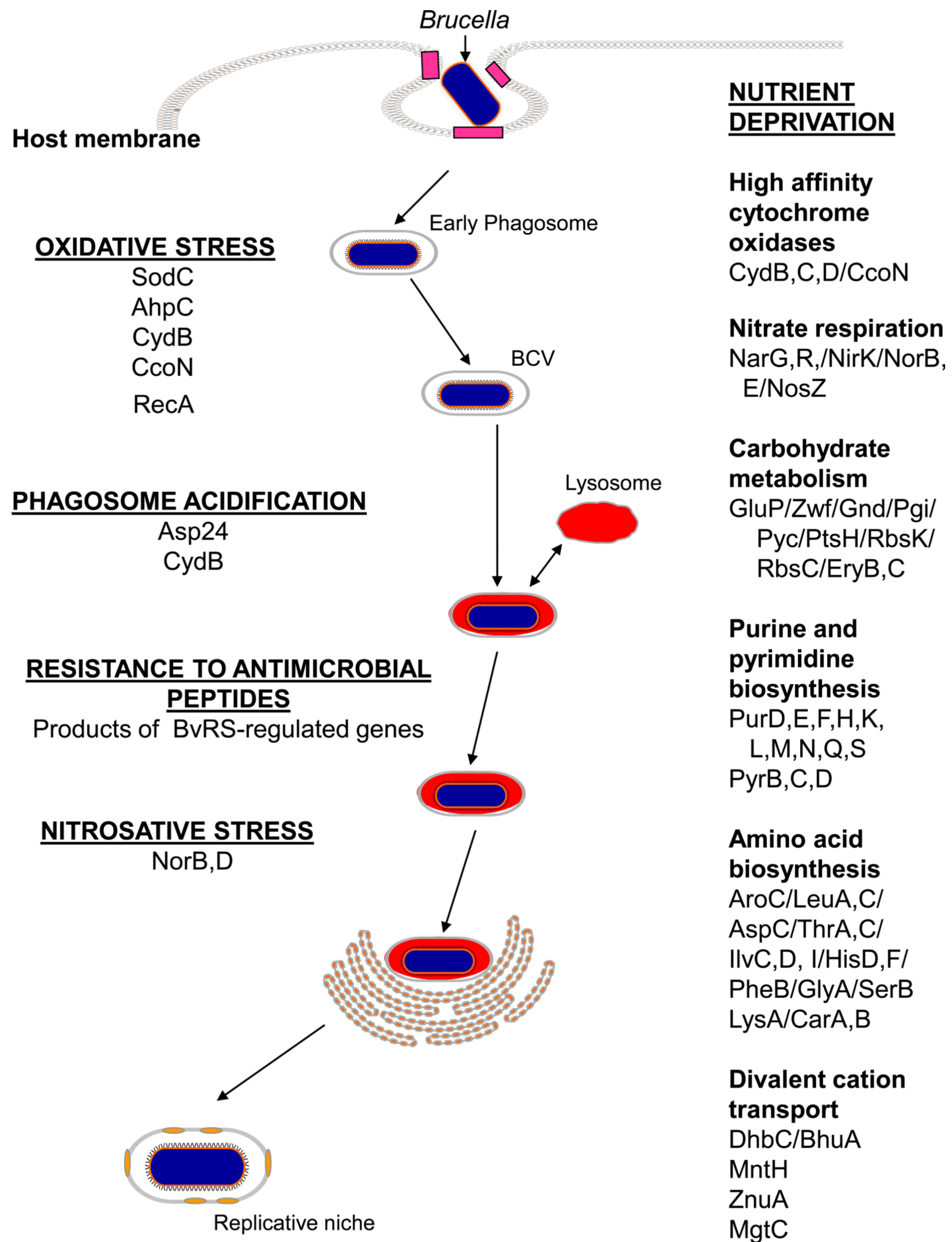

Figure 3.

Gene products that play important roles in allowing *Brucella* strains to resist the environmental stresses they encounter during intracellular replication in the host.